THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 17, Issue of April 23, pp. 11881–11888, 1999

Printed in U.S.A.

Roles of Trp^{31} in High Membrane Binding and Proinflammatory Activity of Human Group V Phospholipase A_2*

(Received for publication, December 14, 1998, and in revised form, January 19, 1999)

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Group V phospholipase A_2 is a recently discovered secretory phospholipase A_2 (PLA_2) that has been shown to be involved in eicosanoid formation in inflammatory cells, such as macrophages and mast cells. We have demonstrated that human group V PLA_2 ($hsPLA_2$-V) can bind phosphatidylcholine (PC) membranes and hydrolyze PC substrates much more efficiently than human group IIa PLA_2, which makes it better suited for acting on the outer plasma membrane (Han, S.-K., Yoon, E. T., and Cho, W. (1998) *Biochem. J.* 331, 353–357). In this study, we demonstrate that exogenous $hsPLA_2$-V has much greater activity than does group IIa PLA_2 to release fatty acids from various mammalian cells and to elicit leukotriene B_4 formation from human neutrophils. To understand the molecular basis of these activities, we mutated two surface tryptophans of $hsPLA_2$-V to alanine (W31A and W79A) and measured the effects of these mutations on the kinetic activity toward various substrates, on the binding affinity for vesicles and phospholipid-coated beads, on the penetration into phospholipid monolayers, and on the activity to release fatty acids and elicit eicosanoid formation from various mammalian cells. These studies show that the relatively high ability of $hsPLA_2$-V to induce cellular eicosanoid formation derives from its high affinity for PC membranes and that Trp^{31} on its putative interfacial binding surface plays an important role in its binding to PC vesicles and to the outer plasma membrane.

Phospholipase A_2s (PLA_2)[1] are a family of lipolytic enzymes that are found both intra- and extracellularly. Because PLA_2-catalyzed liberation of arachidonic acid from membrane phospholipids leads to the production of potent inflammatory lipid mediators, eicosanoids that include prostaglandins, thromboxanes, leukotrienes, and lipoxins, the elucidation of their regulatory mechanisms is important for understanding the pathogenesis of inflammatory diseases and for developing a new class of anti-inflammatory drugs. Mammalian tissues contain multiple forms of PLA_2s (1), including groups I, IIa, IIc, V, X (2) secretory PLA_2 ($sPLA_2$), group IV cytosolic PLA_2 ($cPLA_2$), and group VI intracellular Ca^{2+}-independent PLA_2. Recent cell studies have indicated that both $cPLA_2$ and $sPLA_2$ are involved in eicosanoid production (3–5). The critical involvement of $cPLA_2$ was demonstrated by recent genetic studies showing that the disruption of the $cPLA_2$ gene results in loss of lipid mediator biosynthesis (6, 7).

The nature of proinflammatory $sPLA_2$s is not fully understood. Group IIa $sPLA_2$ has long been implicated in inflammation based on findings that it is synthesized and secreted by a variety of cells in response to inflammatory cytokines and that it is found in fluids from inflammatory exudation. However, group IIa $sPLA_2$ has extremely low affinity for zwitterionic phosphatidylcholine (PC) vesicles (8, 9). Thus, it is unclear how this secreted protein might act on the extracellular face of plasma membrane of mammalian cells, which is composed largely of zwitterionic phospholipids, PC and sphingomyelin. More recently, group V $sPLA_2$ has been shown to be involved in eicosanoid formation from murine macrophages and mast cells (10, 11). Molecular cloning of group V $sPLA_2$ from different species showed that these enzymes, although homologous to group IIa $sPLA_2$, have some notable variations in amino acid sequence (12, 13). In particular, they contain a few tryptophan residues, some of which are located on their putative interfacial binding surfaces (see Fig. 1), whereas group IIa $sPLA_2$s have none. Those $sPLA_2$s (*e.g.* cobra venom PLA_2s) that show high activity toward PC vesicles and intact cell membranes typically have a number of tryptophans and other aromatic side chains on their interfacial binding surfaces; this finding suggests that group V $sPLA_2$ might be better suited for acting on the outer cell membrane than group IIa $sPLA_2$. Indeed, our recent study demonstrated that human group V $sPLA_2$ ($hsPLA_2$-V) could bind and hydrolyze PC vesicles much more effectively than human group IIa $sPLA_2$ ($hsPLA_2$-IIa) (14). The present study demonstrates that $hsPLA_2$-V also is much more active than $hsPLA_2$-IIa in releasing fatty acids (including arachidonic acid) and in eliciting eicosanoid production from a variety of cells including neutrophils. This study also identifies a surface tryptophan res-

* This work was supported by National Institutes of Health Grants GM52598 (to W. C.), HL36235 (to M. H. G.), HL46368, and HL56399 (to A. R. L.), a Biomedical Science Grant from Arthritis Foundation, a grant from the Arthritis and Rheumatism Council (to D. C. W.), and a grant from Glaxo Wellcome, Inc. (to A. R. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: BLPG, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphoglycerol; PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; $hsPLA_2$-IIa, human secretory class IIa PLA_2; $hsPLA_2$-V, human secretory class V PLA_2; $cPLA_2$, cytosolic PLA_2; diC_6thio-PC, 1,2-dihexanoylthio-1,2-dideoxy-glycero-3-phosphosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; LTB_4, leukotriene B_4; PC, phosphatidylcholine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; pyrene-PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; pyrene-PG, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol; DHPC, 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine.

idue (Trp^{31}) of hsPLA$_2$-V as a structural determinant of its high affinity for PC membranes and for outer cell membranes.

EXPERIMENTAL PROCEDURES

Materials—1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC), and -glycerol (pyrene-PG) were purchased from Molecular Probes (Eugene, Oregon). 1,2-Bis(12-(lipoyloxy)-dodecanoyl)-*sn*-glycero-3-phosphoglycerol (BLPG) was prepared as described elsewhere (15, 16). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and -glycerol (POPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and -methanol (DMPM), and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine were from Avanti Polar Lipids. Polymyxin B sulfate, *Naja naja naja* venom PLA$_2$, 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-*sn*-dioleoylglycerol, cytochalasin B, and *N*-formylmethionyl-leucyl-phenylalanine were from Sigma. Racemic 1,2-dihexanoylthio-1,2-dideoxy-glycero-3-phosphocholine (diC$_6$thio-PC) was prepared as described (17). Phospholipid concentrations were determined by phosphate analysis (18). 1-Stearoyl-2-[^{14}C] arachidonoyl-*sn*-glycero-3-phosphocholine (55 mCi/mmol) was from Amersham Pharmacia Biotech. Styrene-divinylbenzene beads (5.2 ± 0.3 μm diameter) were purchased from Seradyn (Indianapolis, IN). Fatty acid-free bovine serum albumin was from Bayer Inc. (Kankakee, Illinois). 5,5′-Dithiobis(2-nitrobenzoic acid) and sodium sulfite were obtained from Aldrich. 2-Nitro-5-(sulfothio)-benzoate was synthesized from 5,5′-dithiobis(2-nitrobenzoic acid) as described (19). All restriction enzymes, T4 ligase, T4 polynucleotide, kinase and isopropyl β-D-thiogalactopyranoside were obtained from Boehringer Mannheim. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and used without further purification. Recombinant hsPLA$_2$-IIa, which carries the Asn[1] to Ala mutation, was prepared as described (20–22). Porcine pancreatic PLA$_2$ was obtained as a gift from Prof. M. K. Jain (University of Delaware).

Mutagenesis—Mutagenesis was performed using the Sculptor *in vitro* mutagenesis kit from Amersham Pharmacia Biotech and a phagemid DNA prepared from the pSK vector in the presence of helper phage R408 as described previously (23).

Expression and Purification of hsPLA$_2$-V—Proteins were expressed in *Escherichia coli*, refolded, and purified as described previously (14), with some modifications. *E. coli* strain BL21(DE3) was used as a host for protein expression. An 8-liter Luria broth containing 100 μg/ml of ampicillin was inoculated with 80 ml of overnight culture from a single colony and was grown at 37 °C. When the absorbance of the medium at 600 nm reached 0.2, additional ampicillin was added to a final concentration of 1 mM, and 0.5 mM isopropyl β-D-thiogalactopyranoside was added when the absorbance at 600 nm reached 0.8. After an additional 4 h at 37 °C, cells were harvested at 3000 × *g* for 10 min at 4 °C and frozen at –20 °C. Cells were resuspended in 100 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM EDTA, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% (v/v) Triton X-100, and 0.4% (w/v) sodium deoxycholate and stirred at 4 °C. The suspension was sonicated on ice using a Sonifier 450 (Branson) in pulse mode for 10 pulses of 15 s each. The inclusion body pellet was obtained by centrifugation at 17,000 × *g* for 20 min. The pellet was resuspended in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM EDTA, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.8% (v/v) Triton X-100, and 0.8% (w/v) sodium deoxycholate, sonicated as described above, and the suspension was centrifuged. The pellet was resuspended in 100 ml of the same buffer solution and stirred for 30 min at room temperature. The pellet was collected by centrifugation as described above and washed in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 5 M urea and 5 mM EDTA, and the suspension was centrifuged. The inclusion body protein was solubilized in 20 ml of 50 mM Tris-HCl, pH 8.5, containing 8 M guanidinium chloride and 0.3 M sodium sulfite, and stirred vigorously at room temperature for 30 min. Eight ml of 2-nitro-5-(sulfothio)-benzoate solution (50 mM) was then added, and the modification was monitored spectrophotometrically at 412 nm. After the modification was complete (approximately 20 min), the mixture was further stirred for 20 min, and any insoluble matter was removed by centrifugation at 100,000 × *g* for 15 min at room temperature. The reaction mixture was loaded onto a Sephadex G-25 column (2.5 × 45 cm) equilibrated with 25 mM Tris-HCl buffer, pH 8.0, containing 5 M urea and 5 mM EDTA at room temperature, and the second major protein peak was collected (120 ml) and dialyzed against water and then against 0.3% (v/v) glacial acetic acid to precipitate the sulfonated protein. The precipitated protein was resuspended in 100 ml of deionized water, and the suspension was centrifuged at 17,000 × g for 10 min at 4 °C. The protein pellet was resuspended in 10 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA and 5 M guanidinium chloride. The clear solution was loaded onto a HiLoad 16/60 Superdex 200 column (Amersham Pharmacia Biotech) attached to a fast protein liquid chromatography system at 4 °C (Amersham Pharmacia Biotech). The major protein peak was collected (ca 30 ml), and to this solution of sulfonated protein, 30 ml of 50 mM Tris-HCl, pH 8.5, containing 10% (v/v) glycerol, 8 mM reduced glutathione and 7 mM oxidized glutathione, were added dropwise with stirring (120 rpm) over 3 h. The solution was kept at room temperature for 20 h, at which point, the protein solution was dialyzed at room temperature against 3 volumes of 4 liters of 25 mM Tris-HCl buffer containing 0.2 M guanidinium chloride and 10% (v/v) glycerol, pH 7.5. For wild type hsPLA$_2$-V, the clear solution of folded protein was fractionally precipitated with 30–40% ammonium sulfate at room temperature. The resulting protein precipitate was collected by centrifugation at 50,000 × g for 15 min at 4 °C and resuspended in 5 ml of 25 mM Tris (pH 7.5) buffer containing 0.2 M guanidinium chloride. The fractional precipitation of refolded hsPLA$_2$-V by ammonium sulfate not only increased the purity of protein (>90% pure electrophoretically) but also allowed the concentration of protein solution (up to micromolar). For W31A and W78A, the refolded protein solution was dialyzed at room temperature against 25 mM Tris-HCl buffer, pH 7.5, containing 0.2 M guanidinium chloride, then against 25 mM Tris-HCl, pH 7.5, and finally against distilled water. The dialyzed solution was lyophilized and the lyophilized powder was stored at –20 °C. The purity of wild type and mutant proteins assessed by SDS-polyacrylamide gel electrophoresis was consistently higher than 90%. Protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as standard (Pierce).

Kinetic Measurements—PLA$_2$-catalyzed hydrolysis of polymerized mixed liposomes was carried out at 37 °C in 2 ml of 10 mM HEPES buffer, pH 7.4, containing 0.1 μM pyrene-containing phospholipids (1 mol %) inserted in 9.9 μM BLPG, 2 μM bovine serum albumin, 0.16 M NaCl, and 10 mM $CaCl_2$ (15, 16). The progress of hydrolysis was monitored as an increase in fluorescence emission at 378 nm using a Hitachi F4500 fluorescence spectrometer with the excitation wavelength set at 345 nm. Spectral bandwidth was set at 5 nm for both excitation and emission. Values of k_{cat}^*/K_m^* were determined from reaction progress curves as described previously (22). Enzymatic hydrolysis of DMPC and DMPM vesicles was monitored with the pH-stat method (24). Sonicated small unilamellar vesicles of DMPM were prepared as described (24). Sonicated small unilamellar DMPC vesicles were prepared by suspending 10 mg of lipid in 1 ml of water and sonicating as described (for about 10 min to give an almost clear suspension). The vesicles were annealed by incubating the solution at 50 °C for 90 min and then kept at 37 °C during use over 1 day. DMPM reaction mixtures contained 4 ml of 1 mM NaCl, 2.5 mM $CaCl_2$, 20 μg of polymyxin B sulfate, 240 μM DMPM at 21 °C and pH 8.0. DMPC reaction mixtures contained 100 mM NaCl, 1 mM $CaCl_2$, 77 mM DMPC in a volume of 4 ml at 25 °C and pH 8.0. Reactions were started by the addition of enzyme and monitored as the consumption of 3 mM NaOH titrant. Assays were calibrated to give nmol of product as described (24). Assays with monomeric substrate racemic diC$_6$thio-PC were carried out as described (17).

Binding of sPLA$_2$ to Phospholipid-coated Beads and Vesicles—To circumvent the complication due to low pelleting efficiency of PC vesicles, PC-coated styrene-divinylbenzene beads that can be rapidly and completely separated from the solution by low speed centrifugation were used (9). Phospholipid-coated beads were prepared as described previously (9). Phospholipid-coated beads were suspended in 3 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 0.16 M NaCl and 0.1 mM EDTA (or 10 mM $CaCl_2$; see under "Results"). Final bulk phospholipid concentration was 100–150 μM. Aliquots (20–140 μl) of bead suspension was incubated at room temperature for 15 min in the same buffer (total volume, 150 μl) containing 1 μM of bovine serum albumin and varying concentrations of PLA$_2$. Controls contained the same mixtures minus phospholipid-coated beads. Mixtures were centrifuged for 2 min at 12,000 × *g,* and aliquots of the supernatants were assayed for PLA$_2$ activity using 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine/POPG/1,2-*sn*-dioleoylglycerol (10:9:1 in mol ratio) mixture as a substrate as described (25). Values of n and K_d were determined by nonlinear least-squares analysis of the $[E]_b$ *versus* $[E]_o$ plot using the following equation,

$$[E]_b = \frac{[E]_o + K_d + [PL]_o/n - \sqrt{([E]_o + K_d + [PL]_o/n)^2 - 4[E]_o[PL]_o/n}}{2} \quad \text{(Eq. 1)}$$

where $[PL]_o$, $[E]_o$, and $[E]_b$ are total phospholipid, total enzyme, and bound enzyme concentrations, respectively. This equation assumes that

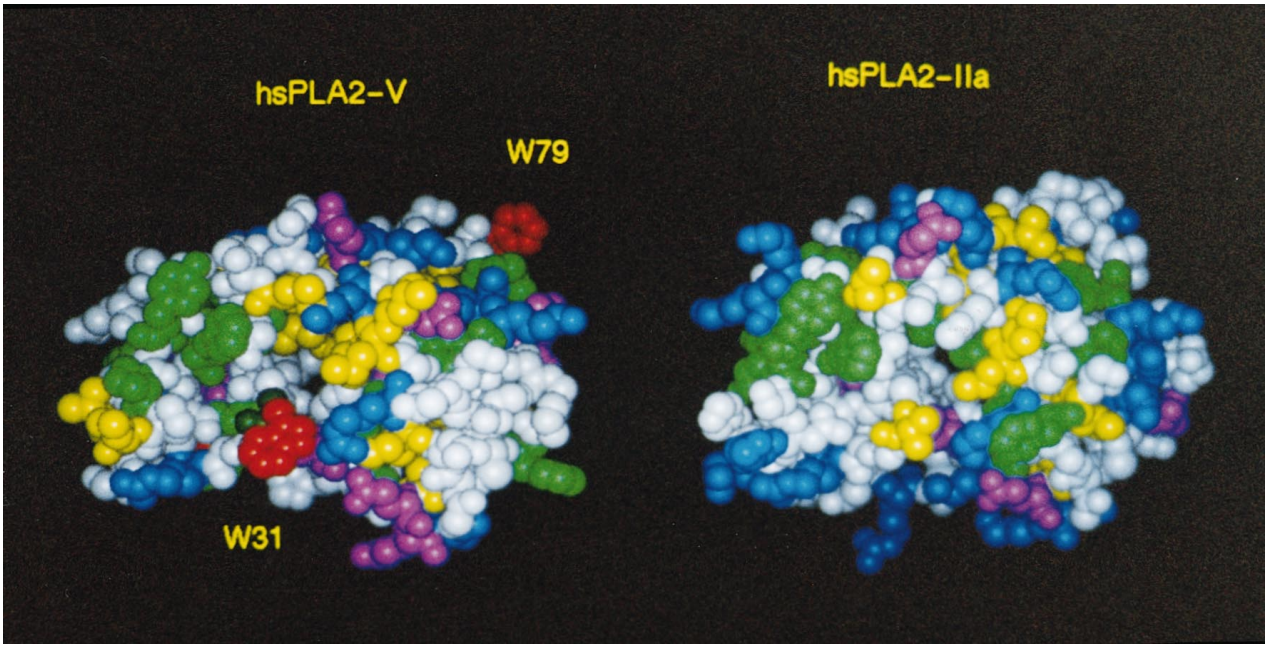

FIG. 1. **A model structure of hsPLA$_2$-V based on homology to hsPLA$_2$-IIa.** The model structure of hsPLA$_2$-V shown as a space filling representation is built on the backbone of hsPLA$_2$-IIa (42, 43) with side chain replacements using the Biopolymer program (Molecular Simulations). The molecules are oriented with their (putative) interfacial binding surfaces facing the viewer. Two mutated surface tryptophans of hsPLA$_2$-V are shown in *red* and labeled. Aliphatic side chains are shown in *yellow,* aromatic side chains in *green,* cationic side chains in *blue,* and anionic side chains in *pink*. Polar side chains and the peptide backbone are shown in *white*.

each enzyme binds independently to a site on the interface composed of n phospholipids with a dissociation constant of K_d. For anionic PG, the binding was measured in the presence of 1 mM EDTA using both POPG-coated beads and sucrose-loaded POPG vesicles. The binding to sucrose-loaded POPG vesicles was measured as described previously (22).

Monolayer Experiments—Surface pressure (π) of monolayers was measured at room temperature using a du Nouy ring as described previously (26, 27). DHPC was spread onto the subphase (20 mM Tris-HCl, pH 7.5, 0.16 M NaCl, and 10 mM Ca^{2+}) to form a monolayer with a given initial surface pressure (π_o). Then, PLA$_2$ was injected into the subphase and penetration was measured by monitoring the change in surface pressure ($\Delta\pi$). At a given π_o of phospholipid monolayer, the maximal $\Delta\ \pi$ value depended on the protein concentration in the subphase and reached a saturation when the protein concentration was above a certain value (approximately 1.5 μg/ml of hsPLA$_2$-V at π_o = 5 dyn/cm). Protein concentrations in the subphase were therefore maintained above such values to ensure that an observed $\Delta\pi$ value represents a maximum at a given π_o. The analysis of monolayer penetration data obtained under this condition was described in detail previously (25).

Fatty Acid Release from Mammalian Cells—Fatty acid release from CHO-K1 and RAW 264.7 cells and from human peripheral blood neutrophils by exogenously added PLA$_2$s was measured using a real-time fluorometric assay based on rat liver fatty acid-binding protein as described (28).

Eicosanoid Production from Neutrophils—Human neutrophils were prepared from heparinized venous blood collected from healthy medication-free donors by fractionation through centrifugation on Percoll solution for 20 min at 1000 $\times$ g, and remaining red blood cells were removed by hypotonic lysis as described previously (29). Neutrophils (1×10^6 cells/ml) were incubated at 37 °C in 250 μl of Hanks' balanced salt solution containing $CaCl_2$ (1.2 mM) and increasing concentrations (1–100 nM) of hsPLA$_2$-V, W31A, W79A, or hsPLA$_2$-IIa. Control cells were treated with Hanks' balanced salt solution. Thereafter cells were centrifuged at 8000 $\times$ g for 2 min. Leukotriene levels were determined using a leukotriene B_4 (LTB_4) enzyme immunoassay kit from Cayman Chemical Co. (Ann Arbor, MI). Typically, LTB_4 secretion reached a maximal value within 30 min of incubation under this condition. The maximal value for each incubation mixture was then corrected for a background signal from control cells.

RESULTS

Kinetic Activities of hsPLA$_2$-V and Mutants—hsPLA$_2$-V has four tryptophan residues, of which at least two are solvent-exposed according to the model structure of hsPLA$_2$-V, based on homology between it and hsPLA$_2$-IIa (Fig. 1). To assess the contribution of surface tryptophan residues of hsPLA$_2$-V to its unique ability to act on PC membranes, two surface tryptophans, Trp^{31} and Trp^{79}, were mutated to Ala. Both mutants, W31A and W78A, were refolded more effectively than wild type, suggesting that these surface tryptophans might interfere with the *in vitro* refolding of recombinant hsPLA$_2$-V. Enzymatic activities of wild type hsPLA$_2$-V and the mutants were then rigorously compared using different types of substrates: monomers, anionic and zwitterionic vesicles, and polymerized mixed liposomes. We first measured initial velocities for hsPLA$_2$-V-catalyzed hydrolysis of the soluble substrate racemic diC$_6$thio-PC (see Table I). The substrate concentration, 0.5 mM, is well below the critical micelle concentration for this short-chain phospholipid (17). The turnover numbers for hsPLA$_2$-V and its two mutants are comparable to that measured with *N. n. naja* PLA$_2$, suggesting that the recombinant hsPLA$_2$-V and mutants are correctly refolded. It is not clear whether hydrolysis of this substrate occurs via a truly monomeric enzyme-substrate complex or whether enzyme and substrate interact to form a enzyme-substrate microaggregate (30).

The turnover numbers calculated from the initial velocities for the hydrolysis of anionic DMPM vesicles by hsPLA$_2$-V, W31A and W79A, and hsPLA$_2$-IIa are given in Table I. In these assays, the cationic cyclic peptide polymyxin B was included. This additive causes rapid intervesicle exchange of DMPM, which keeps the mole fraction of nonhydrolyzed DMPM in enzyme-containing vesicles near 1 so that the initial velocity can be easily measured (24). The progress curves were linear for at least 15 min (not shown). The turnover number under these conditions for hsPLA$_2$-V is 13-fold smaller than that for hsPLA$_2$-IIa, and the mutation of Trp^{31} and Trp^{79} to Ala has only a modest effect on the catalytic efficiency. When the concentration of DMPM was doubled from 240 to 480 μM, the initial velocities changed by <5%, which proves that all of the proteins are fully bound to vesicles. Thus, the differences in the turnover numbers reported in Table I reflect differences

TABLE I
Rate constants for in vitro hydrolysis of substrates by PLA$_2$s

Turnover numbers (k_{cat}) for diC$_6$thio-PC, DMPC, and DMPM were determined from initial velocities of hydrolysis as described under "Experimental Procedures." Specificity constants ($k_{cat}*/K_m*$) for polymerized mixed liposomes were determined from the nonlinear least-squares analysis of reaction progress curves.

Enzyme	diC$_6$thio-PC	DMPM	DMPC	Pyrene-PC/BLPG	Pyrene-PG/BLPG
		k_{cat} (s^{-1})		$10^6 \times (k_{cat}*/K_m*)(M^{-1}\ s^{-1})$	
hsPLA$_2$-V	0.33 ± 0.02	1.5 ± 0.2	2.2 ± 0.2[a]	7 ± 2	7 ± 3
W31A hsPLA$_2$-V	0.26 ± 0.02	1.1 ± 0.1	0.05 ± 0.01	2 ± 1	1.5 ± 0.3
W79A hsPLA$_2$-V	0.34 ± 0.02	1.0 ± 0.1	2.4 ± 0.2[a]	6 ± 2	6 ± 3
hsPLA$_2$-IIa	ND[b]	20 ± 6	≈0.0003	≈0.2	35 ± 10
N. n. naja PLA$_2$	0.54 ± 0.03	ND	ND	ND	ND

[a] Measured after the short lag phase (see Fig. 1).
[b] ND, not determined.

in the catalytic efficiencies of the enzymes at the vesicle interface.

Fig. 2 shows the reaction progress curves for the hydrolysis of DMPC vesicles by PLA$_2$s, and Table I gives the turnover numbers. As expected from earlier studies, hsPLA$_2$-IIa showed very poor activity on zwitterionic vesicles, and this was due in part to poor binding to vesicles that lack negative charge (8, 21). The progress curve with porcine pancreatic PLA$_2$ shows the classical lag phase, which is due in part to poor binding of enzyme to non-hydrolyzed vesicles and product-dependent binding of enzyme leading to rate acceleration (31). Even in the presence of 13.5 μg of porcine pancreatic PLA$_2$, the initial rate was barely detectable, and then the velocity accelerated dramatically after about 10 min (Fig. 2). Interestingly, wild type and W79A hsPLA$_2$-V were highly active on DMPC vesicles, and only a very short lag was seen (Fig. 2). The turnover numbers for both proteins measured after the short lag were approximately 7000-fold larger than that for hsPLA$_2$-IIa (Table I). This is in marked contrast to the results with DMPM vesicles, in which hsPLA$_2$-IIa is the more active enzyme. Mutation of Trp^{31} to Ala reduced the turnover number by 44-fold. Thus, Trp^{31} seems to be a key residue for promoting high activity of hsPLA$_2$-V on zwitterionic vesicles.

We also measured the activity of hsPLA$_2$-V and mutants on anionic polymerized mixed liposomes. In polymerized mixed liposome system, it is possible to accurately determine the head group specificity of PLA$_2$ by varying the head group structure of hydrolyzable phospholipids in an inert polymerized matrix. Two phospholipids, pyrene-PC and pyrene-PG, were used as inserts in the BLPG polymerized matrix. As reported previously (14), hsPLA$_2$-V has comparable activities on pyrene-PC and pyrene-PG, whereas hsPLA$_2$-IIa has much lower activity on pyrene-PC. As a result, hsPLA$_2$-V is about 35 times more active than hsPLA$_2$-IIa on pyrene-PC/BLPG polymerized mixed liposomes and 5 times less active than hsPLA$_2$-IIa on pyrene-PG/BLPG polymerized mixed liposomes. Most importantly, the effects of W31A and W79A mutations on the activities of hsPLA$_2$-V on the two polymerized mixed liposomes are comparable, indicating that the mutations have no effect on the head group specificity of hsPLA$_2$-V. Thus, the effects of W31A mutation on kinetic activities of hsPLA$_2$-V are solely due to the reduced interfacial binding. All together, the data in Table I indicate that the dramatically lower activity of hsPLA$_2$-IIa on PC *versus* anionic PG interfaces is due to a combination of its poor interfacial binding to zwitterionic interfaces and lower preference of its catalytic site for PC. Also, the data indicate that hsPLA$_2$-V has much greater activity on PC than does hsPLA$_2$-IIa because of its higher affinity for zwitterionic interfaces and comparable affinity of its active site for PC and anionic phospholipids.

Membrane Affinities of hsPLA$_2$-V and Mutants—To further study how Trp^{31} promotes the high activity of hsPLA$_2$-V on zwitterionic PC vesicles, we measured the binding affinity of wild type and mutants for PC- and PG-coated beads. Phospholipid-coated hydrophobic beads have been shown to be useful in determining the membrane affinity of PLA$_2$s (9). In particular, this model membrane allows rapid and accurate measurement of PC affinity, which normally is difficult to achieve with PC vesicles due to their low pelleting efficiency compared with anionic vesicles (data not shown).

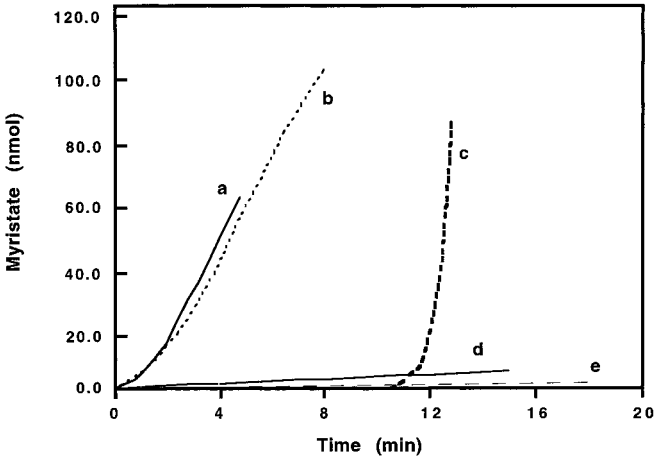

FIG. 2. **Reaction progress curves for the hydrolysis of DMPC vesicles by PLA$_2$s.** *Curve a,* 2 μg of W79A hsPLA$_2$-V; *curve b*, 2 μg of wild type hsPLA$_2$-V; *curve c,* 13.5 μg of porcine pancreatic PLA$_2$; *curve d,* 2 μg of W31A hsPLA$_2$-V; *curve e,* 60 μg of hsPLA$_2$-IIa. Reaction conditions are given under "Experimental Procedures."

We first measured the binding affinity of hsPLA$_2$-V for beads coated with DHPC, a nonhydrolyzable ether analog of PC. Note that K_d is expressed in terms of molarity of enzyme binding sites composed of n phospholipids (Equation 1). Thus, nK_d is the dissociation constant in terms of molarity of lipid molecules and the relative binding affinity can be best described in terms of relative values of ($1/nK_d$). As shown in Fig. 3, hsPLA$_2$-V had relatively high affinity for DHPC-coated beads in the presence and absence of Ca^{2+}; nK_d = 1.5 ± 0.3 μM with 10 mM Ca^{2+} and = 1.8 ± 0.3 μM with 0.1 mM EDTA. Under the same conditions, hsPLA$_2$-IIa showed much lower affinity (*i.e.* $nK_d >$ 100 μM).

The Ca^{2+} independence of the interfacial binding of hsPLA$_2$-V allowed us to measure the binding to PC- and PG-coated beads in the absence of Ca^{2+} using natural phospholipids instead of ether analogs. hsPLA$_2$-V showed essentially the same affinity for DHPC and POPC-coated beads in the absence of Ca^{2+} (see Fig. 3). Thus, we measured the relative affinity of wild type hsPLA$_2$-V and mutants for beads coated with readily available POPC and POPG in the absence of Ca^{2+} (*i.e.* with 0.1 mM EDTA). nK_d and relative affinity values are summarized in Table II. The relative affinity is calculated as the ratio of $1/nK_d$

value for $hsPLA_2$-V/POPC-coated beads binding to that for other enzyme/lipid combinations. Compared with wild type $hsPLA_2$-V, W31A mutant bound 14 times less tightly to the PC-coated beads, whereas W79A retained about one-half of the wild type affinity. This indicates that Trp^{31} plays an important role in the binding of $hsPLA_2$-V to PC membranes, whereas Trp^{79} is not directly involved in the process. $hsPLA_2$-V showed about 15-fold higher binding for POPG-coated beads than for POPC-coated beads. This is much smaller than the >200-fold increase in binding observed for $hsPLA_2$-IIa. $hsPLA_2$-V had 2-fold higher affinity for PG membranes than did $hsPLA_2$-IIa. Thus, $hsPLA_2$-V has high intrinsic affinity for both zwitterionic and anionic membranes, whereas $hsPLA_2$-IIa has high affinity only for anionic interfaces. Unlike the case with PC-coated beads, the effect of the Trp^{31} to Ala mutation on the affinity of $hsPLA_2$-V for PG-coated beads was not pronounced (approximately a 2.3-fold drop) and comparable to the effect of the Trp^{79} to Ala mutation (3.8-fold decrease). This indicates that Trp^{31} plays a less critical role in the binding of $hsPLA_2$-V to anionic surfaces, which may be driven in part by electrostatic interactions.

Finally, we measured the binding of enzymes to sucrose-loaded POPG to check the validity of our binding data using phospholipid-coated beads. As shown in Table II, all PLA_2s showed 3–4-fold higher affinity for POPG vesicles than for POPG-coated beads, which presumably reflects different surface packing density and curvature of the two model membranes. Importantly, the relative affinity of PLA_2s for POPG vesicles was essentially the same as that for POPG-coated beads, demonstrating the validity of our uses of phospholipid-coated beads to quantify the affinity of PLA_2s to biological membranes.

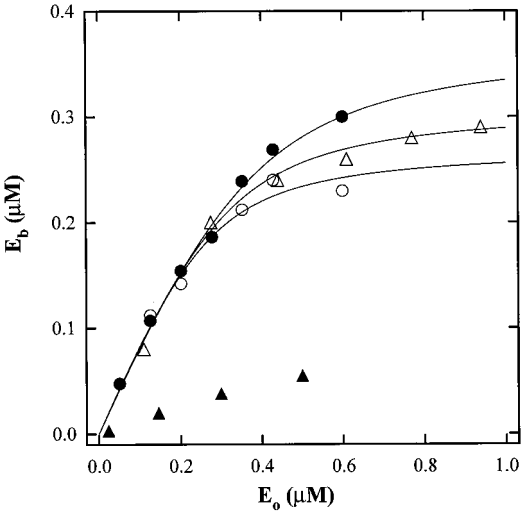

FIG. 3. **Binding isotherms of $hsPLA_2$-V and $hsPLA_2$-IIa.** The binding of $hsPLA_2$-V to DHPC-coated beads (10 μM of bulk phospholipid concentration) was measured in the presence of 10 mM Ca^{2+} (○) or 0.1 mM EDTA (●). The binding of $hsPLA_2$-V (△) and $hsPLA_2$-IIa (▲) to POPC-coated beads (10 μM) was measured in the presence of 0.1 mM EDTA. Each point represents an average of duplicate measurements. *Solid lines* are theoretical curves constructed using Equation 1 with experimentally determined n and K_d values.

Monolayer Penetration of $hsPLA_2$-V and Mutants—To better understand how Trp^{31} contributes to interfacial binding of $hsPLA_2$-V, we measured the penetration of wild type $hsPLA_2$-V and mutants into the DHPC monolayer at the air-water interface. In these studies, a phospholipid monolayer of a given initial surface pressure (π_o) was spread at constant area and the change in surface pressure ($\Delta\pi$) was monitored after injection of protein into the subphase. Fig. 4 shows that W31A penetrates into DHPC monolayer significantly less effectively than wild type and W79A over a wide range of π_o. The monolayer penetration ability of W31A was similar to that of $hsPLA_2$-IIa, which has extremely low activity on PC monolayers and bilayers. This data thus suggests that the higher activity of $hsPLA_2$-V on PC membranes derives from the ability of Trp^{31} to partially penetrate into zwitterionic PC membranes, thereby making favorable interfacial interactions.

Activities of $hsPLA_2$-V and Mutants to Release Fatty Acids and Eicosanoids from Cells—The extracellular face of the plasma membrane of mammalian cells is largely composed of zwitterionic PC and sphingomyelin. Thus $hsPLA_2$-V, which has higher affinity and activity for PC membranes than does $hsPLA_2$-IIa, might show relatively high activity on the outer cell membrane. The activities of $hsPLA_2$-V and mutants as well as $hsPLA_2$-IIa, added exogenously to the mammalian cell lines including RAW264.7 and CHO-K1, were measured by monitoring fatty acid release. Also, *N. n. naja* PLA_2 which is highly active on PC vesicles and intact cells was studied for comparison. As summarized in Table III, $hsPLA_2$-V was 20–30 times more active than $hsPLA_2$-IIa but 15–20-fold less active than *N. n. naja* PLA_2 on these cells. Also, W31A $hsPLA_2$-V had 10–30% of the wild type activity, and W79A showed only a modest decrease in activity. Thus, both the difference in activity between $hsPLA_2$-IIa and $hsPLA_2$-V and the effects of tryptophan mutations on the activity of $hsPLA_2$-V were less pronounced in cell assays than in vesicle assays. It should be noted, however, that there are differences between vesicles and complex cell membranes, including the fact that the latter has some outer layer anionic lipids. Thus, the qualitative correlation between the activities of wild type and mutants determined from cell and vesicle assays supports the notion that the high activity of $hsPLA_2$-V to release fatty acids from mammalian cells derives from its ability to avidly bind PC membranes and that Trp^{31} plays an important role.

We also measured the release of fatty acids and LTB_4 from human neutrophils by various exogenously added $sPLA_2$s, including $hsPLA_2$-V and mutants, to see whether their activity to release fatty acids from the outer cell membrane is correlated to their ability to elicit cellular eicosanoid production. LTB_4 is a major eicosanoid produced by neutrophils upon activation by inflammatory agonists. All $sPLA_2$s showed comparable activities on neutrophils, RAW264.7, and CHO-K1 cells. As shown in

TABLE II
Membrane binding affinities of wild type and mutant $hsPLA_2$-V and $hsPLA_2$-IIa

Values represent the mean and S.D. of triplicate determinations. Relative affinity is the ratio of $1/nK_d$ value of $hsPLA_2$-V for POPC-coated beads to that for other enzyme/lipid combinations.

Enzyme	POPC-coated beads		POPG-coated beads		POPG vesicles	
	nK_d	Relative affinity	nK_d	Relative affinity	nK_d	Relative affinity
	μM		μM		μM	
$hsPLA_2$-V	1.6 ± 0.3	1	0.13 ± 0.04	12.3	0.04 ± 0.03	40
W31A	24.5 ± 0.5	0.07	0.30 ± 0.05	5.3	0.12 ± 0.03	13.3
W79A	3.8 ± 0.1	0.42	0.50 ± 0.06	3.2	0.3 ± 0.1	5.3
$hsPLA_2$-IIa	>70	<0.02	0.38 ± 0.06	4.2	0.10 ± 0.07	16

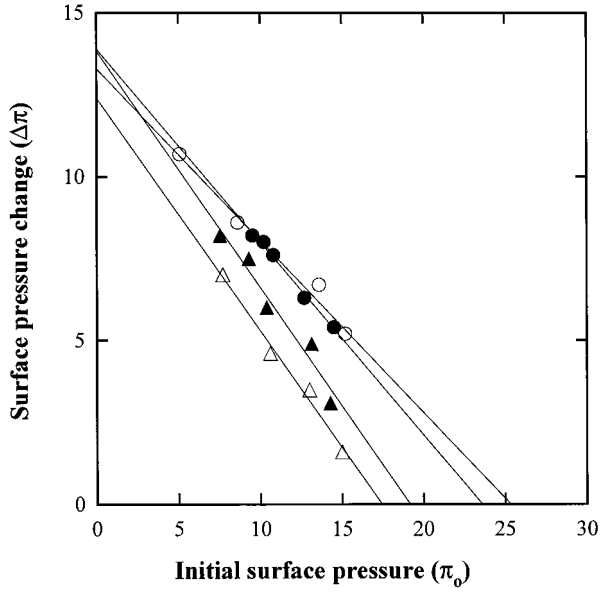

FIG. 4. **Penetration of $hsPLA_2$-V (○), W31A (▲), W79A (●) and $hsPLA_2$-IIa (△) into the DHPC monolayer as a function of initial surface pressure of monolayer.** The subphase contained 20 mM Tris-HCl, pH 7.5, 0.16 M NaCl, and 10 mM Ca^{2+}. Enzyme concentrations were 0.12 μM. *Solid lines* were obtained by the linear regression of experimental data. Each point represents an average of duplicate measurements

TABLE III
Cellular activities of $hsPLA_2$-V, its tryptophan mutants, and $hsPLA_2$-IIa

Values represent the mean of triplicate determinations.

Enzyme	Relative fatty acid release activity			Relative LTB_4 release activity, neutrophils[a]
	RAW264.7	CHO-K1 cells	Neutrophils	
$hsPLA_2$-V	1[b]	1[b]	1[b]	1
W31A	0.31	0.14	0.23	0.29
W79A	0.65	0.75	0.60	0.77
$hsPLA_2$-IIa	0.05	0.03	0.10	0.10
N. n. naja PLA_2	21	16	ND[c]	ND

[a] Determined with 100 nM enzymes; an absolute value for $hsPLA_2$-V was 190 pg/10^6 cells.
[b] Absolute values for $hsPLA_2$-V (μmol of fatty acid released/min/mg of PLA_2): 1.2 ± 0.32 (RAW 264.7 cells), 0.69 ± 0.079 (CHO-K1 cells), and 1.0 ± 0.5 (neutrophils).
[c] ND, not determined.

Fig. 5, $hsPLA_2$-V elicited LTB_4 production in a concentration-dependent manner from unstimulated human neutrophils. In contrast, $hsPLA_2$-IIa showed less than 10% of the $hsPLA_2$-V activity under the same conditions. $hsPLA_2$-V (100 nM) released 190 pg of LTB_4 per 10^6 cells, which is half of the maximal amount of LTB_4 release (400 pg/10^6 cells) caused by the potent activators, f-MLP (1 μM) + cytochalasin B (5 μg/ml), under the same conditions. At the same concentration, W79A and W31A showed 77 and 29% of the wild type activity, respectively. Overall, an excellent correlation was observed between the relative activity of $sPLA_2$s to release of fatty acids and that to elicit LTB_4 production from neutrophils. Taken together, these results indicate that $hsPLA_2$-V has higher activities to release fatty acids, including arachidonic acid, from the outer cell membrane and to elicit cellular eicosanoid production than does $hsPLA_2$-IIa and that Trp^{31} plays an important role in these activities.

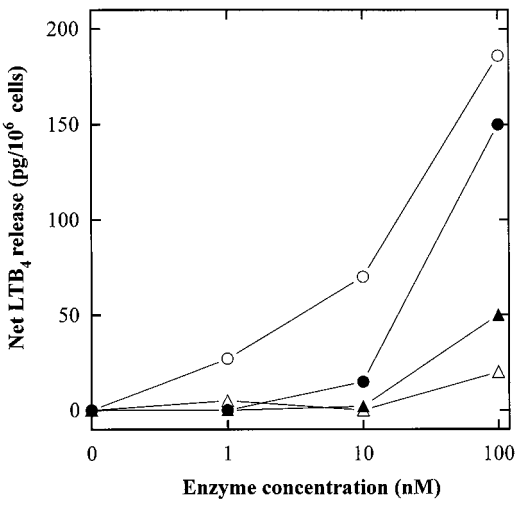

FIG. 5. **Dose-dependent LTB_4 release from human neutrophils by exogenous $hsPLA_2$-V (○), W31A (▲), W79A (●), and $hsPLA_2$-IIa (△).** Incubation mixtures at 37 °C contain neutrophils (0.5×10^6 cells) and varying concentration of enzymes in Hanks' balanced salt solution. LTB_4 levels were determined after 30 min incubation. Each point represents an average of quadruple measurements.

DISCUSSION

This study demonstrates that $hsPLA_2$-V is more active than $hsPLA_2$-IIa by up to 4 orders of magnitude in hydrolyzing PC-rich membranes, including the outer plasma membrane of mammalian cells. $sPLA_2$s have a common interfacial binding surface that is located on the flat external surface surrounding the active site slot. Many $sPLA_2$s, including $hsPLA_2$-IIa, prefer anionic membranes due in part to the presence of cationic residues on the interfacial binding surface. Only a subset of $sPLA_2$s (*e.g.* cobra PLA_2s) that contain a number of aromatic residues, Trp in particular, on their interfacial binding surfaces can effectively bind and hydrolyze PC membranes. For instance, *N. n. naja* PLA_2 has three tryptophans on its putative interfacial binding surface, and this may be the reason it shows the highest activity and affinity for PC membrane and intact cells (32). Also, mutational analyses of several $sPLA_2$s demonstrated the importance of surface tryptophans in interfacial binding (33–35). In particular, the addition of a single tryptophan to the interfacial binding surface of $hsPLA_2$-IIa enhances its activity on PC membranes by more than 2 orders of magnitude (35). Mammalian group V PLA_2s contain multiple tryptophans (three for mouse and rat enzymes and four for $hsPLA_2$-V) among which Trp^{31} and Trp^{43} are conserved (13). The model structure of $hsPLA_2$-V illustrated in Fig. 1 shows that Trp^{31} is located in the center of its putative interfacial binding surface, thereby suggesting its critical involvement in interfacial binding. This study shows that Trp^{31} indeed plays an essential role in the binding of $hsPLA_2$-V to membranes, zwitterionic PC membranes in particular, whereas Trp^{79} located on the opposite face is involved neither in interfacial binding nor in catalytic steps. Reduced enzymatic activity, vesicle binding affinity and monolayer penetration power of W31A compared with wild type enzyme show that Trp^{31} enhances the binding of $hsPLA_2$-V to membranes, whether zwitterionic or anionic, by partially penetrating into membranes and thereby achieving optimal interactions with membranes, which involve a complex combination of hydrophobic and electrostatic interactions (36).

$hsPLA_2$-V also contains several cationic residues in its putative interfacial binding surface (see Fig. 1). The fact that $hsPLA_2$-V prefers anionic PG membranes to PC membranes suggests the importance of these cationic residues in its membrane binding and possibly cell surface binding (5). However, other features in addition to electrostatics are involved in promoting the relatively high affinity of $sPLA_2$s for anionic *versus*

zwitterionic membranes (37). Higher affinity of $hsPLA_2$-V for anionic membranes than $hsPLA_2$-IIa despite the smaller number of cationic interfacial binding residues again underscores the contribution of nonelectrostatic effects. Presumably, Trp^{31} and other non-ionic residues also make a significant contribution to the binding of $hsPLA_2$-V to anionic membranes.

As shown in Fig. 2, the hydrolysis of PC membranes by porcine pancreatic PLA_2 is preceded by a long lag, which corresponds to an accumulation of a critical amount of reaction products, one of which is anionic fatty acid. Jain and Berg (31) have shown that addition of PLA_2 reaction products to PC vesicles greatly promotes the binding of the porcine enzyme to the interfaces, and this binding enhancement provides a basis for the rate acceleration. Cobra venom PLA_2s bind tightly to PC vesicles, and no lag in the hydrolysis of PC vesicle is seen (31). $hsPLA_2$-V binds less tightly to PC membranes than do cobra venom PLA_2s but much more tightly than do $hsPLA_2$-IIa and porcine pancreatic PLA_2, which is consistent with a short lag seen in the hydrolysis of PC vesicles by $hsPLA_2$-V. The unique ability of $hsPLA_2$-V to avidly bind both zwitterionic and anionic membranes would not only shorten the lag for the initial hydrolysis of PC membranes but also allow the enzyme to remain on membrane surfaces as the hydrolysis progresses with the accumulation of anionic fatty acids. This might account for the larger effect of the W31A mutation on the kinetics of DMPC hydrolysis (44-fold) than on the binding to PC membranes (15-fold). Furthermore, $hsPLA_2$-V has an additional kinetic advantage over $hsPLA_2$-IIa in that the catalytic site of the former can accommodate both zwitterionic and anionic phospholipids whereas the catalytic site of the latter discriminates against PC (Table I). Note that the catalytic activity of $hsPLA_2$-V is approximately 13 times and 5 times lower than that of $hsPLA_2$-IIa toward DMPM vesicles and pyrene-PG/BLPG polymerized mixed liposomes, respectively, under conditions where all enzymes are bound to vesicles. This difference in intrinsic catalytic activity on anionic phospholipids is presumably due to their different active site structures. Our previous mutagenesis study of $hsPLA_2$-IIa showed that the mutation of its Lys^{69}, which forms a hydrogen bond with the *sn*-3 phosphate of substrate, to Arg resulted in a 5-fold reduction in catalytic activity toward anionic phospholipids (21). Sequence comparison of $hsPLA_2$-IIa and $hsPLA_2$-V reveals that the latter has Arg in place of Lys in position 69 (13). The origin of unique substrate head group specificity of $hsPLA_2$-V is currently under investigation.

Our data show good correlation between the *in vitro* ability of $sPLA_2$s to act on model membranes, such as vesicles, and their activity on complex cell membranes. Such a correlation has also been observed for the action of a panel of $hsPLA_2$-IIa mutants on vesicles and cell membranes (28). Exogenously added $hsPLA_2$-IIa, due to its low interfacial and active-site affinity for zwitterionic phospholipids, has low activity to release fatty acids from mammalian cells and cannot effectively elicit eicosanoid formation from unstimulated neutrophils. In contrast, exogenous $hsPLA_2$-V can effectively hydrolyze phospholipids in the outer plasma membranes of mammalian cells and can also induce a significant degree of eicosanoid formation from unstimulated neutrophils.

$sPLA_2$s, including $hsPLA_2$-V, have little *sn*-2 arachidonoyl specificity because their active sites can hold only about nine carbons in the *sn*-2 acyl chain (38). Thus, high activity of $hsPLA_2$-V to elicit eicosanoid formation should derive from its ability to bind to the outer plasma membrane and release various fatty acids from membrane phospholipids including arachidonic acid. This notion is consistent with a recent finding that exogenous arachidonic acid is rapidly transported across the neutrophil plasma membrane via a protein-facilitated mechanism (39). Due to the inability of $hsPLA_2$-IIa to directly act on intact cell membranes, several activation mechanisms have been proposed to account for its interactions with cells. They include membrane perturbation, including cell surface exposure of aminophospholipids (*e.g.* phosphatidylserine and -ethanolamine) (40), prior activation of $cPLA_2$ (4, 5), and binding to cell surfaces via heparinoids (41). Our results would suggest that none of these activation steps are essential for the action of $hsPLA_2$-V on CHO-K1, RAW264.7, and human neutrophils, thereby suggesting that under the physiological conditions, $hsPLA_2$-V can effectively act on unprimed mammalian cells. The precise physiological significance of the action of $hsPLA_2$-V on neutrophils was not elucidated in this investigation. Neutrophils are not known to have high $sPLA_2$ activities. However, the close proximity of these cells to mast cells and macrophages, which secrete $sPLA_2$s, including $hsPLA_2$-V during many inflammatory processes (10, 11), and the ability of $hsPLA_2$-V to elicit substantial LTB_4 synthesis and secretion in nanomolar concentrations, suggest a possible paracrine mechanism of neutrophilic inflammation. Further studies are required to elucidate the specific physiological role of this unique PLA_2 isoform in granulocytic inflammation.

Acknowledgment—W. Cho thanks Dr. Marianne Schiffer for suggestions on the folding and stability of mutants.

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