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On the Binding Preference of Human Groups IIA and X Phospholipases A_2 for Membranes with Anionic Phospholipids*

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Mammals contain 9–10 secreted phospholipases A_2 ($sPLA_2$s) that display widely different affinities for membranes, depending on the phospholipid composition. The much higher enzymatic activity of human group X $sPLA_2$ (hGX) compared with human group IIA $sPLA_2$ (hGIIA) on phosphatidylcholine (PC)-rich vesicles is due in large part to the higher affinity of the former enzyme for such vesicles; this result also holds when vesicles contain cholesterol and sphingomyelin. The inclusion of anionic phosphatidylserine in PC vesicles dramatically enhances interfacial binding and catalysis of hGIIA but not of hGX. This is the result of the large number of lysine and arginine residues scattered over the entire surface of hGIIA, which cause the enzyme to form a supramolecular aggregate with multiple vesicles. Thus, high affinity binding of hGIIA to anionic vesicles is a complex process and cannot be attributed to a few basic residues on its interfacial binding surface, as is also evident from mutagenesis studies. The main reason hGIIA binds poorly to PC-rich vesicles is that it lacks a tryptophan residue on its interfacial binding surface, a residue that contributes to the high affinity binding of hGX to PC-rich vesicles. Results show that the lag in the onset of hydrolysis of PC vesicles by hGIIA is due in part to the poor affinity of this enzyme for these vesicles. Binding affinity of hGIIA, hGX, and their mutants to PC-rich vesicles is well correlated to the ability of these enzymes to act on the PC-rich outer plasma membrane of mammalian cells.

Secreted phospholipases A_2 ($sPLA_2$)[1] are 14–18-kDa calcium-dependent, disulfide-rich enzymes that liberate free fatty acids from the *sn*-2 position of glycerophospholipids (1). Most mammalian tissues contain one or more members of the $sPLA_2$ family (10 gene products in mice and 9 genes plus one pseudogene in humans) (2). There has been intense interest in the role of $sPLA_2$s in the liberation of arachidonic acid from cellular phospholipids for the formation of the eicosanoids (3, 4). Also, given the molecular diversity of $sPLA_2$s and their distinct tissue distribution patterns, it is highly likely that these enzymes are involved in other physiological responses besides inflammation. Some of these additional functions are known and include digestion of dietary phospholipids in the gastrointestinal track by pancreatic (group IB) $sPLA_2$ (5) and possibly other $sPLA_2$s (6), bactericidal action of human group IIA $sPLA_2$ in phagocytic cells, and in human tears (7–11) and release of free fatty acids for formation of the permeability barrier of skin (12, 13).

$sPLA_2$s have also been extensively studied as a paradigm for enzymes that act at the lipid-water interface (interfacial enzymes) (14, 15). Kinetic studies of $sPLA_2$s in the scooting mode establish that these enzymes bind to the membrane surface as a prelude to the loading of the active site with a single phospholipid molecule for the lipolysis reaction (16). The x-ray structure of several $sPLA_2$s reveal a deep active site surrounded by a nearly planar protein surface (17). The latter has been shown to function as the interfacial recognition surface (also called IBS) (18–20). By studying the properties of porcine pancreatic and cobra venom $sPLA_2$s, it was realized early on that $sPLA_2$s display large variation in their affinities for membranes of differing phospholipid composition (21). This leads to phenomena that are characteristic of interfacial enzymes. For example, in considering the substrate specificities of $sPLA_2$s, one must distinguish between the phospholipid binding specificity of the active site slot *versus* the IBS (22). Another example is the lag phase in the onset of phospholipid hydrolysis when porcine pancreatic $sPLA_2$ is added to PC vesicles (14). This lag is due, in part, to the fact that this $sPLA_2$ binds very weakly to vesicles of pure PC (zwitterionic head group) compared with vesicles containing phospholipids with reaction products (fatty acid plus lysophospholipid). Thus the reaction proceeds initially very slow as most of the enzyme is in the aqueous phase, and product formation accelerates as enzyme accumulates on product-containing vesicles.

It is becoming clear that differential binding of mammalian $sPLA_2$s to membranes of differing phospholipid composition has physiological significance. For example, human tears contain a high concentration of human group IIA $sPLA_2$ (hGIIA), and this enzyme is the principal bactericidal factor against Gram-positive bacteria in this fluid (8). Yet the enzyme does not degrade the outer plasma membrane of a number of mammalian cells (23–25). This is probably because of the fact that the outer plasma membrane of mammalian cells are rich in PC, and hGIIA displays virtually no enzymatic activity on PC-rich

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[1] The abbreviations used are: $sPLA_2$, secreted phospholipase A_2; CH, cholesterol; DOPC and DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine or -serine, respectively; $DO_{et}PC$ and $DO_{et}PS$, 1,2-dioleyl-*sn*-glycero-3-phosphocholine or -serine, respectively; hGIIA and hGX, human group IIA- and X-secreted phospholipase A_2, respectively; IBS, interface binding surface; PC, phosphatidylcholine; POPC and POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine or -serine, respectively; PS, phosphatidylserine; SM, sphingomyelin; HEK, human embryonic kidney; MOPS, 4-morpholinepropanesulfonic acid.

liposomes *in vitro* (26, 27). On the other hand human groups V and X $sPLA_2$ display relatively high activity on PC-rich liposomes and are able to liberate free fatty acids including arachidonic acid when added exogenously to a variety of mammalian cells (23, 28, 29). In this sense, groups V and X $sPLA_2$s resemble cobra venom $sPLA_2$s, which have been known for several years to efficiently hydrolyze mammalian cell membranes and PC vesicles without a lag in the onset of the reaction (21). It is interesting to understand in molecular detail the basis for the differential interfacial binding of $sPLA_2$ to membranes. Because a systematic study of the membrane binding properties of the full set of human and murine groups I, II, V, X, and XII $sPLA_2$s shows that hGIIA displays the lowest specific activity for the hydrolysis of the outer plasma membrane of mammalian cells while the human group X $sPLA_2$ (hGX) displays the highest specific activity,[2] we decided to carry out a structure-function study of the interfacial binding properties of hGIIA and hGX.

Previous attempts to quantify the binding of $sPLA_2$s to vesicles of anionic phospholipids have been hampered by the exceptionally high affinity of these enzymes for anionic vesicles. Thus, only limiting values for the equilibrium constant for dissociation of the enzyme from the vesicle into the aqueous phase, K_d, have been obtained (for example, Refs. 18 and 31). In the present study we have applied a new method for accurate quantification of values of K_d that describe the affinity of $sPLA_2$s to vesicles composed of charge-neutral PC containing various concentrations of the anionic phospholipid PS. We have applied this method to study the interfacial binding of hGIIA and hGX as well as their mutants in which IBS residues have been changed. We also correlate the interfacial binding data with interfacial kinetic results to examine the functional consequences of interfacial binding of $sPLA_2$s to vesicles.

EXPERIMENTAL PROCEDURES

Materials—Recombinant hGIIA was produced as its N1A mutant (to facilitate removal of the initiator methionine in *Escherichia coli*) as described (N1A has the same specific activity as the wild type enzyme when analyzed on a variety of phospholipid vesicles) (32). Recombinant hGX was also produced by expression in bacteria as described (33). Other recombinant $sPLA_2$s used in the study were produced as described (32, 34). hGIIA and hGX mutants were prepared using the QuikChange kit (Stratagene), and the full-length coding regions were sequenced. SDS-PAGE analysis of all proteins showed that they are >98% pure, and electrospray mass spectrometry analysis[2] showed that all disulfide bonds are intact. ^{14}C-SM (55 Ci/mol) and ^{14}C-CH (50 Ci/mol) were from American Radiochemicals Inc. The diether phospholipids $DO_{et}PC$ and $DO_{et}PS$ were synthesized as described (35).

Interfacial Binding Studies—Diether phospholipids were mixed in chloroform, and solvent was removed *in vacuo*. Extrusion buffer (5 mM MOPS, pH 7.4, 176 mM sucrose) was added, and vesicles were prepared by extrusion using a Liposofast device (Avestin Inc.) as described (36) except that vesicles were first extruded through a stack of two 0.8-μm Nucleopore filters and then extruded through a stack of two 0.2-μm filters. A trace amount of 1-palmitoyl-2-[9,10-^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine (American Radiochemicals Inc., 90 Ci/mmol) was present (sufficient amount such that the binding sample containing the lowest phospholipid concentration used contained a total of 500–1,000 cpm) so that the concentration of total phospholipid could be monitored by scintillation counting of a sample aliquot after each step in the process. Yields of phospholipid after extrusion were typically >90%. The diameter and polydispersity of extruded vesicles in binding buffer was examined by using a BIC particle analyzer (Brookhaven Instruments). Typical values were 100–130 nm for the diameter and 0.08–0.095 for the polydispersity. Stock solutions of extruded vesicles were stored at room temperature and used the same day for binding studies. Particle size analysis scattering indicated no change in vesicle size and polydispersity over the storage period.

The extruded vesicle solution was diluted 6-fold with binding buffer (5 mM MOPS, pH 7.4, 0.1 M KCl, 2 mM $CaCl_2$), and the solution was centrifuged in a 1.5-ml polyallomar microcentrifuge tube at 100,000 × *g* at 21 °C for 1 h in a Kompsin vertical rotor. Most of the supernatant was removed with a pipettor, and the same volume of binding buffer was added to the remaining vesicle pellet. Vesicles were resuspended by gentle up and down passage with a pipettor. This step ensures removal of vesicles that do not pellet (perhaps disrupted vesicles). An aliquot of the resuspended vesicle solution was submitted to scintillation counting to obtain the total phospholipid concentration. In some experiments, the binding buffer contained 0.1 mM EGTA and no $CaCl_2$.

Binding reactions were prepared in polyallomar tubes containing 100 μl of binding buffer with the desired concentration of sucrose-loaded vesicles and 0.5 μg of $sPLA_2$ (proportionally less enzyme was used for vesicles concentrations less than 0.1 mM). In the case of hGIIA-V3W, 50 ng of enzyme was used per binding reaction, and the binding buffer also contained 1 mg/ml γ-globulin (Sigma catalog number G4386) to prevent nonspecific binding of enzyme to the polyallomar tube. Tubes were centrifuged as above, and most of the supernatant was immediately transferred to new tubes. A portion of this supernatant was submitted to scintillation counting to determine the fraction of vesicles that pelleted (typically >90%). A second portion of supernatant was diluted into 3% bovine serum albumin, to minimize loss of enzyme to the tube wall, and an aliquot of this solution was submitted to an $sPLA_2$ enzymatic assay using 1-palmitoyl-2-(10-pyrenedecanoyl)-*sn*-glceryo-3-phosphomethanol (37). Because the specific activity of the different $sPLA_2$s varies considerably, the amount of supernatant taken and the extent of its dilution with 3% bovine serum albumin in water were chosen such that the reaction velocity measured in the assay was at least 5-fold higher than the minus $sPLA_2$ background rate.

In some experiments, CH and SM where included in vesicles. By doping the vesicles with trace amounts of ^{14}C-CH and ^{14}C-SM (in separate experiments), it was found that the yields of these lipids was high (>95%) after extrusion. This rules out the possibility that CH, SM, $DO_{et}PC$, and $DO_{et}PS$ pass through the membrane filters to different extents (because of differential solubility of lipids in the aggregates). Vesicles containing $DO_{et}PC$/SM/CH had a diameter of 214 nm and a polydispersity of 0.151.

The percent $sPLA_2$ remaining in the supernatant, based on enzymatic activity (100% is the amount of $sPLA_2$ activity in the supernatant of a sample processed as above but in the absence of vesicles), was plotted *versus* the total phospholipid present in the binding reaction. The curve was fit to the standard equation for equilibrium dissociation: $100 \times (E/E_T) = K_d/(L + K_d)$, where E is the concentration of $sPLA_2$ in the aqueous phase, E_T is the concentration of total enzyme (free and vesicle bound) in the binding reaction, L is the total phospholipid concentration in the binding reaction (expressed as total moles of phospholipid, $DO_{et}PC + DO_{et}PS$ divided by the volume of binding reaction sample), and K_d is the equilibrium constant for the dissociation of vesicle-bound enzyme into the aqueous layer. The recovery of all $sPLA_2$s used in the study from centrifuged samples that lacked vesicles was >80%. Thus, no correction was needed for loss of enzyme to the walls of the polyallomar tubes.

Light Scattering Studies—Right angle scattering of visible light was used to explore $sPLA_2$-dependent aggregation of vesicles. Binding buffer (1 ml, see above) was placed in a fluorescence cuvette, and scattering was monitored in a fluorimeter (excitation at 505 nm and emission set to 502 nm, 6 nm slit widths). Sucrose-loaded vesicles, prepared as described above, were added to give 50 μM phospholipid in the cuvette, and the signal was recorded for ~1 min with stirring at room temperature. $sPLA_2$ (5 μg) was added, and the signal was recorded for 20–30 min. Some scattering studies were done in the absence of calcium. In this case, binding buffer contained 0.1 mM EGTA in place of $CaCl_2$.

Interfacial Kinetic Studies with Vesicles—POPC or POPG unilamellar vesicles of 0.1-μm diameter were prepared by extrusion (36) (because low millimolar concentrations of phospholipids were extruded, the initial extrusion through 0.8-μm filters, see above, was omitted). As above, vesicles were doped with a trace amount of tritiated PC. The rate of phospholipid hydrolysis by $sPLA_2$ was measured by monitoring the displacement of a fluorescent fatty acid analog from fatty acid-binding protein (23, 27). The reaction mixture contained 30 μM phospholipid in 1.3 ml of Hanks' balanced salt solution with 1.27 mM Mg^{2+} and 0.90 mM Ca^{2+} (Invitrogen) containing 10 μg of liver fatty acid-binding protein (23) and 1 μM 11-dansylundecanoic acid (Molecular Probes Inc.). Reactions were monitored at 37 °C with magnetic stirring in a fluorimeter (emission at 500 nm, excitation at 350 nm, 10 nm slit widths). Assays were calibrated to give moles of oleic acid released by adding a known

[2] Singer, A. G., Ghomashchi, F., LeCalvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, E., Lazdunski, M., Lambeau, G., and Gelb, M. H. (2002) *J. Biol. Chem.* **277,** 48535–48549.

TABLE I
Specific activities for the hydrolysis of vesicles by hGIIA and hGX and their i-face mutants

sPLA2	V_o (POPG)[a]	V_o (POPC)[a]	V_o (20% DOPS in DOPC)	*sn-2* Fatty acyl chain specificity (20:4/16:0)[b]
		μmol min^{-1} mg^{-1}		
hGIIA	150 ± 15	Lag (≈6 min), 0.7 ± 0.1	4 ± 0.5	0.8 ± 0.2
hGIIA-R7G/K10G	153 ± 16	Lag (≈1 min), 1.6 ± 0.2	No data	0.8 ± 0.1
hGIIA-V3W	293 ± 30	Lag (≈1 min), 1.3 ± 0.2	1.7 ± 0.2	0.7 ± 0.2
hGIIA-V3W/R7G/K10G	310 ± 20	1.7 ± 0.2	No data	0.8 ± 0.2
hGIIA-V3W/R7G/K10G/R33H/K115F	120 ± 15	1.1 ± 0.2	No data	0.7 ± 0.1
hGX	36 ± 4	30 ± 4	10 ± 2	2.3 ± 0.3
hGX-W67A	33 ± 3	2.4 ± 0.4	1.4 ± 0.2	2.2 ± 0.3

[a] Initial velocities, V_o, are given. In some cases, as noted, a lag was observed in the onset of phospholipid hydrolysis after the addition of sPLA2 to the reaction mixture. The duration of the lag is given in parentheses, and the listed specific activity is that observed after the lag.
[b] Measured by the double isotope method as described in the text. Estimated errors are based on triplicate analyses.

amount of oleic acid and monitoring the drop in fluorescence. Studies to measure the preference of the $sPLA_2$s for the fatty acyl chain at the *sn*-2 position were carried out as described (26) using POPG vesicles containing 1-palmitoyl-2-[9,10-^{3}H]palmitoyl phosphatidylcholine and 1-stearoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine.

Release of Arachidonic Acid from Mammalian Cells—Arachidonic acid release from mammalian cells (human embryonic kidney cells, HEK293) was measured by determining the counts/min of radioactivity released into the culture medium when [^{3}H]arachidonic acid-labeled cells were incubated with $sPLA_2$ for 3 h at 37 °C as described (23). Arachidonic acid release studies were also carried out using Hanks' balanced salt solution (with calcium and magnesium, see above) instead of complete medium.

Some studies were carried out with SV40-transformed human corneal epithelial cells (38) (obtained as a gift from Dr. Rajiv R. Mohan, Department of Ophthalmology, University of Washington). These cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1/1 ratio, Invitrogen) supplemented with 15% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 10 μg/ml streptomycin sulfate, 5 ng/ml insulin, 0.1 ng/ml cholera toxin, 10 ng/ml human epidermal growth factor, and 0.5% dimethyl sulfoxide at 37 °C under humidified air containing 5% CO_2. Cells were placed in 1 ml of complete medium at 5×10^5 cells/ml, seeded in each well of a 24-well plate, and incubated overnight to near confluence. [^{3}H]Arachidonic acid (0.1 μCi) was added to each well, and the cells were incubated overnight at 37 °C. The labeled cells were washed two times with complete medium and then placed in 1 ml of fresh medium. The cells were treated with various amounts of $sPLA_2$s at 37 °C for 6 h. After the medium was removed and briefly microcentrifuged to remove any detached cells, 0.5 ml of the medium was analyzed by scintillation counting. The remaining cells were dissolved in 1 ml of $CHCl_3/CH_3OH$ (2/1), and the sample was submitted to scintillation counting after removal of the organic solvent *in vacuo*. [^{3}H]Arachidonic acid release is expressed as percentage of the cell medium counts/min to the total counts/min (medium + organic extract).

RESULTS

Approach to Study Interfacial Binding of $sPLA_2$s—Interfacial binding of $sPLA_2$ to 0.1-μm unilamellar vesicles of varying phospholipid composition was quantified by sedimenting vesicles and measuring the fraction of $sPLA_2$ remaining in the supernatant. Vesicles are loaded with sucrose so that they can be sedimented in an ultracentrifuge (39). Sucrose-loaded vesicles are not under osmotic stress because of the presence of 0.1 M KCl in the binding buffer outside of the vesicles (39). Also, the addition of 0.1 M KCl to the binding buffer brings the ionic strength to a value close to the physiological one. The sedimentation method has a number of advantages over other methods. It does not require that the protein contain a spectral probe (*i.e.* a fluorophore), it is applicable under a wide range of vesicle concentrations (scattering from vesicles hampers the use of fluorescence methods with phospholipid concentrations greater than about 50–100 μM), and it is inexpensive compared with methods such as surface plasmon resonance. One problem that arises with all methods to measure vesicle-protein binding is nonspecific binding of protein to the walls of the centrifuge tube; this problem is more pronounced when low protein concentrations are used in an attempt to measure small K_d values. To avoid using enzyme concentrations less than about 10 nM, we have adjusted the mole percent of anionic $DO_{et}PS$ in $DO_{et}PC$ vesicles such that accurate values of K_d can be obtained in the ~1–3,000 μM range. Additionally, inclusion of 1 mg/ml γ-globulin in the binding buffer, but not bovine serum albumin, prevented nonspecific binding of hGIIA and hGIIA-V3W to the polyallomar tubes when using enzyme concentrations down to 10 nM.

The phospholipid:$sPLA_2$ mole ratio in the interfacial binding reactions was at least 200. This was chosen to avoid crowding of enzyme on the vesicle surface. Approximately 100 phospholipids per enzyme lie on the enzyme-accessible outer leaflet of the vesicles, and each $sPLA_2$ binds to about 30–50 phospholipids. Thus when 50% of the $sPLA_2$ is bound to vesicles, the surface coverage by protein is ~25% of maximal coverage. Under these conditions, the simple binding equation (see "Experimental Procedures") is appropriate, *i.e.* the depletion of enzyme binding sites on vesicles during the binding titration can be ignored. Also, the effect of interaction between proteins co-localized on vesicles can most likely be ignored so that K_d is a reflection of the energetics of an isolated enzyme sitting on the vesicle dissociating into the aqueous phase (although see below for studies with hGIIA). Measurement of such K_d values seems most appropriate for interpreting the role of IBS amino acid residues in supporting interfacial binding of $sPLA_2$s.

To best correlate the interfacial binding and phospholipid hydrolysis kinetic data, binding studies were carried out in the presence of saturating calcium. This requires the use of diether phospholipid vesicles to avoid $sPLA_2$-catalyzed generation of reaction products because the latter can modulate the K_d (14). Values of K_d measured in the presence of calcium are apparent values as they reflect binding of enzyme to the interface and the binding of a single phospholipid molecule in the active site of the interface-bound enzyme. The latter step requires calcium (40) and helps bind the enzyme to vesicles by mass action (however, see below for studies with hGIIA). Some binding studies were carried out in the absence of calcium using both diether and diester phospholipid vesicles.

Interfacial Binding and Kinetics of Wild Type hGIIA and hGX—As reported previously, the initial velocity for the action of hGIIA on POPC vesicles is >1,000-fold lower than that measured with anionic POPG vesicles (23) (Table I). hGIIA readily hydrolyzes PC when enzyme is tightly bound to anionic phosphatidylmethanol vesicles (26, 30), which shows that PC is well accommodated in the catalytic site. Thus, the low activity on POPC vesicles is predicted to be due either to poor interfacial binding to these charge-neutral vesicles or to catalytically nonproductive binding or both.

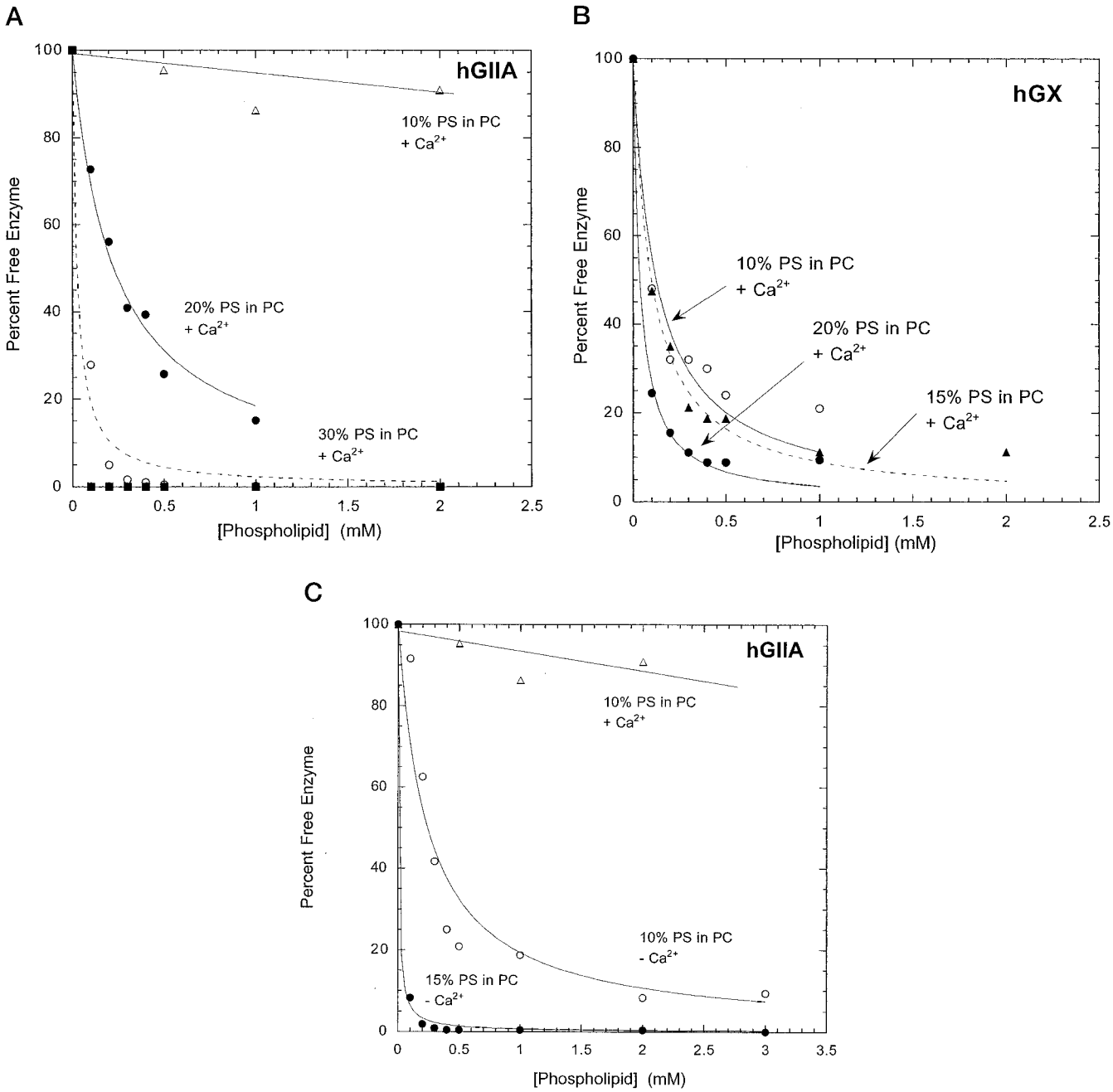

FIG. 1. *A*, binding of hGIIA to $DO_{et}PS/DO_{et}PC$ vesicles in the presence of calcium. The *filled squares* are the data obtained with 50% $DO_{et}PS/DO_{et}PC$ vesicles (*points* overlap at 2 mM). *B*, binding of hGX to $DO_{et}PS/DO_{et}PC$ vesicles in the presence of calcium. *C*, binding of hGIIA to $DO_{et}PS/DO_{et}PC$ vesicles in the absence and presence of calcium as noted.

As shown in Fig. 1*A*, binding of hGIIA to PC-rich vesicles (10% $DO_{et}PS$ in $DO_{et}PC$) in the presence of calcium is weak ($K_d \gg 2$ mM). The rate of hydrolysis of POPC vesicles by hGIIA was studied with the fatty acid-binding protein assay that directly measures the formation of free oleic acid. In the presence of 30 μM POPC vesicles, virtually all of the hGIIA is in the aqueous phase (based on $K_d \gg 30$ μM for $DO_{et}PC$ vesicles), and the reaction velocity is vanishingly small as shown in Fig. 2 and Table I. As also shown in Fig. 2, hGIIA is able to hydrolyze POPC vesicles but only after a lag phase. No lag phase was observed when enzyme was added to POPC vesicles containing 10 mol % reaction products (1/1 mixture of oleic acid and 1-palmitoyl-*sn*-glycero-3-phosphocholine) (not shown). Presumably the reaction products promote the binding of hGIIA to POPC vesicles as has been reported for pig pancreatic $sPLA_2$ (41), but this was not investigated further.

In marked contrast to hGIIA, hGX shows high initial velocity on 30 μM POPC vesicles and no lag was observed (Table I) (see also, Ref. 23). Because hGX, when tightly bound to the interface of anionic vesicles, does not hydrolyze PC in preference to phospholipids with other polar head groups (23), the high activity of this enzyme on POPC vesicles is predicted to be because of a value of K_d much lower than that for hGIIA. Indeed, Fig. 1*B* shows that hGX readily binds to 10% $DO_{et}PS/DO_{et}PC$ vesicles; the K_d value is 0.13 mM (Table I).

As shown in Fig. 1*A*, the binding of hGIIA to vesicles is dramatically enhanced by increasing the mole percent of $DO_{et}PS$ in the $DO_{et}PC$ vesicles. K_d drops more than 10-fold when $DO_{et}PS$ is increased from 10 to 20 mol %, and decreases a further 10-fold when $DO_{et}PS$ is further increased to 30 mol %. With 50 mol % $DO_{et}PS$ in $DO_{et}PC$ vesicles, all of the enzyme is bound at the lowest lipid concentration tested of 0.1 mM (Fig.

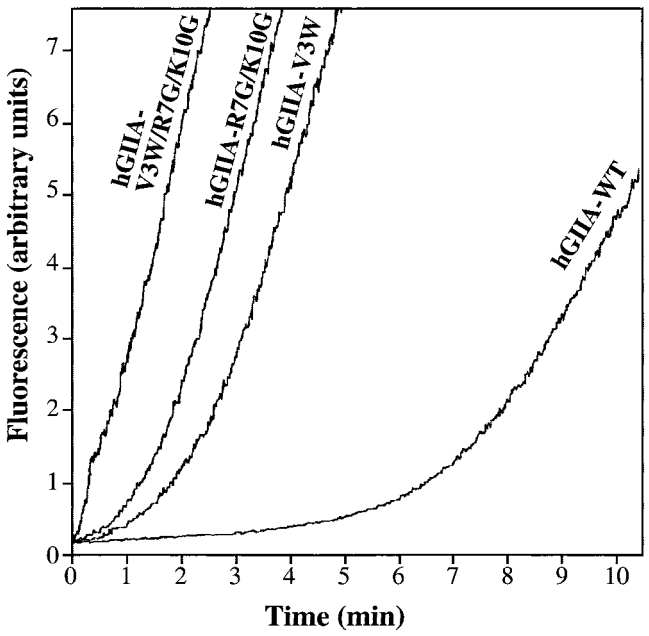

FIG. 2. **Reaction progress curves for the hydrolysis of POPC vesicles by wild type and mutant hGIIA sPLA$_2$s.** Enzymes were added to vesicles at time 0, and the formation of free oleic acid was monitored by the fatty acid-binding protein assay (see "Experimental Procedures").

1*A*), thus $K_d \ll 0.1$ mM. Increasing the mole percent of $DO_{et}PS$ in $DO_{et}PC$ vesicles leads to a much more modest enhancement in the binding of hGX to vesicles (Fig. 1*B*, Table II).

Remarkably, hGIIA binds to $DO_{et}PS/DO_{et}PC$ vesicles more tightly in the absence of calcium than in the presence of this divalent cation (Fig. 1*C*, data listed in parentheses in Table II along with the plus calcium results). For example, in the absence of calcium, the K_d measured with 10% $DO_{et}PS/DO_{et}PC$ vesicles is 0.24 ± 0.04 mM compared with ≫2 mM in the presence of calcium. This result was unexpected as the presence of calcium is expected to increase the apparent affinity of enzyme for vesicles. This is because the binding of a single phospholipid molecule to the catalytic site of interface-bound hGIIA requires calcium, and such binding should bring more enzyme to the vesicle interface by mass action. As in the presence of calcium, binding increases dramatically as the mole fraction of DOetPS is increased (Fig. 1*C*).

Given the unusual finding that the presence of calcium apparently decreases the affinity of hGIIA for vesicles, we suspected that the interfacial binding reaction has anomalous features. Thus, right angle light scattering studies were carried out to probe the interfacial binding reaction in more detail. These studies were carried out with concentrations of phospholipid vesicles and enzyme similar to those used in vesicle binding studies. The right angle light scattering of $DO_{et}PS/DO_{et}PC$ vesicles alone did not increase when hGX was added. In contrast, addition of 5 μg of hGIIA to 10% $DO_{et}PS/DO_{et}PC$ (50 μM phospholipid) in the presence of 2 mM calcium led to a 1.2-fold increase in light scattering above the scattering from vesicles alone. The scattering increased 2-fold when enzyme was added to 20% $DO_{et}PS/DO_{et}PC$ vesicles. In the presence of 30% $DO_{et}PS/DO_{et}PC$, addition of hGIIA led to the formation of a visible particulate, and an accurate change in light scattering could not be obtained (because of instability of the signal). In the absence of calcium, addition of hGIIA to $DO_{et}PC$ vesicles containing 10–30 mol % $DO_{et}PS$ led to visible particulate formation. The dramatic increase in the binding of hGIIA to vesicles as the mole percent of $DO_{et}PS$ is increased from 10 to 30% in $DO_{et}PC$ vesicles in the presence of calcium, as measured by the centrifugation method (Fig. 1*A*), correlates with a large increase in light scattering. The ability of hGIIA to bind to 10% $DO_{et}PS/DO_{et}PC$ vesicles in the absence of calcium (Fig. 1*C*, no binding occurs to these vesicles in the presence of calcium) correlates with the large increase in light scattering seen even with $DO_{et}PC$ vesicles containing a small amount of anionic phospholipid (10 mol % $DO_{et}PS$). Thus, binding of hGIIA to $DO_{et}PS/DO_{et}PC$ is not a simple process involving the formation of a monodisperse enzyme-vesicle complex, and the K_d measured by the centrifugation method is an apparent constant reflecting the onset of formation of a large vesicle-protein aggregate. Thus, the apparent paradoxical results showing that interfacial binding of hGIIA is enhanced by exclusion of calcium is accounted for by the larger amount of protein-vesicle aggregation in the absence of divalent cation. More information about the structure of this aggregate is provided under "Discussion."

Studies with Vesicles Containing Cholesterol and Sphingomyelin—The extracellular face of the plasma membrane of most, if not all, mammalian cells is thought to be rich in PC, whereas most of the anionic phospholipids (PS and phosphatidylinositol) are on the inner monolayer. SM, which like PC has a zwitterionic head group, is also mainly in the outer membrane leaflet. Cell membranes also contain a significant quantity of CH. The outer leaflet of the erythrocyte plasma membrane is composed of 23% PC, 23% SM, and 43% CH (42, 43). Thus, we also measured the binding of hGIIA and hGX to sucrose-loaded, 0.1-μm unilamellar vesicles of $DO_{et}PC$/SM/CH (1/1/1 mol ratio) containing various amounts of $DO_{et}PS$, and binding data are summarized in Table III. Unlike sucrose-loaded, pure $DO_{et}PC$ vesicles, which did not fully pellet, sucrose-loaded $DO_{et}PC$/SM/CH (1/1/1 mole ratio), which lacked $DO_{et}PS$, pelleted completely, and thus binding data is also reported for these vesicles that lack negative charge. The inclusion of CH and SM may have slightly enhanced the interfacial binding of hGIIA in that this enzyme bound to $DO_{et}PC$/SM/CH vesicles containing 10% $DO_{et}PS$ with a K_d of ~2 mM (Tables II and III), whereas no binding occurred to 2 mM 10% $DO_{et}PS/DO_{et}PC$ vesicles. No binding of hGIIA to 2.5 mM vesicles without $DO_{et}PS$ occurred. Thus the inclusion of SM and CH does not allow hGIIA to bind well to zwitterionic vesicles. hGX bound well to PC-rich vesicles with or without SM and CH (Tables II and III).

Table IV summarizes the kinetics of hGIIA and hGX action on vesicles containing SM and CH. The presence of SM and CH does not significantly alter the kinetics. For example, the specific activity of hGIIA on DOPC/CH/SM vesicles is 3-fold lower than that for the hydrolysis of DOPC vesicles (both measured after the lag). Considering that the surface concentration of DOPC in the mixed lipid vesicles is one-third of that in pure DOPC vesicles, this reduction in rate is presumably because of surface dilution of substrate. Likewise, hGX hydrolyzes DOPC/CH/SM vesicles at about one-third the rate of pure DOPC vesicles, and both specific activities are much higher than those for hGIIA on charge-neutral vesicles. The inclusion of increasing amounts of anionic DOPS leads to a progressive shortening of the lag in the onset of hydrolysis by hGIIA as well as a significant increase in the specific activity (a similar trend to that seen for DOPC/DOPS vesicles, Table I).

Interfacial Binding and Kinetics of hGIIA and hGX Mutants—hGIIA is a highly basic protein, calculated pI = 9.4, because of the presence of 22 lysine and arginine residues scattered over its entire surface including the surface that surrounds the opening to the active site slot (putative IBS). Arg-6 and Lys-10 lie on the putative IBS close to the opening to the active site slot. An obvious working hypothesis is that this

TABLE II
Binding of sPLA2s to PC/PS vesicles

sPLA2	K_d (mM)[a]			
	10% $DO_{et}PS$ in $DO_{et}PC$	20% $DO_{et}PS$ in $DO_{et}PC$	30% $DO_{et}PS$ in $DO_{et}PC$	50% $DO_{et}PS$ in $DO_{et}PC$
hGIIA	No binding at 2	0.23 ± 0.01	0.023	All bound at 0.1
	0.24 ± 0.04 (no Ca^{2+})	≈0.02 (no Ca^{2+})		
	0.9 ± 0.2 (POPC/POPS, no Ca^{2+})	0.08 ± 0.3 (POPC/POPS, No Ca^{2+})		
hGIIA-R7G/K10G	≈8	0.32 ± 0.06	0.052 ± 0.008	All bound at 0.1
hGIIA-V3W	0.6 ± 0.2	0.0026 ± 0.0008	All bound at 0.1	No data
	<0.03 (no Ca^{2+})			
hGIIA-V3W/R7G/K10G	0.09 ± 0.005	<0.03	All bound at 0.1	No data
hGIIA-V3W/R7G/K10G/R33H/K115F	0.1 ± 0.04	<0.03	All bound at 0.1	No data
hGIIA-R7E/K10E/K16E	No binding at 2	2.7 ± 0.7	0.23 ± 0.05	≈0.01
hGIIA-K53E/R54E/K57E/R58E	No data	No binding at 2	1.5 ± 0.5	No data
hGX	0.13 ± 0.025	0.10 ± 0.009	0.036 ± 0.004	No data
	0.5 ± 0.2 (10% POPS in POPC, hGX-H46Q)	0.5 ± 0.1 (20% POPS in POPC, hGX-H46Q)		
hGX-W67A	No data	0.8 ± 0.04	0.3 ± 0.03	No data

[a] All values of K_d are in units of millimolar total phospholipid (inner plus outer monolayer) present in the binding mixture. Most K_d values were measured in the presence of 2 mM Ca^2. Some studies were carried out without Ca^{2+} (as noted) and some with POPC/POPS diester vesicles in the absence of Ca^{2+} in the case of hGIIA and in the presence of Ca^{2+} but with a catalytic site mutant, in the case of hGX (as noted). See text for explanation of K_d values preceded by ≈ and < symbols.

pair of basic residues significantly contributes to the stringent dependence of hGIIA interfacial binding on the presence of anionic $DO_{et}PS$ in $DO_{et}PC$ vesicles (Fig. 1*A*). This hypothesis is further supported by the fact that hGX, which is much less dependent on anionic phospholipid for interfacial binding (Fig. 1*B*), contains glycine residues at positions analogous to those occupied by Arg-7 and Lys-10 of hGIIA. Thus, a simple electrostatic model involving the interaction of cationic amino acid side chains with the anionic head group of PS could account for the differences in interfacial binding properties between hGIIA and hGX.

To investigate this hypothesis, we prepared the double mutant hGIIA-R7G/K10G, *i.e.* we interchanged hGIIA residues with hGX residues. Because hGIIA binds tightly to anionic phosphatidylglycerol vesicles ($K_d \ll 1$ μM) and because multisite charge reversal hGIIA mutants also bind tightly to these vesicles (31), we first measured the specific activity of all $sPLA_2$ mutants on POPG vesicles to assess the effect of mutation on the catalytic activity of the proteins under conditions in which they are fully bound to vesicles. Results are summarized in Table I. The specific activity of hGIIA and hGIIA-R7G/K10G are identical within experimental error, indicating that these two basic residues are not critical for enzyme binding to anionic vesicles and that the active site has not been perturbed by mutation of these IBS residues.

Mutation of Trp-31 on the putative IBS of human group V $sPLA_2$ to alanine reduces the activity of this enzyme on PC vesicles and reduces binding to PC-coated polymer beads (28). Replacement of Val-3 of hGIIA by tryptophan increases the specific activity of this enzyme on PC vesicles by at least 2 orders of magnitude (44). These results show that nonelectrostatic effects can be important for modulating interfacial binding of $sPLA_2$. Thus, we also prepared the mutant hGIIA-V3W and measured its interfacial binding and kinetic properties. Wild type hGX contains a single tryptophan on its putative IBS, and we also mutated this residue to alanine (hGX-W67A). We also prepared the 3-site mutant hGIIA-V3W/R7G/K10G. The surface of hGIIA that includes Arg-7 and Lys-10 also includes the additional basic residues Arg-33 and Lys-115. hGX contains His and Phe at positions analogous to Arg-33 and Lys-115 of hGIIA, and thus we also prepared the mutant hGIIA-V3W/R7G/K10G/R33H/K115F. The specific activity of all of the $sPLA_2$s on anionic POPG vesicles are within a factor of 2 of each other (Table I), indicating no major perturbation of structure due to residue changes.

Fig. 3*A* shows the interfacial binding curves for the hGIIA mutants interacting with vesicles of $DO_{et}PC$ containing 10% $DO_{et}PS$. Also included is the charge reversal mutant hGIIA-R7E/K10E/K16E prepared previously (31). Values of K_d are listed in Table II. No significant binding of hGIIA and hGIIA-R7E/K10E/K16E to these vesicles is seen up to 2 mM phospholipid. hGIIA-R7G/K10G may show a small amount of binding (Fig. 3*A*), and only an approximate K_d of ~8 mM could be obtained (higher phospholipid concentrations are difficult to obtain because the sucrose-loaded vesicles have to be diluted after extrusion into buffer without sucrose). This binding is significantly weaker than the binding of wild type hGX to these vesicles (K_d = 0.13 mM, Fig. 1*B*), indicating that basic residues at positions 7 and 10 of hGIIA *versus* glycine at these positions in hGX is not the critical factor that prevents hGIIA from binding to PC-rich vesicles and allows hGX to bind to these vesicles. Mutation of Val-3 to Trp in hGIIA produces a much larger binding enhancement than does conversion of Arg-7 and Lys-10 to Gly (Fig. 3*A*). To accurately quantify the difference in interfacial binding of hGIIA *versus* hGIIA-V3W under conditions where detectable binding of hGIIA occurs, we carried out

TABLE III
Binding of sPLA$_2$s to CH- and SM-containing DO$_{et}$PC/DO$_{et}$PS vesicles in the presence of Ca^{2+}

sPLA2	DO$_{et}$PC/CH/SM (1/1/1)		
	K_d	K_d	K_d
	0% DO$_{et}$PS[a]	10% DO$_{et}$PS	30% DO$_{et}$PS
hGIIA	No binding at 2.5 mM	≈2 mM	0.05 ± 0.01 mM
	(no data)	(no binding at 2 mM)	(0.023 mM)
hGX	≈2 mM	0.08 ± 0.01	All bound at 0.1 mM
	(no data)	(0.13 ± 0.025)	(0.036 ± 0.004)

[a] Vesicles are composed of x/3 mole fraction of DO$_{et}$PC, CH, and SM, and 1-x mole fraction of DO$_{et}$PS. Numbers in parentheses are in the absence of CH and SM (from Table II).

TABLE IV
Specific activities for the hydrolysis of CH- and spingomyelin-containing vesicles by hGIIA and hGX

sPLA2	V$_o$					
	DOPC 0% DOPS	DOPC/CH/SM 0% DOPS	DOPC 10% DOPS	DOPC/CH/SM 10% DOPS	DOPC 30% DOPS	DOPC/CH/SM 30% DOPS
	μmol min^{-1} mg^{-1}					
hGIIA	Lag (≈3 min)	Lag (≈2 min)	Lag (≈2 min)	Lag (≈0.6 min)	Lag (≈0.6 min)	No lag
	0.8 ± 0.3	0.3 ± 0.03	3.0 ± 0.5	4.0 ± 0.5	5.0 ± 0.5	12 ± 2
hGX	No lag	No lag	No data	No data	No lag	No lag
	85 ± 10	35 ± 8			72 ± 12	33 ± 7

[a] Initial velocities are given. In some cases, as noted, a lag was observed in the onset of phospholipid hydrolysis after the addition of enzyme to the reaction mixture. The duration of the lag is given in parentheses, and the listed velocity is that observed after the lag.

binding studies with low concentrations of 20% DO$_{et}$PS/DO$_{et}$PC vesicles. The results in Fig. 3*B* show that hGIIA-V3W binds with a K_d of 0.0026 ± 0.0008 mM. Thus, this mutant binds 90-fold tighter than hGIIA to these vesicles. Remarkably, hGIIA-V3W binds tighter than hGX to DO$_{et}$PC vesicles containing 20 mol % DO$_{et}$PS (Table II). Trp-67 of hGX is important for interfacial binding because substitution with alanine increased K_d by 8-fold with 20% DO$_{et}$PS/DO$_{et}$PC vesicles (Fig. 4, Table II).

The triple mutant hGIIA-V3W/R7G/K10G binds 6-fold tighter than does hGIIA-V3W to 10% DO$_{et}$PS/DO$_{et}$PC vesicles. hGIIA-V3W/R7G/K10G and hGIIA-V3W/R7G/K10G/R33H/K115F display virtually identical values of K_d for interaction with 10% DO$_{et}$PS/DO$_{et}$PC vesicles indicating that basic residues at positions 33 and 115 do not significantly modulate the affinity of hGIIA to vesicles. In light of this result, we did not prepare the hGIIA mutant in which only positions 33 and 115 were mutated. These multisite mutants bound more tightly to vesicles as the mole % DO$_{et}$PS was increased in DO$_{et}$PC. In these cases, only upper limit K_d values are given (Table II) as we did not carry out binding studies at low micromolar phospholipid concentrations.

We also studied the binding of hGIIA-K53E/R54E/K57E/R58E in which a cluster of 4 basic residues on the face of the enzyme that is opposite the putative IBS have been charge reversed (initially as a control experiment, however, see "Discussion"). Remarkably, this protein bound significantly weaker to DO$_{et}$PS/DO$_{et}$PC vesicles than did wild type enzyme. The K_d for the mutant is at least 10-fold higher than that for the wild type with 20% DO$_{et}$PS/DO$_{et}$PC vesicles, and the difference is ~60-fold with 30% DO$_{et}$PS/DO$_{et}$PC vesicles (Table II).

Some of the binding studies were also carried out with POPC/POPS vesicles (*i.e.* diester vesicles) in the absence of calcium so that enzyme does not hydrolyze the vesicles. hGIIA consistently showed a 3–4-fold decrease in affinity to diester *versus* diether phospholipid vesicles (data listed in parentheses in Table II along with the diether phospholipid results). hGX-H46Q, which has <3% of wild type catalytic activity on POPG vesicles and thus could be studied in the presence of calcium (His-46 is a key catalytic residue), bind to diester POPC/POPS vesicles about 4-fold less tightly than to DO$_{et}$PC/DO$_{et}$PC diether vesicles in the presence of calcium (data listed in parentheses in Table II along with the results for wild type hGX). Thus, these sPLA$_2$s bind modestly more tightly to the more hydrophobic diether vesicles, and the data validate the use of diether phospholipid vesicles to study the interfacial binding of hGIIA and hGX.

As noted above, hydrolysis of POPC vesicles by wild type hGIIA occurs only after a several minute lag (Fig. 2). hGIIA-V3W and hGIIA-R7G/K10G display significantly shorter lags, and vesicle hydrolysis starts immediately upon addition of the triple-site mutant hGIIA-V3W/R7G/K10G (Fig. 2). Table I gives the specific activities for the hydrolysis of vesicles by the hGIIA and hGX mutants. In those cases where a lag is seen, the specific activity is calculated from the steady-state reaction slope after the lag phase. Regardless of whether a lag is seen, the specific activity of all of the hGIIA mutants acting on POPC vesicles are well below those for hGX (Table I). Addition of 20% DOPS to DOPC vesicles increases the specific activity of hGIIA from ~0 (immediately after adding enzyme to vesicles) to 4 mmol min^{-1} mg^{-1}, whereas hGX displays comparable specific activities on POPC and 20% DOPS/DOPC vesicles (Table I). Thus, as for interfacial binding, the enzymatic activity of hGIIA but not hGX increases dramatically when anionic phospholipid is added to the zwitterionic interface. Replacement of Trp-67 with alanine causes a 12-fold reduction in specificity activity on POPC vesicles, a 7-fold reduction on 20% DOPS/DOPC vesicles, and no reduction on POPG vesicles (Table I).

Arachidonic Acid Release from Mammalian Cells by Wild Type and Mutant hGIIA and hGX sPLA$_2$s—The extracellular face of the plasma membrane of mammalian cells is thought to be rich in zwitterionic phospholipids (PC and SM) and poor in acidic phospholipids (PS and phosphatidylinositol). It was thus of interest to test hGIIA and its mutants for their ability to release arachidonic acid when added exogenously to cells. Data for the release of [^{3}H]arachidonic acid from HEK293 cells are summarized in Fig. 5. Addition of 1 or 10 μg of hGIIA to 1 ml of complete culture medium containing HEK293 cells resulted in essentially no release of [^{3}H]arachidonic acid. On the other hand, fatty acid release was readily detected when hGIIA-R7G/K10G, hGIIA-V3W, hGIIA-V3W/R7G/K10G/R33H/K115F, and especially hGIIA-V3W/R7G/K10G were added to HEK293 cells. In general the amount of [^{3}H]arachidonic acid released correlates well with the specific activity of this set of proteins on POPC vesicles (Table I). These experiments were also carried out by addition of hGIIA and its mutants to HEK293 cells in

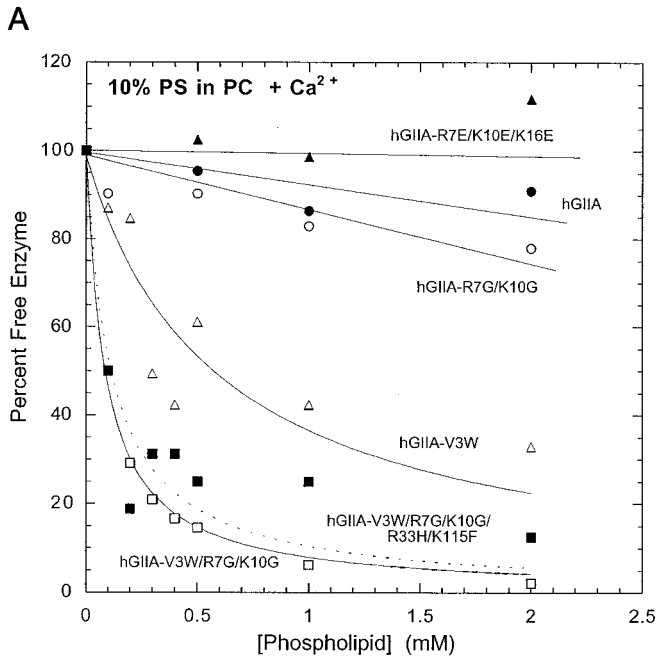

FIG. 3. *A*, binding of hGIIA and hGIIA mutants to $DO_{et}PS/DO_{et}PC$ vesicles in the presence of calcium. *B*, binding of hGIIA-V3W to $DO_{et}PS/DO_{et}PC$ vesicles in the presence of calcium. Note the lower than typical phospholipid concentration used. Binding buffer contained γ−globulin to prevent loss of hGIIA-V3W to the tube walls.

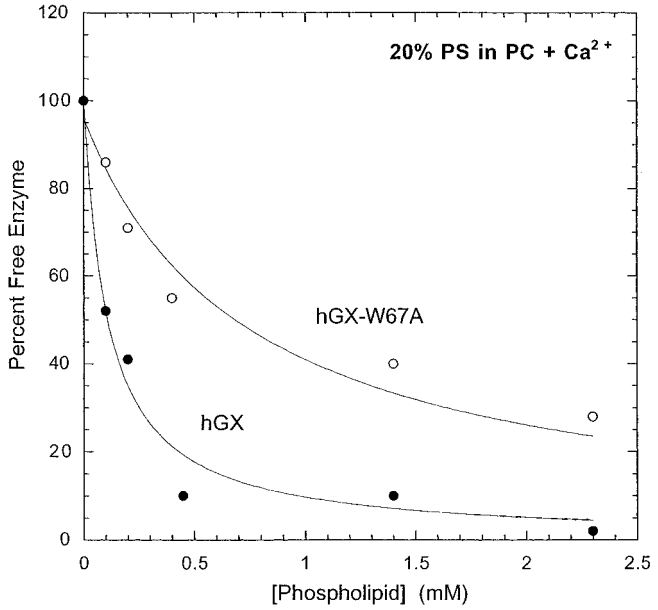

FIG. 4. **Binding of hGX and the hGX-W67A mutant to $DO_{et}PS/DO_{et}PC$ vesicles in the presence of calcium.**

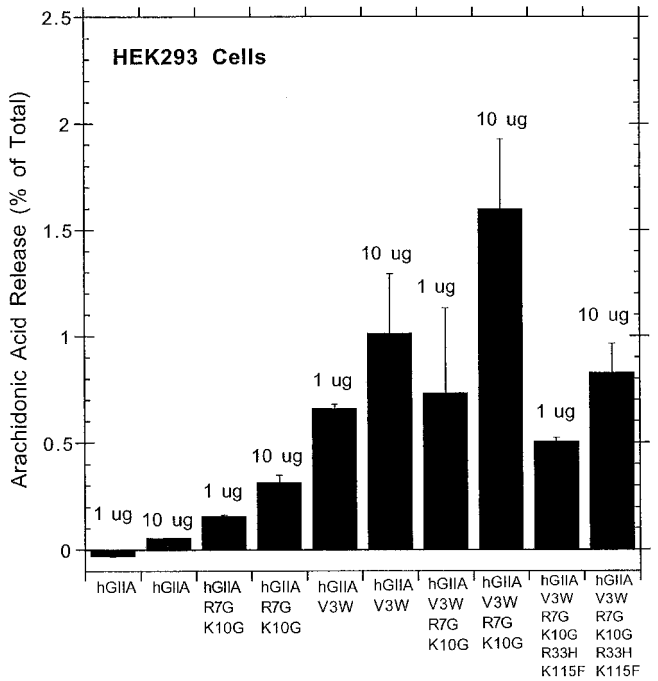

FIG. 5. **Release of [^{3}H]arachidonic acid from HEK293 cells into the culture medium by hGIIA and hGIIA mutants.** Release is expressed as a percentage of the total counts/min of tritium (medium and cell associated) after 3 h incubation with enzyme (corrected for release in the absence of added $sPLA_2$). *Error bars* are the standard deviation from three independent experiments.

balanced salt solution containing calcium and magnesium. For all proteins, the results obtained were the same (within 10%) as those obtained in the presence of complete medium (not shown). Thus, differential binding of $sPLA_2$s to components of the complete medium, *i.e.* albumin, is not the basis for their differential ability to release arachidonic acid from mammalian cells.

We also tested hGIIA and hGIIA-V3W for their ability to release [^{3}H]arachidonic acid from a human corneal epithelial cell line (38). As shown in Fig. 6, 10 μg of wild type hGIIA produced little, if any, fatty acid release, whereas hGIIA-V3W was much more active in this assay. The low activity of hGIIA on these cells can presumably explain why the human corneal epithelium is spared even though human tears contain a large concentration (30 μg/ml) of hGIIA (8).

hGX and hGX-W67A were tested for their ability to release [^{3}H]arachidonic acid from HEK293 cells and from the human corneal epithelial cell line (Fig. 7). Consistent with earlier studies (23, 29), hGX is much more active on mammalian cells than is hGIIA. hGX-W67A is about 10-fold less active than hGX on HEK293 and corneal epithelial cells (Fig. 7).

When the complete medium from HEK293 and corneal epithelial cells treated with hGIIA, hGX, and their mutants were tested for $sPLA_2$ enzymatic activity (fluorimetric assay (37)) after a 3-h incubation at 37 °C, >90% of the enzyme was recovered in the culture medium in all cases. This result shows that the differences in specific activity for the action of these $sPLA_2$s on mammalian cells is not because of differential binding of the enzymes to a nonmembrane cell surface

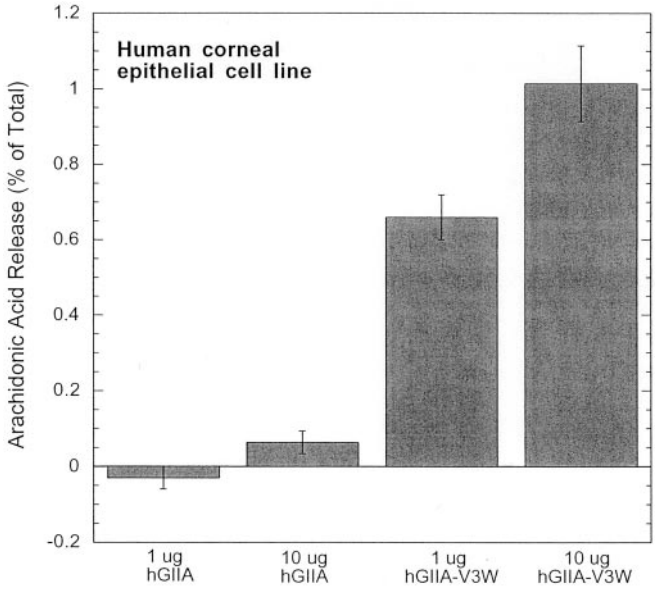

FIG. 6. **Release of [^{3}H]arachidonic acid from a human corneal epithelial cell line by hGIIA and hGIIA mutants.** Release is expressed as a percentage of the total counts/min of tritium (medium and cell associated) after 3 h incubation with enzyme (corrected for release in the absence of added $sPLA_2$). *Error bars* are the standard deviation from three independent experiments.

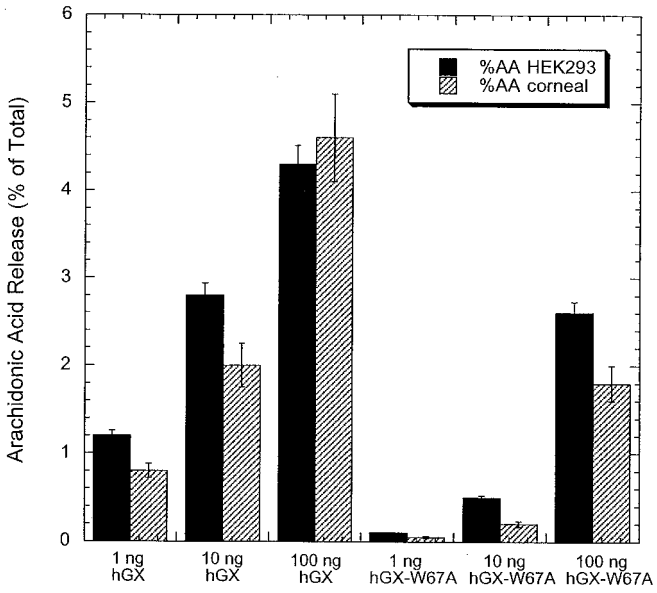

FIG. 7. **Release of [^{3}H]arachidonic acid from HEK293 cells (*black bars*) and a human corneal epithelial cell line (*gray bars*) by hGX and hGX mutants.** Release is expressed as a percentage of the total counts/min of tritium (medium and cell associated) after 3 h incubation with enzyme (corrected for release in the absence of added $sPLA_2$). *Error bars* are the standard deviation from three independent experiments.

component such as heparan sulfate. This result is consistent with earlier studies showing that hGIIA is mainly in the extracellular culture medium in the presence of wild type Chinese hamster ovary cells and mutant cells lacking glycosaminoglycans (32).

We measured the specificity of hGIIA, hGX, and their mutants toward *sn*-2 palmitoyl *versus sn*-2 arachidonyl chains by using the double isotope method (26) in which the ratio of tritiated to carbon-14 product is measured when vesicles of POPG containing 1-palmitoyl-2-[9,10-^{3}H]palmitoyl phosphatidylcholine and 1-stearoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine are submitted to limited hydrolysis by the enzyme. Results, summarized in Table I, show that hGX shows modest selectivity (2.3-fold) toward the *sn*-2 arachidonyl chain *versus* the *sn*-2 palmitoyl chain, whereas hGIIA displays essentially no selectivity. Mutation of IBS residues does not noticeably effect these specificities (Table I). These results show that the dramatically higher ability of hGX *versus* hGIIA to release arachidonic acid from mammalian cells cannot be explained based on differences in *sn*-2 fatty acyl chain specificity and that the results obtained with the IBS mutants are not influenced by a change in *sn*-2 fatty acyl chain specificity (wild type *versus* mutant).

DISCUSSION

IBS Basic Residues Are Not the Critical Factor Governing Interfacial Binding of hGIIA and hGX—Previous studies have clearly shown that the specific activity of hGIIA on PC-rich vesicles is extremely low compared with that for hGX even though both enzymes are able to efficiently hydrolyze PC when this substrate is co-dispersed in anionic phospholipid vesicles (23, 26, 27). The simplest explanation for these results is that hGX binds much more tightly than hGIIA to PC-rich vesicles. The present studies clearly show this to be the case. The data in Table II shows that hGX binds $\gg$15-fold tighter than hGIIA to 10% $DO_{et}PS/DO_{et}PC$ vesicles. Only a lower limit estimate can be given because no binding of hGIIA to up to 2 mM 10% $DO_{et}PS/DO_{et}PC$ could be detected. A more accurate estimate of the difference in affinity of hGX *versus* hGIIA for PC vesicles cannot be given based on specific activities because the initial velocity for the hydrolysis of POPC vesicles by hGIIA is too slow to be detected (hydrolysis is detected only after a lag phase, because of the slow build up of reaction products). As the mole fraction of $DO_{et}PS$ in $DO_{et}PC$ vesicles is increased, it is clear that binding of hGIIA is enhanced much more than is hGX.

The obvious difference in the putative IBS of hGIIA and hGX is that hGX contains neutral residues at locations that are occupied by lysine and arginine residues of hGIIA. However, this difference does not account for the difference in interfacial binding properties of hGIIA and hGX. This is based on the observation that hGIIA-R7G/K10G displays interfacial binding affinity for $DO_{et}PS/DO_{et}PC$ vesicles that is only modestly different from that of wild type hGIIA (Table II, Fig. 3*A*). Additionally, replacement of the basic residues of hGIIA, Arg-33 and Lys-155, with hGX residues, His and Phe, respectively, also has essentially no effect on interfacial binding (Table II, Fig. 3*A*). These results are surprising in light of the fact that interfacial binding of hGIIA, but not hGX, requires a critical amount of anionic $DO_{et}PS$ in $DO_{et}PC$ vesicles (Fig. 1, *A* and *B*).

The interpretation of the effect of mutation of hGIIA IBS residues on interfacial binding must take into account the large increase in right angle light scattering that occurs when enzyme is added to vesicles. In the presence of calcium, the scattering increases as the amount of $DO_{et}PS$ is increased from 10 to 30 mol % in $DO_{et}PC$ vesicles, and this is correlated with the dramatic increase in the amount of hGIIA bound to vesicles. In the absence of calcium, light scattering measurements reveal a large increase even with 10% $DO_{et}PS/DO_{et}PC$ vesicles, and binding studies show that hGIIA binds to these vesicles (Fig. 2*C*). The increase in scattering is not seen when hGX binds to vesicles, as expected for the formation of a monodisperse vesicle-protein complex. Thus, it is clear that addition of hGIIA to anionic vesicles leads to the formation of a vesicle-protein supramolecular structure containing multiple vesicles per aggregate.

Additional details about the structure of this hGIIA-anionic vesicle aggregate comes from our recent electron paramagnetic resonance studies to monitor the exposure of nitroxide spin

labels attached to hGIIA to the water-soluble spin relaxants tris(oxalato)chromate(III) and nickel(EDTA) (45). We placed 13 spin labels in a near uniform distribution over the entire surface of hGIIA. In the absence of anionic vesicles, all of the nitroxides collide with the spin relaxants present in the aqueous phase. Remarkably, when vesicles are added, all 13 spin labels become fully protected from the spin relaxants. Such results show not only that the IBS of hGIIA is in contact with vesicles but that multiple surfaces of the protein are not in contact with the bulk aqueous phase. The increase in light scattering is much larger than expected for a simple model in which a single hGIIA bridges two vesicles together. For the same reason, the scattering data also rules out a mechanism of spin relaxant protection involving a monodisperse enzyme-vesicle complex in which the vesicle "wraps" around the enzyme (such wrapping also seems unlikely because of the high degree of vesicle deformation required). Our proposed model is that several enzymes segregate into a patch that forms a vesicle-vesicle junction site and that each vesicle contains 6 of these patches that hold the supramolecular structure together into a hexagonal packed array of spherical vesicles (45).

Electron paramagnetic resonance and light scattering studies show that addition of 200 mM NaCl reduces the size of the aggregates, causes spin labels on the face of hGIIA opposite the IBS to become partly exposed to aqueous phase spin relaxants, but does not cause spin labels on the IBS to become exposed to these spin relaxants (45). Thus, it seems that there is a significant electrostatic component holding this hGIIA-vesicle aggregate together, which is perhaps not surprising given that the aggregate forms only with vesicles that contain a critical mole fraction of anionic phospholipid and that hGIIA is a highly basic protein (pI = 9.4). The fact that the complex dissociates with increasing concentration of salt while hGIIA remains bound to vesicles via its IBS argues that binding via the IBS has a significant nonelectrostatic component.

In the context of these observations, it must be stated that the K_d value measured by the centrifugation method, or any method, for the interaction of hGIIA with anionic vesicles (Fig. 2, *A* and *C*) is an apparent value that reflects supramolecular structure formation and is not the dissociation equilibrium constant for the desorption of a single enzyme molecule from a monodisperse enzyme-vesicle particle. The lack of a significant effect on aggregate formation and vesicle binding (centrifugation method) of replacement of Arg-7 and Lys-10 with glycine (Table I, Fig. 3*A*) is presumably because of the fact that these basic residues are only 2 of the 22 lysine and arginine residues that cover the surface of hGIIA. This is further supported by the electron paramagnetic resonance studies that show that multiple hGIIA surfaces are involved in aggregate formation. On the other hand, replacement of these two residues with anionic glutamates (hGIIA-R7E/K10E) produces a larger effect on the apparent K_d (Table II), presumably because of electrostatic repulsion of anionic vesicles by these negatively charged residues. The hGIIA-vesicle aggregation model also explains the remarkable observation that charge reversal mutation of hGIIA residues that are on the face of the protein that are opposite the IBS also increases the value of the apparent K_d (hGIIA-K53E/R54E/K57E/R58E, Table II). Clearly, the formation of hGIIA-anionic vesicle aggregates complicates our ability to dissect the role of individual amino acids in modulating interfacial binding; however, the following conclusions can be drawn. The aggregation of anionic vesicles by hGIIA clearly has a significant electrostatic effect involving multiple lysine and arginine residues; this is based on the mutagenesis data and the observation of aggregation reversal by high salt. The binding of the IBS to anionic vesicles has a significant nonelectrostatic component. *Finally, the data establish conclusively that tight interfacial binding of hGIIA to anionic vesicles is not the result of electrostatic interactions between a few IBS basic residues and the anionic head groups of acidic phospholipids.*

Our results are seemingly at odds with a previous study of the binding of venom $sPLA_2$ (*Agkistrodon piscivorus piscivorus*, D49 isoform) to anionic vesicles of polymerized phosphatidylglycerol. In these earlier studies it was stated that Lys-7 and Lys-10 on the IBS of this $sPLA_2$, together, account for nearly half of the total interfacial binding energy (46). This is based on the following observations. The value of K_d for wild type enzyme dissociating from anionic vesicles was measured to be 2×10^{-8} M (this value must be approximate because it was measured with phospholipid concentrations of a few micromolar or higher), corresponding to a binding free energy of $\Delta G^0 = 10.6$ kcal/mol at room temperature. The K_d for the K7E/K10E mutant is 9.6×10^{-6} M, corresponding to $\Delta G^0 = 6.9$ kcal/mol in binding free energy. The difference in these two free energies, $\Delta\Delta G^0 = 3.7$ kcal/mol, is nearly half of the ΔG^0 for wild type enzyme suggesting that interfacial binding is in large part determined by electrostatic interaction between these two lysines and anionic phospholipids. This argument is incorrect for the following reason. We can consider ΔG^0 for binding to be equal to the sum $\Delta G_s + \Delta G_i$, where ΔG_s is the free energy change associated with the loss of translational and rotational entropy that occurs when enzyme in solution binds to the vesicle surface, and ΔG_i is the intrinsic binding energy (the binding energy available if enzyme does not have to give up translational and rotational entropy) (14, 47). Because ΔG_s is expected to be nearly the same when wild type and mutant $sPLA_2$ binds to vesicles, $\Delta\Delta G^0 = 3.7$ kcal/mol calculated above represents the difference in intrinsic binding energies for wild type *versus* mutant. This cannot be compared with $\Delta G^0 = 10.6$ kcal/mol for binding of wild type $sPLA_2$ to vesicles because the latter includes ΔG_s. Because ΔG_s is always a positive number (unfavorable for enzyme to lose translational and rotational freedom when it settles down on the vesicle), ΔG_i for binding of wild type $sPLA_2$ to vesicles is significantly greater than 10.6 kcal/mol, and thus the contribution of Lys-7 and Lys-10 to interfacial binding represents significantly less than half of the total binding energy. As suggested from the present studies with hGIIA, presumably $\Delta\Delta G^0$ would be significantly less than 3.7 kcal/mol for a Lys-7/Lys-10 double mutant in which the basic residues were replaced with neutral residues rather than with acidic residues. Thus, it is clear the electrostatic interaction of Lys-7 and Lys-10 of the venom $sPLA_2$ with acidic phospholipids represents only a very small portion of the total interfacial binding energy, as we report for IBS lysines and arginines of hGIIA.

Gadd and Biltonen (48) studied the binding of the same venom $sPLA_2$ to vesicles of dipalmitoyl phosphatidylcholine containing various amounts of dipalmitoyl phosphatidylglycerol. A value of $K_d = 5$ μM was obtained for pure anionic vesicles, and a value of $K_d = 2{,}800$ μM was obtained for pure zwitterionic vesicles (by extrapolation of data obtained from vesicles with 0.05 to 1 mol fraction of phosphatidylglycerol). A value of $\Delta\Delta G^0 = 3.6$ kcal/mol was obtained for the difference in PC and phosphatidylglycerol interfacial binding energies. Because ΔG_i for binding of the venom $sPLA_2$ to PC vesicles is larger than $\Delta G^0 = 3.5$ kcal/mol (calculated from $K_d = 2{,}800$ μM), again because of the positive value of ΔG_s, these authors correctly state, "thus the interaction to the zwitterionic surface is stronger than the enhancement achieved by replacing them with anionic lipids. Although the binding does have an electrostatic component, another thermodynamic component, likely of

a hydrophobic or van der Waals type, must be playing a major role in the interaction."

The inclusion of SM has been reported to reduce the activity of hGIIA acting on PC vesicles (49) and lipoproteins (50). The inhibition by SM is offset by addition of CH to vesicles (51). It is highly suggestive from the present study that the reduction in velocity for the hydrolysis of PC vesicles because of the addition of SM and CH is because of surface dilution, *i.e.* a reduction in the mole fraction of substrate that the bound enzyme encounters (52). It is clear that SM and CH are not potent inhibitors of hGIIA. Additionally, the higher affinity of hGX *versus* hGIIA for PC-rich vesicles still holds when vesicles contain SM and CH. It has been reported that hGIIA selectively hydrolyzes vesicles derived from erythrocytes that have their intracellular surface facing outwards over those that have the reverse orientation (49). Based on the data in Tables I-IV, it is probably the PS in the intracellular leaflet of the erythrocyte membranes rather than the SM in the extracellular leaflet (49) that determines this preference.

Tryptophan on the IBS of $sPLA_2$ Potently Promotes Interfacial Binding and Catalysis—If the presence of IBS basic residues is not the basis for the enhanced binding of $sPLA_2$s to PS-rich *versus* PS-poor vesicles, how do we explain the difference in interfacial binding properties of hGIIA *versus* hGX? Initial thoughts were based on studies with human group V $sPLA_2$, which like hGX is able to bind to PC-rich membranes much more strongly than hGIIA. Mutagenesis studies have shown that replacement of Trp-31 of human group V $sPLA_2$ with alanine leads to a substantial loss in binding to PC vesicles (28). These studies were inspired by earlier observations that cobra venom $sPLA_2$s display high specific activity on PC-rich vesicles, and that chemical modification of 1–2 tryptophans on the IBS of these enzymes dramatically reduces catalytic activity even though these tryptophans were not part of the active site slot (see for example, Ref. 53).

In the present study we have found that addition of a single tryptophan to the IBS of hGIIA (at position 3 on the N-terminal α-helix) dramatically enhances interfacial binding to $DO_{et}PS$/$DO_{et}PC$ vesicles. From the data in Table II, it is seen that hGIIA-V3W binds about 90-fold tighter than wild type hGIIA to 20% $DO_{et}PS/DO_{et}PC$ vesicles. Baker *et al.* (44) found that the specific activity of hGIIA-V3W for the hydrolysis of 1,2-dioleoylphosphatidyl-PC vesicles is about 200–1,000-fold higher than that for wild type enzyme (44). This number is only an approximate difference as it depends on the initial velocity for the action of hGIIA on pure PC vesicles. Because of the lag in the reaction progress for the action of hGIIA on PC vesicles (Fig. 2), this initial velocity is difficult to measure accurately, and it will depend critically on the presence of trace amounts of reaction products present as in impurity in PC vesicles prior to the addition of enzyme. The fact that hGIIA and hGX-V3W show similar specific activity when tightly bound to POPG vesicles is consistent with Trp-3 mainly effecting the fraction of enzyme bound to the interface of PC-rich vesicles.

hGX, like hGV, displays high activity on PC vesicles relative to hGIIA. The only Trp on the face of hGX that includes the opening to the active site slot is Trp-67. Mutation of this residue to alanine causes an 8-fold reduction in interfacial binding to PC-rich vesicles and a corresponding decrease in the specific activity for the hydrolysis of PC-rich vesicles. This effect of mutation is presumably due only to a change in interfacial binding because both wild type hGX and hGX-W67A display the same specific activity when tightly bound to POPG vesicles. This more modest effect of tryptophan removal in the case of hGX *versus* hGIIA may be due to the fact that Trp-3 of hGIIA-V3W lies closer to the catalytic slot than does Trp-67 of hGX, and thus may be in a better position for optimal penetration into the membrane surface. It would appear that the lack of tryptophan on the IBS of hGIIA is the major reason that this enzyme binds poorly to PC-rich vesicles. Indeed, hGIIA-V3W binds tighter than hGX to PC-rich vesicles (Table II). Recent studies have shown that each of 2 tryptophans on the surface of bacterial phospholipase C near the opening to the active site contribute about 10-fold to the interfacial binding of this enzyme (54).

The Basis of the Lag Seen in the Hydrolysis of POPC Vesicles by hGIIA—The simplest explanation for the lag seen in the onset of hydrolysis of POPC vesicles by hGIIA is that the build up of reaction products in the vesicle interface promotes interfacial enzyme binding, thus leading to an acceleration in the reaction progress as the reaction proceeds. This explanation is consistent with the observation that addition of reaction products to POPC vesicles eliminates the lag (not shown), as also reported for porcine pancreatic $sPLA_2$ (14). Also, hGIIA-V3W, that binds tighter than wild type enzyme to PC-rich vesicles displays a shorter lag (Table II, Fig. 2). However, the lag cannot be fully explained by product-dependent interfacial binding. hGIIA-V3W binds tighter than hGX to PC-rich vesicles and yet the former shows a slight lag, whereas the latter displays no lag (Table II, Fig. 2). Additionally, the specific activity of hGIIA-V3W on POPC vesicles is much higher than that of wild type enzyme, but it is still 40-fold lower than that for hGX. These results suggest that interfacial binding is important but not sufficient for full activation of hGIIA on PC-rich vesicles. This conclusion is also consistent with data obtained with additional hGIIA mutants. hGIIA-R7G/K10G and wild type hGIIA display virtually identical interfacial binding properties (Table II, Fig. 3*A*) and yet the mutant displays a shortened lag, comparable with that for hGIIA-V3W, and the multisite mutant hGIIA-V3W/R7G/K10G hydrolyzes POPC vesicles without a discernable lag (Fig. 2). These results suggest that although the IBS basic residues do not significantly contribute to interfacial binding, they do play a role in supporting the catalytic turnover of interface-bound hGIIA.

All together the results suggest that the reaction products in the interface of POPC vesicles have two effects on interfacial catalysis by hGIIA. The first is to increase the fraction of enzyme at the interface, K_d effect, and the second is to activate the interface-bound enzyme, interfacial allosteric effect. By dissection of all of the kinetic and equilibrium parameters for the interfacial catalytic cycle of porcine pancreatic $sPLA_2$, Jain and co-workers (55) have convincingly shown that this enzyme displays enhanced binding to product-containing vesicles and that anionic amphiphiles in the interface of PC vesicles also lead to allosteric, k_{cat} type, activation of the enzyme at the interface, the latter is dependent on IBS basic residues. This dual activating effect of the reaction products in PC vesicles has also been reported for water moccasin venom $sPLA_2$ (56). Our results with hGIIA are consistent with these earlier findings with other $sPLA_2$s, but a full molecular dissection of possible interfacial allosteric activation of hGIIA is beyond the scope of the present study.

Action of hGIIA and hGX on Mammalian Cells—It has been suggested that mammalian cells are generally poor substrates for the action of extracellular, exogenously added hGIIA because the enzyme cannot bind to the PC-rich outer plasma membrane (32). This hypothesis is further supported by the demonstration in the present study that mutants of hGIIA that display higher activity on PC-rich vesicles *in vitro* are also more efficient at releasing arachidonic acid when added exogenously to HEK293 cells and to a human corneal epithelial cell line (Figs. 5 and 6). As already mentioned under "Results," the

poor activity of hGIIA when added exogenously to mammalian cells is not because of capture of enzyme on nonphospholipid membrane components of the *in vitro* system. On the other hand hGX is highly efficient at releasing fatty acids when added exogenously to a variety of mammalian cells (Fig. 7 and Refs. 23, 25, 29, and 57) and to PC-rich lipoproteins (30). This is likely to the relatively high affinity of hGX for PC-rich vesicles. This is suggested by the results of Fig. 7 showing that mutation of hGX residue Trp-67 to alanine, which reduces binding to and activity on PC-rich vesicles, also reduces the specific activity for arachidonic acid release from mammalian cells. These results strongly suggest that hGIIA and hGX have distinct physiological functions. The studies described here provide a molecular understanding of the different catalytic efficiencies of hGIIA and hGX on PC-rich vesicles.

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