

## Action of Human Group IIa Secreted Phospholipase A<sub>2</sub> on Cell Membranes

VESICLE BUT NOT HEPARINOID BINDING DETERMINES RATE OF FATTY ACID RELEASE BY EXOGENOUSLY ADDED ENZYME\*

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Human group IIa phospholipase A<sub>2</sub> (hIIa-PLA<sub>2</sub>) is a highly basic protein that is secreted from a number of cells during inflammation and may play a role in arachidonate liberation and in destruction of invading bacteria. It has been proposed that rodent group IIa PLA<sub>2</sub> is anchored to cell surfaces via attachment to heparan sulfate proteoglycan and that this interaction facilitates lipolysis. hIIa-PLA<sub>2</sub> contains 13 lysines, 2 histidines, and 10 arginines that fall into 10 clusters. A panel of 26 hIIa-PLA<sub>2</sub> mutants were prepared in which 1–4 basic residues in each cluster were changed to glutamate or aspartate (charge reversal). A detailed analysis of the affinities of these mutants for anionic vesicles and for heparin and heparan sulfate *in vitro* and of the specific activities of these proteins for hydrolysis of vesicles *in vitro* and of living cell membranes reveal the following trends: 1) the affinity of hIIa-PLA<sub>2</sub> for heparin and heparan sulfate is modulated not by a highly localized site of basic residues but by diffuse sites that partially overlap with the interfacial binding site. In contrast, only those residues on the interfacial binding site of hIIa-PLA<sub>2</sub> are involved in binding to membranes; 2) the relative ability of these mutants to hydrolyze cellular phospholipids when enzymes were added exogenously to CHO-K1, NIH-3T3, and RAW 264.7 cells correlates with their relative *in vitro* affinity for vesicles and not with their affinity for heparin and heparan sulfate. 3) The rates of exogenous hIIa-PLA<sub>2</sub>-catalyzed fatty acid release from wild type CHO-K1 cells and two mutant lines, one lacking glycosaminoglycan and one lacking heparan sulfate, were similar. Thus basic residues that modulate interfacial binding are important for plasma membrane fatty acid release by exogenously added hIIa-PLA<sub>2</sub>. Binding of hIIa-PLA<sub>2</sub> to cell surface heparan sulfate does not modulate plasma membrane phospholipid hydrolysis by exogenously added hIIa-PLA<sub>2</sub>.

The phospholipase A<sub>2</sub> (PLA<sub>2</sub>)<sup>1</sup> family of enzymes hydrolyze the *sn*-2 ester of glycerophospholipids to produce a fatty acid and a lysophospholipid (1–3). Based on amino acid sequences, 10 groups of PLA<sub>2</sub>s have been identified, including eight from mammals (4, 5). The human group IIa secreted PLA<sub>2</sub> (hIIa-PLA<sub>2</sub>) is secreted from a number of activated cells including platelets (6), whereas the macrophage-like cell line P3881 secretes a related enzyme, the recently discovered group V PLA<sub>2</sub> (7, 8). Extensive studies have shown that hIIa-PLA<sub>2</sub> is pro-inflammatory when injected into animals (9, 10). After regulated secretion of these PLA<sub>2</sub>s from cells, they are capable of liberating arachidonic acid from membrane phospholipids for eicosanoid production in the same cell (8, 11, 12) or in other cells such as fibroblasts (13). One of the main questions in the field is to understand the molecular features of PLA<sub>2</sub>s that control their binding to target cell membranes.

Detailed kinetic and structural studies have shown that secreted PLA<sub>2</sub>s contain functionally and topographically distinct interfacial binding and active site surfaces (2, 14–16). The interfacial binding surface, which allows a desolvated contact of the enzyme with membranes (17) consists of residues that surround the opening of a deep active site slot (Fig. 1). A substrate molecule must leave the membrane plane to travel about 15 Å into the active site slot to reach the catalytic residues (18). Most, if not all, secreted PLA<sub>2</sub>s bind several orders of magnitude tighter to vesicles of anionic phospholipids (phosphatidylmethanol and phosphatidylserine) than to vesicles of charge-neutral phospholipids (phosphatidylcholine) (19), and this differential binding is very pronounced for hIIa-PLA<sub>2</sub> (20, 21). This is due in part to the presence of basic amino acids on the putative interfacial binding surface of hIIa-PLA<sub>2</sub> as shown by recent directed mutagenesis studies in which lysines and arginines were changed to glutamates (charge reversal) (22). A more detailed discussion of the molecular elements of interfacial binding of secreted PLA<sub>2</sub>s including the important role of hydrophobic residues has been presented (23, 24).

Group IIa PLA<sub>2</sub> from several species also binds to the anionic sulfated glycosaminoglycans heparin and heparan sulfate (referred to below as heparinoids), and high salt elution of enzyme from heparin-Sepharose provides a key step for the purification of this enzyme (21, 25). It has been proposed that group IIa PLA<sub>2</sub> is tethered to cell surfaces by binding to heparan sulfate proteoglycan, and that this binding is important for the hydro-

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<sup>1</sup> The abbreviations used are: PLA<sub>2</sub>, phospholipase A<sub>2</sub>; DAUDA, 11-(dansylamino)undecanoic acid; hIIa-PLA<sub>2</sub>, human secreted group IIa phospholipase A<sub>2</sub>; N1A, mutant of hIIa-PLA<sub>2</sub> with asparagine-1 changed to alanine.

lysis of cell phospholipids. Rat liver-derived BRL-3A cells secrete group IIA PLA<sub>2</sub>, and appreciable amounts of this enzyme are found in the extracellular medium only when free heparin, heparan sulfate, or dextran sulfate are added to the culture medium (26), which is consistent with the observation that group IIA PLA<sub>2</sub> binds to these anionic polymers. Treatment of the cells with heparitinases results in a reduction of prostaglandin production, which suggests that binding of group IIA PLA<sub>2</sub> to cell surface proteoglycan augments arachidonic acid release (26). Similar findings have been reported for prostaglandin production in human umbilical vein endothelial cells (11) and for degranulation of mast cells induced by exogenous rodent group IIA PLA<sub>2</sub> (27). Cell surface anchoring of group IIA PLA<sub>2</sub> to heparan sulfate proteoglycan is not without precedence. It has been well established that proteins such as fibroblast growth factor and antithrombin III are anchored to cell surfaces by binding to proteoglycan (28). Lipoprotein lipase is bound to heparan sulfate proteoglycan on the surface of capillary endothelial cells where it acts on lipid esters present in circulating chylomicrons and very low density lipoproteins (28). Additionally, cellular uptake of lipoproteins is thought to be facilitated by their capture by cell-surface bound lipoprotein lipase (28). Addition of exogenous heparin results in release of the lipase from cell surfaces so that it now binds to circulating lipoproteins in plasma.

One possible model is that group IIA PLA<sub>2</sub> is bound simultaneously to cell surface proteoglycan via a putative heparin-binding site and to membranes via its interfacial binding site. There are reasons to propose that group IIA PLA<sub>2</sub> contains such distinct binding sites. Two monoclonal antibodies have been described that prevent binding of rat group IIA PLA<sub>2</sub> to heparin-Sepharose but do not inhibit the lipolytic activity of this enzyme on phospholipid vesicles (29). Detailed kinetic studies of the effect of heparin on porcine pancreatic PLA<sub>2</sub> catalyzed vesicle hydrolysis show that heparin can bind to the enzyme without displacing it from anionic vesicles (30). We have shown that hIIA-PLA<sub>2</sub> bound to heparin-Sepharose is able to bind inhibitors in its active site while the tethered enzyme remains bound to micelles of hexadecylphosphatidylcholine.<sup>2</sup>

hIIA-PLA<sub>2</sub> is a highly basic protein that contains 13 lysines and 10 arginines scattered over its surface, and presumably some of these are involved in binding to sulfated glycosaminoglycans. In the present study, we have mutated many of the surface cationic residues to anionic glutamates or aspartates. The *in vitro* binding of these mutants to anionic phospholipid vesicles and to heparin and heparan sulfate was studied. In addition, the rate of fatty acid release from three different mammalian cells treated exogenously with mutant hIIA-PLA<sub>2</sub>s was quantified. The results show that *in vitro* vesicle binding affinity rather than heparinoid binding affinity is a main determinant for controlling the rate of fatty acid release from intact cells treated exogenously with hIIA-PLA<sub>2</sub>. The data is discussed in terms of previous elegant studies of rodent group IIA PLA<sub>2</sub>-heparinoid binding including recent mutagenesis analysis of the mouse enzyme (31).

#### EXPERIMENTAL PROCEDURES

**Materials**—The following were purchased from commercial sources: guanidinium chloride (Amresco, catalog E424); restriction enzymes, T4 ligase, T4 polynucleotide kinase (New England Biolabs or Life Technologies, Inc.); isopropyl-β-D-thiogalactopyranoside (Boehringer Mannheim); oligonucleotides (Integrated DNA Technologies, Coralville, IA). DAUDA was purchased from Molecular Probes. All other reagents were purchased from Sigma.

**Construction of Mutant hIIA-PLA<sub>2</sub> Genes**—The synthetic gene for hIIA-PLA<sub>2</sub> (32) was subcloned into the pET-21a vector (Novagen) and

the construct designated as pYS (22). This synthetic gene carries the Asn to Ala mutation (N1A) at the amino terminus to facilitate the removal of the initiator Met by the endogenous methionine aminopeptidase (32). Mutagenesis was performed as described (22) using the following oligonucleotides: K100E, GGTAGCTGCTGCTCGTCGCATTC; R108E, GGTGGTGTCTCAGCGAAGCAG; R132E, CTATTAGCACTCGGGGTCGACCCACG; K124A/R127A, CGCGGGGTCGACCCAGCGCAGTGTGCGTTAGAGTAG. The mutant K53E/R54E/R58E was prepared first by introducing K58E with ACCGCAACCTTCTTCTCTAGACGTTTG followed by addition of K53E/R54E with CTCTTCTCGAGCTC TTCGTAGCAGCAG. The mutant K53E/R54E/K57E/R58E was prepared by addition of K57E with GCAACCTTCTTCTCGAGCTCTTC starting from K53E/R54E/R58E. The mutant K38E/R110E/K115E/K116E was prepared sequentially from K38E/K116E (22) using GGTATTCTTCGTTGTAGGTGGTTTCGTTACGAGCGAAG to introduce K110E/K115E. In the above DNA sequences, the underlined bases indicate the location of the charge-reversal mutations, and boldfaced bases indicate the location of silent mutations introduced to either eliminate or create a diagnostic restriction site. The single italicized base is a mutation introduced to remove a *Nci*I site, whose presence would interfere with mutagenesis. To make K38E/K124E/R127D, plasmids containing K38E and K124E/R127D (22) were digested with *Kpn*I and *Mlu*I, and the respective fragments were ligated using standard methods. Restriction fragments were purified by agarose electrophoresis followed by GeneClean (Bio 101, Inc.). The five-site mutant K53E/R54E/R58E/K124E/R127D was prepared by digesting plasmids containing K53E/R54E/R58E and K124E/R127D with *Nru*I and *Mlu*I, and ligating the respective fragments together. Mutant R7E/K10E/K16E/K124E/R127D was prepared by digesting R7E/K10E/K16E and K124E/R127D containing plasmids with *Kpn*I and *Hind*III, followed by ligating the fragments together. DNA sequences of the entire coding regions of all mutants were verified using a Sequenase 2.0 kit (Amersham) or by machine sequencing using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Individual recombinant pYS vectors were transformed into *Escherichia coli* strain BL21(DE3) (Novagen) for protein expression.

**Bacterial Protein Expression and Purification**—Most proteins were expressed, refolded, and purified as described (32), except K74E/K87E/R92E which was obtained by a modified procedure (22). The enzymatic activity of each mutant was measured using the fluorimetric assay with 1-palmitoyl-2-(10-pyrenedecanoyl)-sn-glycero-3-phosphomethanol or the pH stat assay with 1,2-dimyristoyl-sn-glycero-3-phosphomethanol (22, 33). Protein concentrations were obtained from the absorbance at 280 nm using A<sub>1%</sub> of 9 (20). Relative protein concentrations obtained from the absorbance at 280 nm were further confirmed by the Bradford protein assay (Bio-Rad) and by visual inspection of SDS-polyacrylamide electrophoresis gels stained with Coomassie Brilliant Blue.

All yields of purified single-site mutants are comparable to that obtained for N1A. In general, the yields roughly decreased as the number of charge-reversal mutations increased. The range of yields is 5 mg of purified protein per liter of culture for N1A to 0.3 mg/liter for K53E/R54E/R58E/K124E/R127D.

**Interaction of hIIA-PLA<sub>2</sub> with Heparinoids**—Purified N1A and mutant proteins (typically 25–30 μg) were analyzed for heparinoid binding using heparin-Sepharose (Pharmacia) and heparan sulfate-Sepharose. The latter was prepared by coupling heparan sulfate (Sigma catalog H7640) to cyanogen bromide-activated Sepharose 4B (Sigma catalog C9142) (34). Cold 1 mM HCl was added to 1 g of cyanogen bromide-activated Sepharose 4B, and the gel suspension was kept at 4 °C for 30 min. Gel was washed with 200 ml of 1 mM HCl followed by 50 ml of distilled water, and finally with 10 ml of 0.1 M NaHCO<sub>3</sub>. Immediately after the last wash, 3.7 mg of heparan sulfate in 1 ml of 0.1 M NaHCO<sub>3</sub> was added. Final volume was adjusted to 7 ml with 0.1 M NaHCO<sub>3</sub>. The reaction was mixed on a rocking platform at 4 °C for 16 h. Ethanolamine (0.4 ml) was added, and the gel was rocked for 4 h to block unreacted groups. Gel was washed consecutively with 50 ml of water, 30 ml of 0.5 M NaCl, and 150 ml of water. Gel was equilibrated in 20 mM Tris, pH 7.4. Gel treated in the same way but without heparan sulfate was used as a control.

Typically 25–30 μg of hIIA-PLA<sub>2</sub> was loaded onto a 1-ml column of heparin-Sepharose or heparan sulfate-Sepharose which was developed at 1 ml/min with a linear gradient (total volume 60 ml) of 0–0.6 M KCl in 20 mM Tris-HCl, pH 7.4, at 4 °C. Column fractions were assayed for hIIA-PLA<sub>2</sub> activity with 1-palmitoyl-2-(10-pyrenedecanoyl)-sn-glycero-3-phosphomethanol as described above.

**Binding of hIIA-PLA<sub>2</sub> to Anionic Vesicles**—Binding of hIIA-PLA<sub>2</sub> to anionic vesicles of polymerized 1,2-bis[12-(lipoyloxy)dodecanoyl]-sn-glycerol-3-phosphoglycerol was quantified as described (22).

<sup>2</sup> M. D. Bryant and M. H. Gelb, unpublished observations.

**Cell Culture and Fatty Acid Release Studies**—RAW 264.7 and NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium containing fetal bovine serum (10%), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and 2 mM glutamine. CHO-K1 cells (ATCC CRL-9618) and pgsD-677 cells (ATCC CRL-2244) were grown in F-12 medium containing fetal bovine serum (10%), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and 2 mM glutamine. pgsA-745 cells (ATCC CRL-2242) were grown in F-12K medium containing fetal bovine serum (10%), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and 2 mM glutamine. All cells were grown at 37 °C in a humidified atmosphere with 5%  $CO_2$ .

NIH-3T3 cells were grown to 70–90% confluence before harvesting. Cell culture medium was decanted, and cells were dislodged with non-enzymatic dissociation solution (Sigma catalog C5789). Cell suspension was mixed with an equal volume of calcium-containing Hanks' balanced salt solution (Life Technologies, Inc. catalog 14025-084). After pelleting, cells were resuspended in balanced salt solution. For RAW 264.7 cells, culture medium and floating cells were poured off. The cells were washed twice with balanced salt solution and resuspended by scraping into a one-third volume of balanced salt solution. The cells were then pelleted by centrifugation, and resuspended in 1 ml of balanced salt solution. CHO-K1, pgsA-745, and pgsD-677 cells were harvested with non-enzymatic dissociation solution, washed in balanced salt solution, and then resuspended in the same solution. Cell counts were performed in a hemocytometer in the presence of trypan blue to measure viability.

Fatty acid release by exogenously added *hIIa*-PLA $_2$  was measured using a continuous fluorescence displacement assay in which the released long chain fatty acid displaces the fluorescent probe DAUDA from recombinant rat liver fatty acid-binding protein (35). All experiments were carried with 1 ml of cell suspension in balanced salt solution containing 1 mM  $CaCl_2$  (see above). Whole cell fluorescence assays using RAW 264.7 cells have been described elsewhere (36). Briefly, a typical assay contained RAW 264.7 cells ( $4 \times 10^5$  per ml) or NIH-3T3 cells ( $2.5 \times 10^4$  per ml), or CHO-K1 cells or mutant cell lines ( $1.7 \times 10^5$  per ml), DAUDA (1  $\mu$ M), and fatty acid-binding protein (10  $\mu$ g) in balanced salt solution with 1 mM  $CaCl_2$  at 37 °C with magnetic stirring. The decrease in fluorescence (excitation 350 nm, emission at 500 nm) after addition of *hIIa*-PLA $_2$  to the cell suspension in the cuvette was recorded for 6–7 min, and the initial rate of release was obtained from the slope during the first minute. The rate of decrease was calibrated in terms of moles of fatty acids released by addition of a standard oleic acid solution to a cell suspension in the absence of *hIIa*-PLA $_2$ . In this system, a 50% decrease in fluorescence corresponds to 1–1.5 nmol/ml oleic acid. Amounts of each enzyme added were adjusted to provide measurable rates under conditions where the rate was proportional to the amount of enzyme. Typically, these ranged from 1  $\mu$ g/ml for N1A to 60  $\mu$ g/ml for some of the mutants.

The total phospholipid of the membrane that is available for enzymatic hydrolysis was assessed by measuring fatty acid release following hydrolysis by excess *Naja naja* PLA $_2$  (typically 2  $\mu$ g, Sigma catalog P6139) using the fluorescence displacement assay as above. Differing amounts of membrane were diluted into 1 ml of reaction mixture, and the hydrolysis was allowed to go to completion (2 min). Addition of more venom PLA $_2$  at the end of the reaction did not lead to more fatty acid release, but addition of oleic acid led to a further decrease in the fluorescence showing that sufficient fatty acid-binding protein was present to detect additional fatty acid.

**Radiometric Analysis of Arachidonate Release**—Wild type CHO-K1 cells were labeled with [ $^3H$ ]arachidonic acid (200 Ci/mmol, American Radiochemicals Inc. catalog ART 196) by incubating a 10-cm dish of ~50% confluent cells with 2  $\mu$ Ci in 10 ml of medium for 20–22 h. Medium was removed, and cells were washed twice with balanced salt solution containing 0.1% bovine serum albumin (Sigma catalog A-7906). Cells were dissociated as described above. For competition experiments, labeled cells ( $4 \times 10^5$ ) were mixed with various numbers of non-labeled wild type cells or proteoglycan-deficient cells (pgsA-745) in a total volume of 1 ml of balanced salt solution containing 0.1% albumin. N1A (10  $\mu$ g) was added, and the mixture was incubated at 37 °C for 15 min. Cells were quickly pelleted in a microcentrifuge, and 900  $\mu$ l of supernatant was submitted to scintillation counting. To the remaining 100  $\mu$ l, medium containing the cell pellet was added 1 ml of  $CHCl_3/MeOH$  (2/1). The tube was vortexed, the lower organic layer was transferred to a scintillation vial. Solvent was allowed to evaporate prior to scintillation counting. In this way, the amount of arachidonate release was expressed as a percentage of the total counts/min present in each sample.

## RESULTS

**Preparation and Kinetic Characterization of *hIIa*-PLA $_2$  Mutants**—A reasonable working hypothesis is that binding of *hIIa*-PLA $_2$  to heparinoids requires one or more basic residues on the surface of the enzyme. Since *hIIa*-PLA $_2$  contains 13 lysines, 10 arginines, and 2 histidines scattered over its surface, it is a formidable task to identify those that are involved in heparin binding. After visual inspection of the *hIIa*-PLA $_2$  x-ray structure (37), basic residues were grouped into clusters based on spatial proximity. In this way all 25 basic residues were grouped into 10 clusters (A–J) (Table I, Fig. 1). The numbering of all residues is based on the common numbering scheme for secreted PLA $_2$ s (37). Cluster I consists only of Lys-69, and this cluster was not considered further because the  $\epsilon$ -amino group of this residue directly hydrogen bonds to the *sn*-3 phosphate of active site-bound phospholipid substrate (37). Cluster A is a potential site for heparinoid binding. It is a large patch of positive charge, residues 53 and 57 lie on the same face of an  $\alpha$ -helix as do 54 and 58, it is certainly too far from the opening of the active site slot to be involved in interfacial binding (see Introduction), and it contains three residues, Arg-54, Lys-57, and Arg-58, that are well conserved among different species (Table I). Clusters B, D, E, and F also contain conserved basic residues. Cluster J was not considered further because only the human enzyme contains a cationic residue, a weakly basic histidine. Based on these considerations, single site charge-reversal mutants were made in those clusters containing 1–2 basic residues (clusters E and F), double or triple site mutations were made in those clusters containing 3 basic residues (B, C, D, G, and H), and triple and quadruple site mutations were made in cluster A, the largest cluster. All of the mutants are listed in Table II. The likelihood that each basic residue lies on the interfacial binding site of *hIIa*-PLA $_2$ , based on visual inspection of the x-ray structure, is listed in Table I.

The specific activity of all mutants was measured using enzymatic assays with anionic vesicles (see "Experimental Procedures") and are listed in Table II. Given that the specific activity relative to that of N1A for all mutants lie within the range 50–133%, it can be assumed that the mutants are properly folded, although local perturbation of structure by mutations is possible.

**Binding of *hIIa*-PLA $_2$  to Anionic Vesicles**—The binding of N1A and some of the charge-reversal mutants to polymerized anionic phosphatidylglycerol vesicles was studied previously (22), and binding data for remaining mutants were obtained in the present study using the identical protocol. Since phospholipids in these vesicles are cross-linked to each other via disulfide linkages between fatty acyl chains, it is impossible for the amphiphile in the interface to slide into the active site slot of bound *hIIa*-PLA $_2$ . In fact these polymerized phospholipids are not hydrolyzed by bound enzyme in the presence of calcium. Use of such vesicles allows the interfacial binding step to be quantified without contribution from the binding of a phospholipid molecule to the active site of interface bound *hIIa*-PLA $_2$  (38).

It is reasonable to propose that the interfacial binding site of secreted PLA $_2$ s including *hIIa*-PLA $_2$  lies somewhere on the enzyme surface that surrounds the opening to the active site slot (15, 39, 40). Recent studies using a new electron paramagnetic resonance technique has defined the interfacial binding site of bee venom PLA $_2$  as a portion of the enzyme's surface that surrounds the active site slot (14). In general, the anionic vesicle binding data for *hIIa*-PLA $_2$  fits well with the expected location of the interfacial binding site. Cluster C lies on the putative interfacial binding site closest to the active site slot opening, and the triple mutant R7E/K10E/K16E binds 290-fold

TABLE I  
Clusters of basic residues on the surface of hIIa-PLA2

Cluster name	Residues	Homology <sup>a</sup>	Interfacial binding <sup>b</sup>	Color <sup>c</sup>
A	Lys-53	N,K,D,—,—	NL	Green
	Arg-54	R,S,R,—,—	NL	
	Lys-57	K,K,K,—,—	NL	
	Arg-58	R,S,R,—,—	NL	
	Arg-132	S,K,S,S,—	NL	
B	Arg-34	R,K,K,R,K	?	Red
	Lys-124	K,M,G,N,—	?	
	Arg-127	K,K,R,S,—	?	
C	His-6	G,G,T,R,R	VL	Orange
	Arg-7	Q,E,E,K,K	VL	
	Lys-10	L,R,K,R,K	VL	
D	Lys-74	K,K,R,K,—	VL	Yellow-green
	Lys-87	N,N,K,—,—	VL	
	Arg-92	R,Q,Q,—,—	VL	
E	Arg-81	Q,Q,S,—,—	NL	Pink
	Lys-100	K,K,K,K,—	NL	
F	Arg-43	W,R,R,—,—	NL	Purple
	Arg-108	R,R,A,—,—	NL	
G	Lys-16	K,K,K,K,K	?	Cyan
	Lys-110	K,K,L,—,—	?	
	Lys-115	L,L,R,—,—	VL	
H	Lys-38	K,K,K,K,K	VL	Brown
	Lys-116	K,K,R,K,—	VL	
I	Lys-69	K,K,K,K,—	NL <sup>d</sup>	
J	His-125	F,F,L,R,—	VL	Blue-gray

<sup>a</sup> For each residue, the corresponding residue in the homologous group IIa enzyme is given in the following order: human (37), rat (58), mouse (59), guinea pig (60), rabbit (25), and pig (61). Only partial sequences are available for the rabbit and pig enzymes, and unknown residues are denoted by a dash.

<sup>b</sup> The interfacial binding site of hIIa-PLA2 is thought to be the planar surface that surrounds the active site slot. Residues on this surface close to the active site slot are considered very likely to be involved in interfacial binding and are designated VL. Those on the planar surface but further from the active site slot are designated by a question mark, and those residues not likely to be involved in interfacial binding are designated NL.

<sup>c</sup> The cluster colors are those used in Fig. 1.

<sup>d</sup> The  $\epsilon$ -amino group of Lys-69 binds directly to the *sn*-3 phosphate of substrate phospholipids (62) and thus is probably not involved in heparinoid binding.

weaker than N1A to anionic vesicles (Table II). Cluster D likely lies on the interfacial binding site, and the cluster D triple mutant K74E/K87E/R92E binds 200-fold weaker than N1A. Three residues of the quadruple mutant K38E/K110E/K115E/K116E (clusters G and H) are likely on the interfacial binding site, and this mutant binds 200-fold weaker to anionic vesicles than N1A. Cluster B appears to lie on the edge of the interfacial binding site, and the double mutant K124E/R127D binds 60-fold weaker than N1A to vesicles (the triple mutant R34E/K124E/R127D did not refold). The charge neutralization mutant K124A/R127A bound to anionic vesicles with an affinity intermediate between that of K124E/R127D and N1A, which is consistent with an electrostatic interaction role of these basic residues in supporting interfacial binding. The cluster A triple mutant K53E/R54E/R58E is on the side of the protein opposite the putative interfacial binding site, and this protein binds only 4-fold weaker than N1A to anionic vesicles. The reason for the slight reduction in membrane affinity is unknown, but the effect is very small in energetic terms. The five-site mutant K53E/R54E/R58E/K124E/R127D, as expected, binds with similar affinity as K124E/R127D, 90-fold *versus* 60-fold reductions, respectively. Of all of the mutants prepared, the five-site mutant R7E/K10E/K16E/K124E/R127D (clusters B and C) binds weakest to anionic vesicles (2,200-fold reduction). Single site mutations produce less significant effects on membrane binding (Table II). Membrane binding data for K100E and R108E

were not obtained because these residues are far from the putative interfacial binding site.

**Heparinoid Binding Studies**—Because heparin and hIIa-PLA2 associate in solution to form a high molecular weight aggregate (Ref. 30 and “Discussion”), no attempt was made to measure the dissociation equilibrium constant for the protein-heparin complex. Rather, the binding of hIIa-PLA2 mutants to heparin was assessed by monitoring the elution position of enzyme from a column of heparin-Sepharose developed with a linear salt gradient from 0 to 0.6 M KCl in 20 mM Tris-HCl, pH 7.4, at 4 °C. Data are summarized in Table II. As shown in Fig. 2, the elution position of N1A in separate runs is very reproducible, and there is complete separation of N1A from K124E/R127D or K53E/R54E/R58E/K124E/R127D when pairs of proteins are simultaneously loaded onto the column. In the presence of 2 mM CaCl<sub>2</sub> and 10 mM MgCl<sub>2</sub>, N1A eluted from heparin-Sepharose at 400 mM KCl, compared with 420 mM KCl in the absence of divalent metals, indicating that binding of hIIa-PLA2 to heparin is Ca<sup>2+</sup> and Mg<sup>2+</sup> independent. All single site mutants bind to heparin with affinities only marginally lower than that of N1A; the largest difference is for K38E which elutes at 370 mM KCl *versus* 420 mM KCl for N1A. It is clear that no single basic residue is critical for binding of hIIa-PLA2 to heparin.

Multisite mutants in which charge reversal has been introduced into clusters that are involved in interfacial binding,

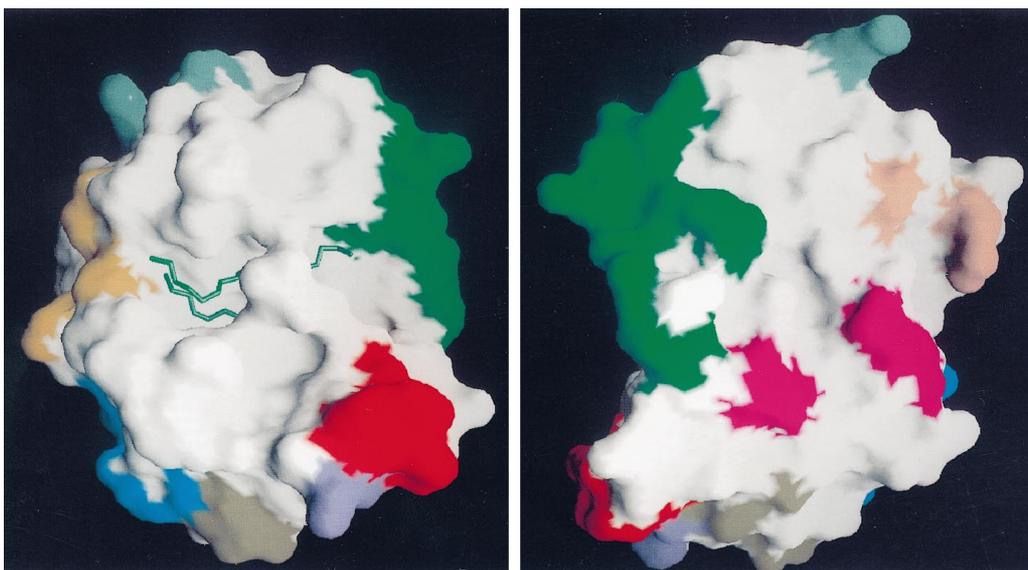


FIG. 1. Clusters of basic residues on the surface of hIIa-PLA<sub>2</sub>. The enzyme is oriented with its putative interfacial binding site toward the viewer (left) or away from the viewer (right). A short chain phospholipid analog inhibitor is shown in blue. The inhibitor is bound in the active site slot which extends all the way through the globular protein. Clusters of basic residues are as follows: cluster A (Lys-53, Arg-54, Lys-57, Arg-58, and Arg-132), green; cluster B (Arg-34, Lys-124, Arg-127), red; cluster C (His-6, Arg-7, Lys-10), orange; cluster D (Lys-74, Lys-87, Arg-92), yellow-green; cluster E (Arg-81, Lys-100), pink; cluster F (Arg-43, Arg-108), purple; cluster G (Lys-16, Lys-110, Lys-115), cyan; cluster H (Lys-38, Lys-116), brown; cluster J (His-125), blue-gray. The structure of the hIIa-PLA<sub>2</sub>-inhibitor complex is from Scott *et al.* (37).

R7E/K10E/K16E, K38E/K110E/K115E/K116E, K74E/K87E/R92E, and K124E/R127D elute from heparin-Sepharose at lower salt concentrations (360, 280, 340, and 340 mM KCl, respectively) than any of the single site mutants. K124A/R127A binds slightly tighter to heparin-Sepharose than K124E/R127D, which is expected for electrostatic binding to heparin. The five-site mutant R7E/K10E/K16E/K124E/R127D binds even weaker to heparin-Sepharose (240 mM KCl). These results show that basic residues on the interfacial binding face of hIIa-PLA<sub>2</sub> partially contribute to heparin binding.

Mutation of residues that are not involved in interfacial binding (K53E/R54E/R58E and K53E/R54E/K57E/R58E, cluster A) reduces heparin binding affinity (elution at 370 and 355 mM KCl, respectively). Combining cluster A and B mutations, both clusters lie on the same face of hIIa-PLA<sub>2</sub> (Fig. 1), that leads to further reduction in heparin affinity (255 mM KCl).

The binding of a subset of the hIIa-PLA<sub>2</sub> mutants to heparan sulfate-Sepharose was studied (Table II). In general the proteins bind weaker to heparan sulfate than to heparin. For example, N1A elutes at 420 mM KCl from heparin-Sepharose, but elutes at 240 mM KCl from heparan sulfate-Sepharose. This may be due to the lower sulfate content of heparan sulfate compared with heparin (28). In general, the trends seen with mutants binding to heparin sulfate-Sepharose are similar to the data obtained with heparin-Sepharose. As with heparin, it seems apparent from the data that hIIa-PLA<sub>2</sub> does not contain a well defined and localized cluster of basic residues that is critical for binding to heparan sulfate.

**Fatty Acid Release from Cells Treated with Exogenous hIIa-PLA<sub>2</sub>**—We have developed a continuous, fluorimetric PLA<sub>2</sub> assay for real-time monitoring of fatty acid release from cells treated with exogenous PLA<sub>2</sub> (35). In this assay, the fluorescent fatty acid analog DAUDA bound to rat liver fatty acid-binding protein becomes less fluorescent when it is dislodged into the aqueous phase by naturally occurring fatty acids derived from PLA<sub>2</sub>-catalyzed hydrolysis of membrane phospholipids. The sensitivity of this fluorimetric assay is comparable to that obtained with procedures in which cells are prelabeled with radioactive fatty acid, and it shows similar sensitivity for

saturated and unsaturated fatty acids including arachidonic acid.

N1A and a number of mutants were tested on three different mammalian cell lines. CHO-K1 cell fatty acid release kinetics were the same within experimental error using cells that were dislodged from the tissue culture dish with trypsin/EDTA or with a non-enzymatic dislodging solution (not shown). Thus, trypsin treatment does not destroy a cell surface component(s) that is required for hIIa-PLA<sub>2</sub>-catalyzed fatty acid release. Fig. 3A shows fatty acid release from CHO-K1 cells following the addition of increasing amounts of N1A. Fatty acid release in the absence of enzyme was negligible, and thus no background correction was necessary. As can be seen, the initial rate of fatty acid release is proportional to the amount of N1A added, and the rate decreases about 6-fold after the first few minutes. The reason for this decrease in rate is not known. In all of these experiments, 1 mM calcium was present in the extracellular fluid. In these experiments, the amount of hIIa-PLA<sub>2</sub>-catalyzed fatty acid release in the first 20 s is typically 1–2% of the maximum releasable fatty acid (defined as total nanomole of fatty acid released with excess cobra venom PLA<sub>2</sub>, typically  $\sim 3$  nmol/ $2.5 \times 10^4$  NIH-3T3 cells). Thus, in experiments with hIIa-PLA<sub>2</sub> the initial velocity is being measured and the membrane is left intact.

Fig. 3B shows representative fluorescence traces for fatty acid release from CHO-K1 cells treated with N1A and four hIIa-PLA<sub>2</sub> mutants. From these data, the specific activity for each protein was obtained, and values are expressed in Table III relative to the specific activity of N1A. Table III also gives relative specific activities for the hydrolysis of NIH-3T3, RAW 264.7, and mutant CHO-K1 cell membranes treated with hIIa-PLA<sub>2</sub> proteins.

Of all the mutants studied, R7E/K10E/K16E, K38E/K110E/K115E/K116E, and R7E/K10E/K16E/K124E/R127D have the lowest specific activities, 46-, 43-, and >200-fold lower than that of N1A, respectively, when hydrolyzing CHO-K1 cell membranes. The two mutants K38E/K116E and K110E/K115E that make up the four-site mutant show intermediate potency ( $\sim 10$ -fold lower than N1A). The two-site and five-site mutants,

TABLE II  
Kinetic and binding properties of hIIa-PLA2 to heparin-Sepharose and anionic vesicles

Mutant	Specific activity <i>in vitro</i> <sup>a</sup>	Anionic vesicle binding	Heparin-Sepharose binding <sup>b</sup>	Heparan sulfate- Sepharose binding <sup>c</sup>
	% versus N1A	Fold weaker than N1A	<i>mm KCl</i>	
Cluster A				
K53E	80		400 (420)	
R54E	96		390 (420)	
K57E	118		400 (420)	
R58E	87		390 (420)	
K53E/R54E/R58E	75	4 ± 2	370 (420)	
K53E/R54E/K57E/R58E	50		355 (420)	
Cluster B				
K124E/R127D	85	60 ± 20	340 (420)	190 (240)
K124A/R127A	60	18 ± 5	360 (420)	
Cluster C				
R7E	52 <sup>d</sup>	19 ± 8	385 (420)	
R7E/K10E	129 <sup>e</sup>	30 ± 15	370 (410)	
K10E/K16E	—	290 ± 50	360 (420)	190 (240)
R7E/K10E/K16E	92 <sup>e</sup>			
Cluster D				
K74E	133	3 ± 1	372 (420)	
K87E	109	1.3 ± 0.3	382 (420)	
R92E	124	3.3 ± 1	380 (420)	
K74E/K87E/R92E	116	200 ± 40	340 (420)	
Cluster E				
K100E	118		400 (420)	
Cluster F				
R108E	130		390 (420)	
Cluster G				
K16E	62 <sup>d</sup>	10 ± 3	390 (420)	
K110E/K115E	111	4 ± 1	350 (410)	195 (240)
Cluster H				
K38E	107		370 (420)	
K38E/K116E	103	10 ± 3	320 (420)	180 (240)
Mixed clusters				
K38E/K124E/R127D	64		300 (420)	
K38E/K110E/K115E/K116E	70	200 ± 30	280 (420)	
K53E/R54E/R58E/K124E/R127D	60	90 ± 20	255 (420)	150 (240)
R7E/K10E/K16E/K124E/R127D	108 <sup>b</sup>	2,200 ± 500	240 (420)	

<sup>a</sup> Enzymatic activity was determined using 1-palmitoyl-2-(10-pyrenedecanoyl)-sn-glycero-3-phosphomethanol, and protein concentration was determined from the absorbance at 280 nm and by two other methods (see "Experimental Procedures"). Error in the numbers is ±15%.

<sup>b</sup> Measured with 10 mM 1-palmitoyl-2-(10-pyrenedecanoyl)-sn-glycero-3-phosphomethanol.

<sup>c</sup> The heparin- and heparan sulfate-Sepharose column (1 ml) elution position of hIIa-PLA2 is given as the concentration of KCl in the gradient when the protein emerges from the column. The corresponding values for N1A measured on the same day as the mutant are given in parentheses. Error in the numbers is ±5 mM. Error in the numbers is ±20%.

<sup>d</sup> Activity measured with anionic polymerized phosphatidylglycerol vesicles (22).

<sup>e</sup> Activity measured with unilamellar vesicles of the anionic 1,2-dimyristoyl-sn-glycero-3-phosphomethanol (22).

K124E/R127D and K53E/R54E/R58E/K124E/R127D, are 14- and 9-fold, respectively, less effective than N1A at releasing fatty acids from CHO-K1 cells. The mutant K124A/R127A shows a specific activity on cells that is intermediate between those of the corresponding charge-reversal mutant and N1A. The three-site mutant K53E/R54E/R58E hydrolyzes CHO-K1 cell membranes at about the same rate as does N1A. Trends seen in the fatty acid release rates with NIH-3T3 and RAW 264.7 cells are similar to those seen with CHO-K1 cells (Table III).

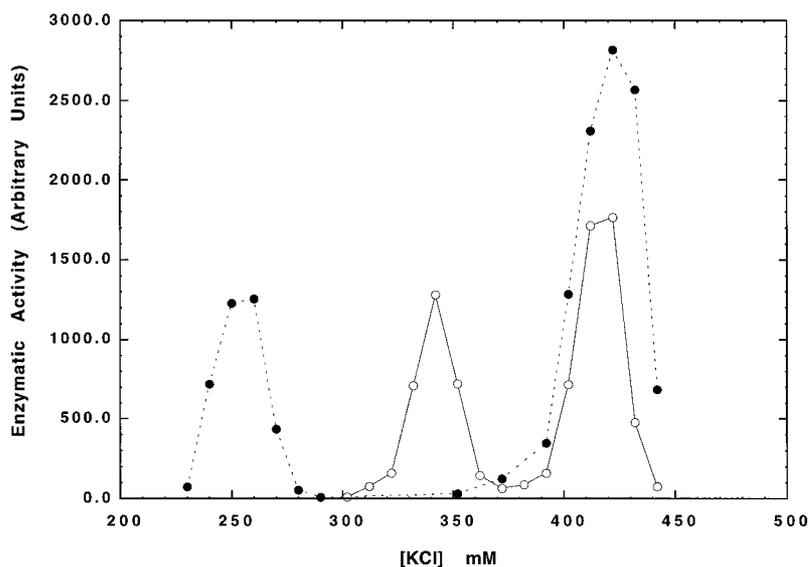
hIIa-PLA2-catalyzed fatty acid release from mutant CHO-K1 cell lines pgsD-677 and pgsA-745 that lack heparan sulfate and glycosaminoglycans, respectively (see "Discussion") was also studied. For N1A and each mutant studied, there was no statistically significant difference in the rate of fatty acid release from wild type *versus* mutant cell lines (Table III). Although this data suggests that proteoglycan is not required for plasma membrane fatty acid release by exogenously added hIIa-PLA2, it is possible that the dissociation constants for the

complexes of enzyme with these cells lines actually differ in magnitude, but that such differences are not manifested in our studies because the concentration of cell membranes exceeds the values of the dissociation constants. In this case hIIa-PLA2 would be fully bound to WT and mutant CHO-K1 cells. To explore this possibility, experiments were carried out in which hIIa-PLA2-catalyzed fatty acid release from wild type CHO-K1 cells prelabeled with [<sup>3</sup>H]arachidonic acid was measured in the absence and presence of various numbers of non-labeled competitor cells. As shown in Fig. 4, non-labeled wild type and proteoglycan deficient (pgsA-745) cells were equally efficient at blocking [<sup>3</sup>H]arachidonate release from wild type radiolabeled CHO-K1 cells. Thus it can be concluded that the dissociation constants for hIIa-PLA2 interacting with wild type and mutant CHO-K1 cells are the same.

#### DISCUSSION

*Diffuse Heparinoid-binding Sites of hIIa-PLA2*—Systematic mutation of at least one basic residue (lysine or arginine) in

FIG. 2. Salt gradient elution of N1A and mutants from heparin-Sepharose. Experiments were performed with a column of 1 ml of heparin-Sepharose as described under "Experimental Procedures." The solid line with open circles is N1A co-injected with K124E/R127D, and the dashed line with filled circles is N1A co-injected with K53E/R54E/R58E/K124E/R127D.



each of the basic clusters of hIIa-PLA<sub>2</sub> (Table I, Fig. 1) coupled with heparin and heparan sulfate binding studies lead to the following conclusions. No single basic cluster is critical for binding to sulfated glycosaminoglycans. It is apparent that a number of cationic clusters on hIIa-PLA<sub>2</sub> make a significant contribution to heparanoid binding. Basic residues that are involved in interfacial binding as well as those that are not on the interfacial binding surface of hIIa-PLA<sub>2</sub> contribute to heparanoid binding. The introduction of multiple charge-reversal mutations is required to produce a significant reduction in heparanoid binding. Multi-site, charge-reversal mutants still bind to heparin and heparan sulfate at low ionic strength, which is perhaps not surprising given that these mutants still have significant net positive charge. Considering only the number of lysines, arginines, aspartates, and glutamates and a bound  $Ca^{2+}$ , the four- and five-site mutants have a net positive charge of +9 and +7, respectively, compared with +17 for N1A.

Murakami *et al.* (31) have recently studied heparin binding of charge-reversal mutants of mouse group IIa PLA<sub>2</sub>. Rather than systematically mutating basic residues in all clusters, they focused on charge-reversal mutation of basic residues that are most conserved among group IIa PLA<sub>2</sub> sequences. Murakami *et al.* (31) designate their weakest heparin binding mutants as KE3 (three-site) and KE4 (four-site), and both of these proteins elute from heparin-Sepharose around 0.23 M NaCl compared with the elution of wild type mouse enzyme at 0.65 M salt. These changes in heparin affinity are similar to what was observed for the four- and five-site hIIa-PLA<sub>2</sub> mutants (Table II). As shown in Fig. 6, KE3 mouse PLA<sub>2</sub> probably corresponds to K53E/R54E/R58E/K124E/R127D hIIa-PLA<sub>2</sub> except that the mouse mutant contains only 1 mutation in cluster A. KE4 mouse PLA<sub>2</sub> contains two mutations in the same region as residues 110, 115, and 116 that were mutated in our four-site mutant (Fig. 6). The other two mutations in KE4 correspond to our 124- and 127-site mutations. The two-site mouse mutant (KE2) corresponds to our K124E/R127D mutant, and both bind only slightly weaker to heparin-Sepharose than do the respective wild type enzymes (Ref. 31 and Table II).

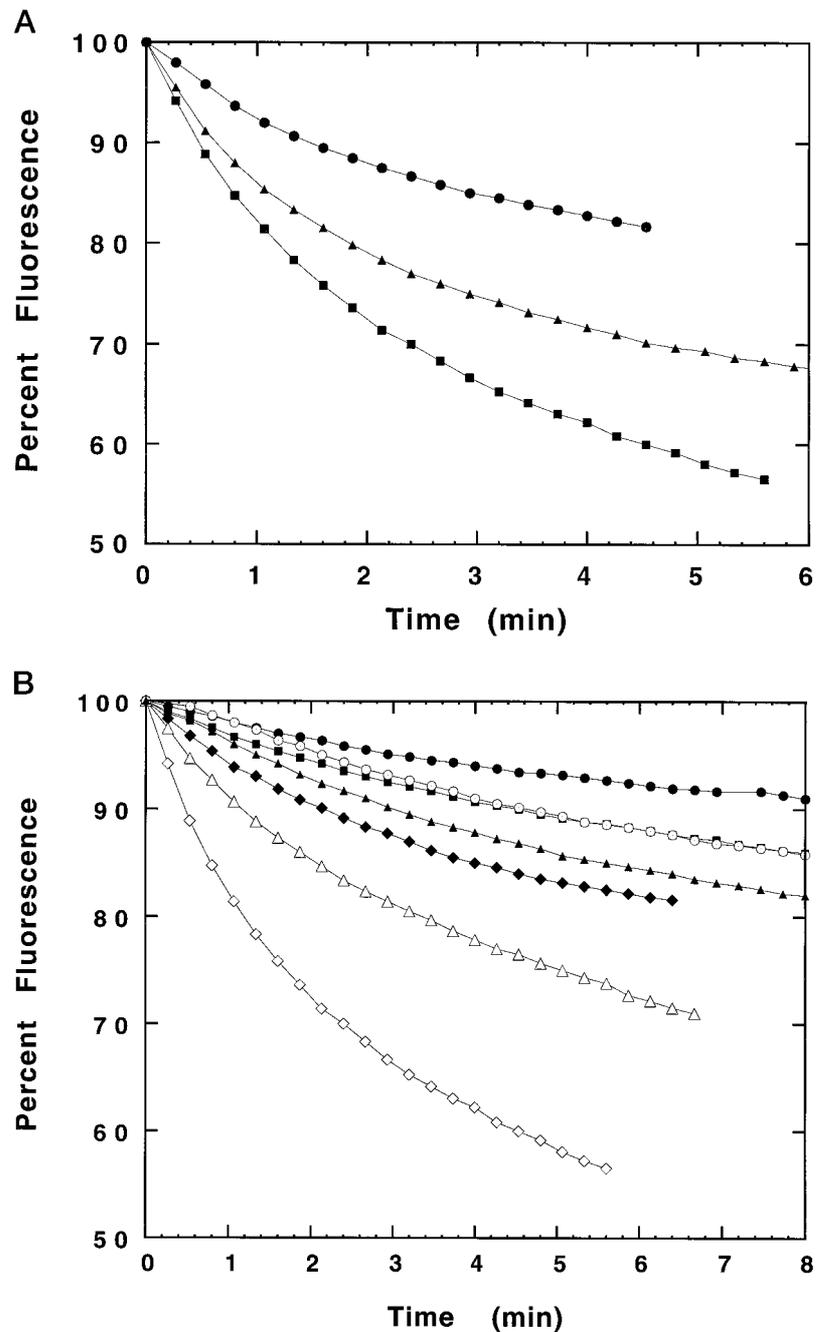
It may be noted that proteins that are known to have localized heparin-binding regions elute from heparin-Sepharose at much higher salt concentrations than does hIIa-PLA<sub>2</sub>. Examples include antithrombin III (elutes at 2.2 M NaCl) (41), basic fibroblast growth factor (elutes at 1.4 M NaCl) (42), and lipoprotein lipase (elutes at 1.0–1.2 M NaCl) (28) versus hIIa-PLA<sub>2</sub> (0.42 M KCl, Table II) and mouse enzyme (0.65 M salt) (31). For

these former three proteins, mutation of even single basic residues to alanine results in severe reduction in heparin binding (41, 42, 43), which is in marked contrast with the present mutagenesis studies of hIIa-PLA<sub>2</sub>. All together, these results indicate that hIIa-PLA<sub>2</sub> does not display features typical of a classical heparinoid-binding protein. hIIa-PLA<sub>2</sub> binds weaker to heparan sulfate-Sepharose (elution at 0.24 M KCl, Table II) than to heparin-Sepharose.

*Binding of hIIa-PLA<sub>2</sub> Mutants to Anionic Vesicles*—The affinity of hIIa-PLA<sub>2</sub> mutants for anionic, polymerized phospholipid vesicles was determined by measuring the amount of enzymatic activity in supernatants after vesicles were pelleted by ultracentrifugation (22) (Table II). After a systematic and thorough study, it has not been possible to find a hIIa-PLA<sub>2</sub> mutant that binds weakly to heparinoids but retains affinity for anionic vesicles similar to that of N1A (Table II). Evidence that the heparinoid-binding site(s) is distinct from the interfacial binding site comes from studies with monoclonal antibodies (29). Monoclonal antibodies MD7.1 and ME6.1 prevent the binding of rat group IIa PLA<sub>2</sub> to heparin but do not inhibit the catalytic activity of the enzyme when assayed with phosphatidylserine vesicles. Monoclonal antibody MB5.2 neutralizes the catalytic activity of the rat enzyme without affecting heparin binding. Although these results are consistent with the notion that rat group IIa PLA<sub>2</sub> has a well defined heparin-binding site that is distinct from its interfacial binding site, studies with antibodies provide much lower resolution structural information than do studies with point mutants. For example, it is likely that MD7.1 or ME6.1 bind only to a subset of the basic residues that are involved in heparin binding. This may be sufficient to weaken heparin binding but may not prevent vesicle binding and catalysis. We favor the evidence from the present mutagenesis studies of hIIa-PLA<sub>2</sub> showing that basic residues that are or are not involved in interfacial binding contribute to heparanoid binding.

It has been reported that solution-phase heparin inhibits the hydrolysis of vesicles by hIIa-PLA<sub>2</sub> (21, 30, 44). As noted by Yu *et al.* such inhibition does not require binding of heparin only to the interfacial binding site. Rather, detailed studies show that heparin forms a high molecular weight aggregate with porcine pancreatic PLA<sub>2</sub> and with hIIa-PLA<sub>2</sub> that disassembles upon the addition of 0.5 M NaCl. Enzyme in this aggregate is not active toward the hydrolysis of anionic vesicle substrate but shows measurable but reduced activity on micellar 1,2-dihexanoyl phosphatidylcholine substrate. We have confirmed these

**FIG. 3. Fatty acid release from CHO-K1 cells treated exogenously with hIIa-PLA2.** A, CHO-K1 cells were treated with 3  $\mu\text{g}$  (filled circles), 6  $\mu\text{g}$  (filled triangles), and 12  $\mu\text{g}$  (filled squares) of hIIa-PLA2, and the decrease in fluorescence was followed (see "Experimental Procedures" for additional details); B, same as A but with 6  $\mu\text{g}$  of K124E/R127D (filled circles), 30  $\mu\text{g}$  of R7E/K10E/K16E (filled squares), 6  $\mu\text{g}$  of K53E/R54E/R58E/K124E/R127D (open circles), 60  $\mu\text{g}$  of R7E/K10E/K16E (filled triangles), 6  $\mu\text{g}$  of K124A/R127A (filled diamonds), 30  $\mu\text{g}$  of K53E/R54E/R58E/K124E/R127D (open triangles), 12  $\mu\text{g}$  of N1A (open diamonds). In these assays, a 50% reduction in fluorescence represents 1.0 nmol of oleic acid as determined by adding a known amount of oleic acid to the cell suspension in the absence of hIIa-PLA2. The assay is continuous, and plotted points were read from the strip-chart recorder.



observations. The data strongly suggest that micelles but not vesicles are able to penetrate into the heparin-enzyme aggregate to reach the entrapped enzyme. These kinetic studies are consistent with the present results showing that heparin can bind to multiple faces of hIIa-PLA2.

Whereas the overall depletion of positive charge appears to be a dominant parameter for heparinoid binding rather than deletion of a specific basic cluster, binding to anionic vesicles is reduced by charge-reversal mutation of basic residues that lie on a well defined surface of hIIa-PLA2, that which surrounds the opening to the active site slot (Fig. 1).

**Basic Clusters of the Interfacial Binding Site Are Most Important for Hydrolysis of Cellular Phospholipids by Exogenously Added hIIa-PLA2**—As shown in Fig. 5A, the release of fatty acids from mammalian cells treated exogenously with N1A and a panel of hIIa-PLA2 mutants correlates well with the affinity of these proteins for anionic vesicles measured *in vitro*. This correlation argues that the surface of hIIa-PLA2 that

supports catalytically productive interfacial binding to vesicles *in vitro* is the same that allows interfacial catalysis on the plasma membrane of living cells. In contrast, cellular fatty acid release by exogenously added hIIa-PLA2 is not correlated with heparanoid affinity (Fig. 5B). For example, mutation of cluster A (K53E/R54E/R58E) clearly decreases heparin binding, marginally affects anionic vesicle binding (as expected since cluster A is far from the putative membrane binding surface), and marginally affects hydrolysis of cell membranes. The two-site and five-site mutants K124E/R127D and K53E/R54E/R58E/K124E/R127D show similar and intermediate affinity for anionic vesicles and hydrolyze cellular phospholipids with intermediate efficacy despite the fact that the five-site mutant binds much weaker to heparinoids than does the two-site mutant. The similar membrane binding affinity of these two-site and five-site mutants makes sense since membrane binding is controlled by K124/R127 and not by K53/R54/R58 (Table II, Fig. 1). The relative behavior of the mutants on CHO-K1,

TABLE III  
Hydrolysis of mammalian cell plasma membrane phospholipids by exogenously added hIIa-PLA<sub>2</sub>s

Mutant	CHO-K1 fatty acid release; wild type, proteoglycan def., heparan sulfate def.	NIH-3T3 cell fatty acid release	RAW 264.7 cell fatty acid release
<i>Fold slower than N1A<sup>a</sup></i>			
Cluster A K53E/R54E/R58E	0.9,0.8,1.0	0.8	0.6
Cluster B K124E/R127D K124A/R127A	14,24,23 3,4.5,4	30 8	14 3
Cluster C R7E/K10E R7E/K10E/K16E	46,64,50	85	10 70
Cluster D K74E/K87E/R92E			16
Cluster G K110E/K115E	9,14,—	11	
Cluster H K38E/K116E	10,14,—	17	8
Mixed Clusters K38E/K110E/K115E/K116E K53E/R54E/R58E/K124E/R127D R7E/K10E/K16E/K124E/R127D	43,—,— 9,10,10 >200 <sup>b</sup> ,—,—	16	53 9 >200 <sup>b</sup>

<sup>a</sup> Error in the numbers is  $\pm 20\%$ .

<sup>b</sup> No fatty acid release seen with 100  $\mu\text{g}$  of this mutant.

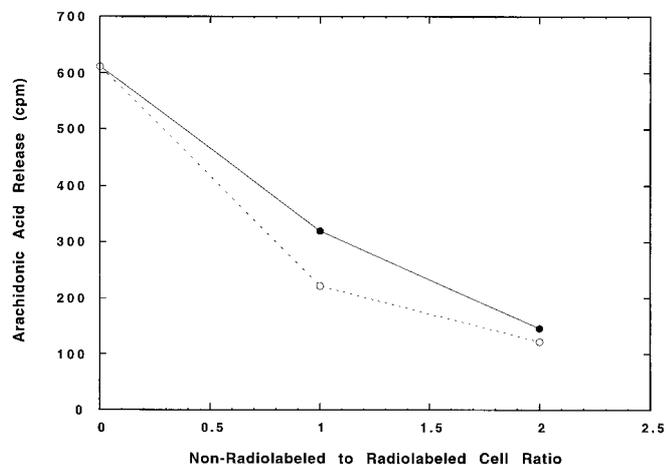


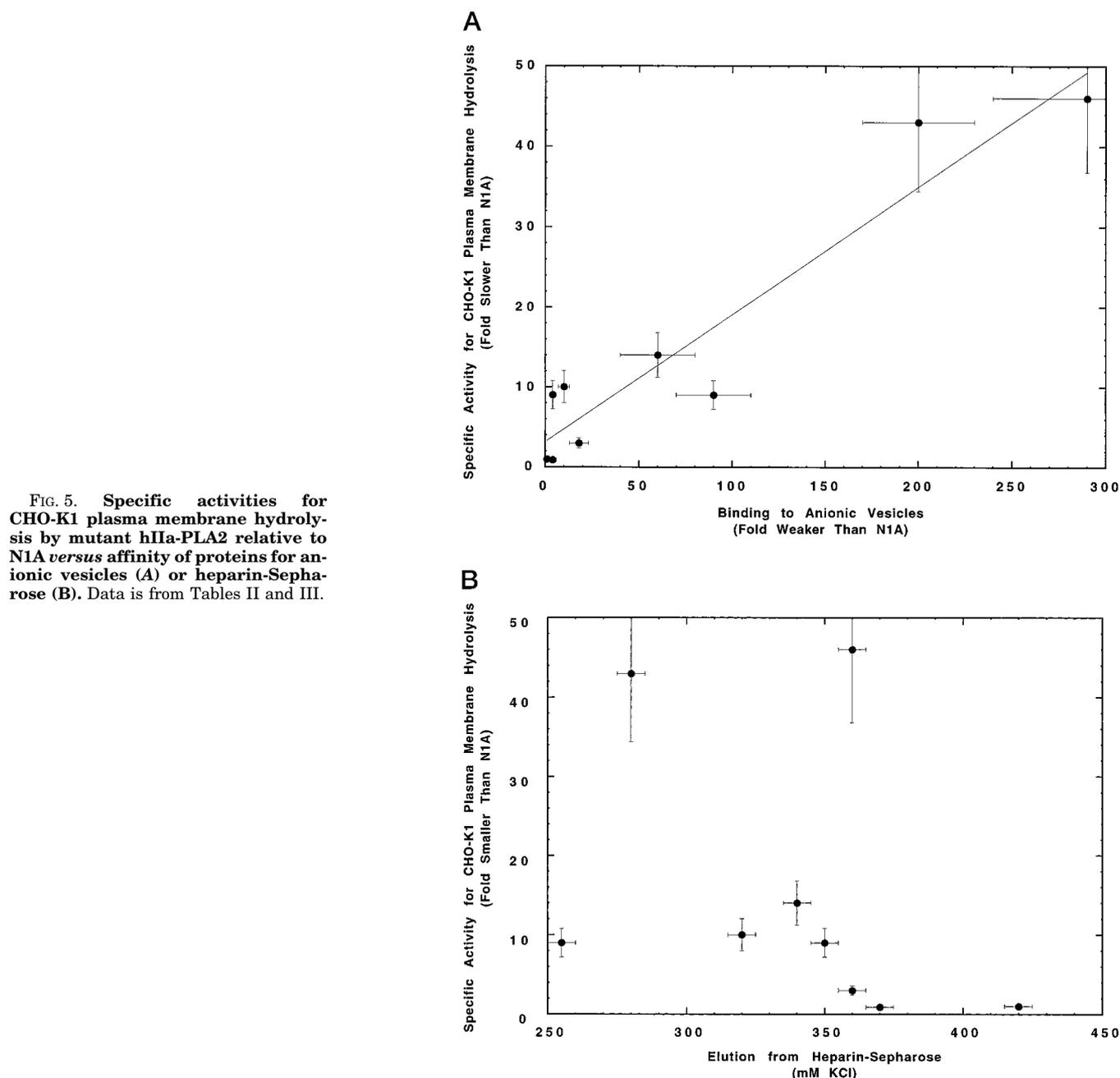
FIG. 4. **Competitive action of hIIa-PLA<sub>2</sub> on radiolabeled and non-labeled CHO cells.** Wild type CHO-K1 cells were prelabeled with [<sup>3</sup>H]arachidonic acid, washed to remove non-esterified fatty acid, and mixed with non-radiolabeled wild type CHO-K1 cells (filled circles, solid line) or non-labeled pgsA-745 CHO cells (open circles, dotted line) at the indicated cell ratio. hIIa-PLA<sub>2</sub> was added last, and the amount of released [<sup>3</sup>H]arachidonic acid was quantified as described under "Experimental Procedures."

NIH-3T3, and RAW 264.7 cells are very similar (Table III). Overall these data strongly suggest that a functional interfacial binding site rather than heparinoid affinity is most important for fatty acid release from cells treated exogenously with hIIa-PLA<sub>2</sub>.

The lack of importance of cell surface heparan sulfate for fatty acid release from mammalian cells treated exogenously with hIIa-PLA<sub>2</sub> is also suggested by comparative studies with wild type and mutant CHO-K1 cells (Table III). The mutant cell line pgsA-745 has a dysfunctional xylosyl transferase. This enzyme puts the  $\beta$ -xyloside residue into the linker that connects the heparinoid to the serine residue of the core protein component of proteoglycans. Radiolabeling studies with

[<sup>35</sup>S]sulfate demonstrate that pgsA-745 cells contain <5% glycosaminoglycan bound to core proteins of proteoglycans (45). Thus these cells have virtually no cell surface heparan sulfate or other glycosaminoglycans. pgsD-677 cells lack both *N*-acetylglucosaminyltransferase and glucuronosyltransferase activity required for biosynthesis of heparan sulfate. These cells contain no heparan sulfate but do express chondroitin sulfate-containing proteoglycan (46). Previously, it has been shown that chondroitin sulfate binds much poorer to group IIa PLA<sub>2</sub> than does heparin and heparan sulfate (26). The results in Table III and Fig. 4 showing that the relative efficiency of fatty acid release catalyzed by N1A and hIIa-PLA<sub>2</sub> mutants is invariant to the cell line used (CHO-K1, pgsA-745, and pgsD-677) strongly argues that cell surface proteoglycans are not important for cell surface lipolysis by exogenous hIIa-PLA<sub>2</sub>.

Recently, Kudo and co-workers have proposed that binding of mouse group IIa PLA<sub>2</sub> to cell surface heparan sulfate augments stimulus-initiated fatty acid release from CHO-K1 cells stably transfected with wild type and mutant mouse group IIa PLA<sub>2</sub> genes and from COS-7 cells transiently transfected with these constructs (31). Mouse group IIa PLA<sub>2</sub> production was initiated by the addition of serum-containing medium, which leads to slow arachidonate release over several hours. For some experiments, interleukin-1 $\beta$  was also added since it augments arachidonate release about 3–4-fold. Their conclusion that binding of enzyme to cell surface heparan sulfate is important for fatty acid release is based on the finding that CHO-K1 cells transfected with mouse group IIa PLA<sub>2</sub> mutants that bind weakly to heparin (KE3 and KE4, see above) show reduced fatty acid production. They did not measure the binding constants for the interaction of their mutants to vesicles *in vitro*. Instead, they reported only that the catalytic activities of their mutants acting on 2  $\mu\text{M}$  1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine were similar. However, this does not imply that the enzymes bind to membranes with the same affinity. We have shown that N1A binds tightly to anionic vesicles (equilibrium dissociation constant,  $K_d$ ,  $\sim 10^{-9}$  M (22)),



and thus with micromolar amounts of substrate, even a mutant that binds several hundredfold weaker to vesicles than N1A will be mostly bound to the interface since the concentration of substrate phospholipid is greater than  $K_d$ . Indeed, all of our charge-reversal mutants show similar catalytic activity when assayed with  $1.3 \mu\text{M}$  1-palmitoyl-2-(10-pyrenedecanoyl)-sn-glycero-3-phosphomethanol even though their values of interfacial  $K_d$  values differ by as much as 290-fold (Table II). Only R7E/K10E/K16E/K124E/R127D, which binds 2,200-fold weaker to vesicles than N1A, requires high substrate concentration,  $10 \mu\text{M}$ , to see full activity. However, as expected, at lower substrate concentration,  $0.1 \mu\text{M}$ , mutants including R7E/K10E/K16E and R7E/K10E/K16E/K124E/R127D that bind weakest to vesicles display <10% of the specific activity of N1A (not shown). Mouse group IIa PLA<sub>2</sub> proteins KE2, KE3, and KE4 contain the mutations K119E and K121E (31), and these appear to be analogous to hIIa-PLA<sub>2</sub> residues Lys-124 and Arg-127 (Fig. 6). Since the two-site hIIa-PLA<sub>2</sub> mutant K124E/

R127D binds 60-fold weaker than N1A to vesicles *in vitro*, one cannot ignore the possibility that the homologous two-site mouse mutant (KE2) displays interfacial binding that is weaker than native mouse enzyme. Thus, it is difficult to know whether reduced arachidonic acid release in cells transfected with mouse group IIa PLA<sub>2</sub> mutants KE3 and KE4 is due to reduced heparanoid binding *versus* reduced membrane binding.

A major difference between our studies and those of Murakami *et al.* (31) is that we added hIIa-PLA<sub>2</sub> exogenously to cells, whereas the previous studies involved mouse enzyme secreted from stably transfected CHO-K1 cells or transiently transfected COS-7 cells. The mechanism of fatty acid release by exogenously added *versus* secreted group IIa PLA<sub>2</sub> may be different. In our studies, measurable fatty acid release over 1 min requires the addition of  $\sim 1 \mu\text{g}$  of N1A to  $1.75 \times 10^5$  CHO-K1 cells per ml (Fig. 3A). Murakami *et al.* (31) estimate that the transfected CHO-K1 cells produce  $\sim 1$  ng mouse group

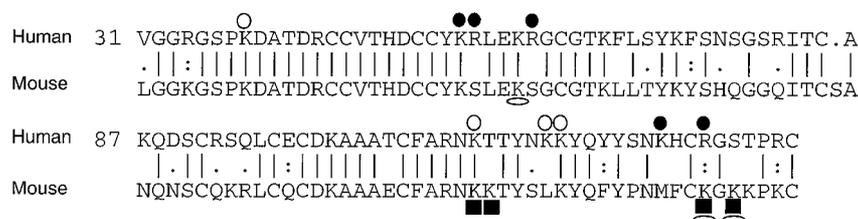


Fig. 6. Heparinoid-binding regions of hIIA-PLA<sub>2</sub> and mouse group IIA PLA<sub>2</sub>. The amino acid sequences of the heparinoid-binding regions of hIIA-PLA<sub>2</sub> and mouse group IIA PLA<sub>2</sub> have been aligned. The secreted PLA<sub>2</sub> common numbering scheme is used (37). The weakest heparinoid binding hIIA-PLA<sub>2</sub> mutants K53E/R54E/R58E/K124E/R127D and K38E/K110E/K115/K116E are shown by filled and open circles, respectively. Mouse group IIA PLA<sub>2</sub> mutants KE3 is shown by open ovals, and KE4 is shown by filled squares (31).

IIA PLA<sub>2</sub> per 10<sup>6</sup> cells. This difference in amount of PLA<sub>2</sub> used in the two studies is not due to the use of the fluorimetric assay since it has already been mentioned that this assay is virtually as sensitive as measurements made with cells labeled with [<sup>3</sup>H]arachidonic acid (~50,000 cpm/10<sup>6</sup> cells) (compare Figs. 3 and 4). The difference is actually due to that fact that in our studies we measure fatty acid release over a few minutes, whereas Murakami *et al.* (31) measured it over 600 min. This argues that the turnover numbers of exogenously added hIIA-PLA<sub>2</sub> acting on CHO-K1 cells and mouse group IIA PLA<sub>2</sub> expressed in transfected CHO-K1 cells are similar. The turnover number for hIIA-PLA<sub>2</sub>-catalyzed release of fatty acids from 10<sup>6</sup> CHO-K1 cells/ml is 0.07 s<sup>-1</sup> (Fig. 3A). The turnover number for the mouse group IIA PLA<sub>2</sub> acting on 10<sup>6</sup> CHO-K1 cells/ml can be estimated to be ~0.1 s<sup>-1</sup> based on the following. Murakami *et al.* find under conditions which give maximal arachidonate release (addition of serum and interleukin-1β) that 4% of the cell-incorporated radiolabeled arachidonate is released over 600 min by ~1 ng of mouse group IIA PLA<sub>2</sub> from 10<sup>6</sup> CHO-K1 cells/ml. A suspension of 10<sup>6</sup> mammalian cells/ml contains ~1 μM outer monolayer plasma membrane phospholipid or about 0.1 μM arachidonyl phospholipid. Since cells were labeled for many hours with [<sup>3</sup>H]arachidonic acid, the specific radioactivity of arachidonate phospholipids is probably nearly constant in all cell membranes. Assuming ~5% of the total cellular arachidonate is in plasma membranes, 4% of the radiolabel release corresponds to ~0.16 nmol released in 600 min by 1 ng of mouse group IIA PLA<sub>2</sub> assuming that most of the liberated arachidonate is released to the medium, which is reasonable in the presence of serum albumin (47). When mast cells are treated exogenously with hIIA-PLA<sub>2</sub>, ~50% of the total fatty acids released is arachidonic acid (48). Thus, the estimated turnover number is ~0.13 s<sup>-1</sup>. This estimate seems reasonable since Murakami *et al.* (31) showed that ~0.007 nmol of prostaglandin E<sub>2</sub> is produced in 600 min from 10<sup>6</sup> CHO-K1 cells, which represents 10% of the total moles of released arachidonate.

These turnover numbers for the action of group IIA PLA<sub>2</sub> on mammalian cell membranes are very low. The *in vitro* turnover number for hIIA-PLA<sub>2</sub> fully bound to anionic vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol is 45 s<sup>-1</sup> (20) and it is 25 s<sup>-1</sup> for rat group IIA PLA<sub>2</sub> fully bound to phosphatidylethanolamine vesicles (49). Thus, it seems clear that the specific activity of 0.07 s<sup>-1</sup> for the action of group IIA PLA<sub>2</sub> on mammalian cell membranes is due to the fact that most of the enzyme does not have its interfacial binding site in direct contact with membrane phospholipid. This is not due to poor accessibility of cellular membrane phospholipid since the specific activity for *N. naja* PLA<sub>2</sub> acting on these cells is 30 s<sup>-1</sup> (not shown), which is 400-fold higher than that for hIIA-PLA<sub>2</sub> (the specific activities of cobra venom and group IIA PLA<sub>2</sub> when fully bound to vesicles *in vitro* are similar). All of these numbers are reasonable when one considers that although group IIA and cobra venom PLA<sub>2</sub> bind with high affinity to anionic

vesicles (19, 20), hIIA-PLA<sub>2</sub> binds very weakly to phosphatidylcholine vesicles compared with the cobra venom enzyme (20, 50–52) and that the outer face of the plasma membrane is poor in acidic phospholipids. Murakami *et al.* (31) show that about 50% of the mouse group IIA PLA<sub>2</sub> released from transfected COS-7 cells is associated with the outer surface of the cells. Based on the fact that most of this bound enzyme is not attached directly to the membrane interface, it seems likely that it is bound via a non-lipid component. The observation that degradation of cell surface heparanoids with heparitinases leads to enhanced release of group IIA PLA<sub>2</sub> into the culture medium (26) strongly suggests that extracellular group IIA PLA<sub>2</sub> is bound to heparan sulfate. Based on the results of the current study, one can conclude that anchoring of exogenously added hIIA-PLA<sub>2</sub> to cell surfaces via heparan sulfate does not augment hydrolysis of phospholipids on the extracellular face of the plasma membrane.

Altogether, the results at hand underscore the possibility that the production of anionic components in the membrane bilayer could greatly activate interfacial catalysis by group IIA PLA<sub>2</sub> since interfacial binding of this enzyme has a strict requirement for anionic interfaces (20, 51, 53). In fact the recent demonstration that cells undergoing apoptosis are more susceptible to membrane hydrolysis by rodent group IIA PLA<sub>2</sub> (54) and the fact that the anionic lipid phosphatidylserine moves to the extracellular face of the plasma membrane during apoptosis (55) is consistent with a membrane component-dependent augmentation of group IIA PLA<sub>2</sub> catalysis. Interestingly mouse group IIA PLA<sub>2</sub> mutant KE4 was as potent as wild type in eliciting arachidonate released from apoptotic cells, and it was suggested that heparan sulfate binding is not required for PLA<sub>2</sub> activity on apoptotic cells (54). A likely explanation in light of the present results is as follows. Even though one expects the membrane binding of KE4 to be perturbed (see above), it is likely that the dissociation constant for the complex of membrane with wild type and KE4 enzymes are both below the concentration of cell membrane lipid used in the studies with apoptotic cells since binding of group IIA PLA<sub>2</sub> to anionic membranes is very tight ( $K_d \sim 10^{-9}$  M, see above). Recall that the specific activity of N1A and all the mutants in Table II acting on 1.3 μM anionic vesicles are very similar despite the fact that affinity for anionic vesicles spans a large range. Thus one would expect the specific activity of wild type and KE4 PLA<sub>2</sub> to approach each other as the amount of anionic membrane in the outer plasma membrane increases.

It is thought that hydrolytic enzymes including proteases are bound to heparin in high molecular weight aggregates in secretory granules of mast cells (28). Such complexation may serve to prevent degradation of granule proteins and autoprolysis. Following degranulation, the protein-heparin complex presumably dissociates due to dilution in the extracellular fluid. It is possible that heparin-induced aggregation of hIIA-PLA<sub>2</sub> (30) protects the granule membrane from degradation. Since group IIA PLA<sub>2</sub> binds much weaker to heparin than do

classical heparin-binding proteins (see above), release of enzyme from the aggregate may occur following degranulation. It has already been mentioned above that high molecular weight heparin-hIIa-PLA2 complex formation is a reversible process (30).

The physiological role of hIIa-PLA2 remains obscure but as an acute-phase protein, it could be attached to the surface of endothelial cells where it would be available to scavenge and assist in the degradation of microvesicles (blebs) released during cell destruction together with bacterial membranes following infection. The highly basic character of hIIa-PLA2 may be important for its ability to penetrate the highly anionic surface layer of bacteria so that the outer monolayer phospholipids can be hydrolyzed (56, 57), a possibility that can now be tested with the availability of the charge-reversal mutants.

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