Roles of Trp$^{31}$ in High Membrane Binding and Proinflammatory Activity of Human Group V Phospholipase A$_2^*$

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Group V phospholipase A$_2$ (PLA$_2$) 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$_2$S (PLA$_2$)$^3$ are a family of lipolytic enzymes

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The abbreviations used are: BLPG, 1,2-bis[12-(lipoyloxy)dodecanoyl]-sn-glycero-3-phosphorylcholine; 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$_6$-thio-PC, 1,2-di(hexadecanoyl)sn-glycero-3-phosphorylcholine; DHPC, 1,2-dihexadecyl-sn-glycero-3-phosphoglycerol; LTB$_4$, leukotriene B$_4$; PG, phosphatidylglycerol; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; pyrene-PC, 1-hexadecanoyl-2-(1-pyrene-decanoyl)-sn-glycero-3-phosphoglycerol; DIPC, 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine; DHPC, 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine.
ide (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-(lipoyl)do-decanoyl]-sn-glycero-3-phosphocholine (BisPG) and -glycerol (BisPG) were from Avanti Polar Lipids. Polyvinyl sulfate, Naja naja naja venom PLA$_2$, 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine (DMPC), and -methanol (DMPM), and 1,2-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine were from Avanti Polar Lipids. Polyvinyl sulfate and sodium sulfite, and stirred vigorously at room temperature for 30 min. The pellet 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 0.25 m guanidinium chloride. The final precipitation protein was monitored spectrophotometrically 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 $k_{o}$/$R_{m}$ were determined from reaction progress curves as described previously (22). Enzymatic hydrolysis of DMPC and DMPM vesicles was monitored by 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. DMPC reaction mixtures contained 4 ml of 1 mM NaCl, 1 mM CaCl$_2$, 20 mM DMPM, 20 mM DMPM at pH 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 diC$_{18}$-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-streoyl-stearoyl-benzenes were allowed to concentrate and completely separated from the solution by low speed centrifugation. Interactions were measured by the PEG precipitation method (20). Final bulk phospholipid concentration was 100–150 μmol. 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 μmol 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-dioleylglycerol (10:9:1 in mol ratio) mixture as a substrate as described (25). Values of $k_{o}$ and $R_{m}$ were determined by nonlinear least-squares analysis of the $[E]_b$ versus $[E]$, plot using the following equation,

$$[E]_b = \left[ [E] + K_e + [PL]/n \right]/\left[ [E] + K_e + [PL]/n \right]^2 - 4[E][PL]/n^2$$

(Eq. 1)

where $[PL]$, $[E]_b$, and $[E]_o$ are total phospholipid, total enzyme, and bound enzyme concentrations, respectively. This equation assumes that...
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 ($\pi$) of monolayers was measured at room temperature using a de Noy 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 CaCl$_2$) to form a monolayer with a given initial surface pressure ($\pi_i$). Then, PLA$_2$ was injected into the subphase and penetration was measured by monitoring the change in surface pressure ($\Delta\pi$). At a given $\pi_i$ 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 $\mu$g/ml of hsPLA$_2$-V at $\pi_i = 5$ 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 $\pi_i$. 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 previously (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 X g. and remaining red blood cells were removed by hypotonic lysis as described previously (29). Neutrophils (1 X 10$^6$ cells/ml) were incubated at 37 °C in 250 $\mu$l of Hanks’ balanced salt solution containing CaCl$_2$ (1.2 mM) and increasing concentrations (1-100 nM) of hsPLA$_2$-V, W31A, W78A, or hsPLA$_2$-IIa. Control cells were treated with Hanks’ balanced salt solution. Thereafter cells were centrifuged at 8000 X 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 efficiently 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. 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 W78A, and hsPLA$_2$-IIa are given in Table I. In these assays, the cationic cyclic peptide polymyxin B was included. This additive causes rapid intervesicular 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 $\mu$M, 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

![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.](image)
indicate that the dramatically lower activity of hsPLA2-IIa on reduced interfacial binding. All together, the data in Table I
hsPLA2-IIa because of its higher affinity for zwitterionic inter-
antly, the effects of W31A and W79A mutations on the activ-
pyrene-PG/BLPG polymerized mixed liposomes. Most impor-
mutually after about 10 min (Fig. 2). Interestingly, wild type
and W79A hsPLA2-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 approxi-
mately 7000-fold larger than that for hsPLA2-IIa (Table I). This
is in marked contrast to the results with DMPM vesicles, in
which hsPLA2-IIa is the more active enzyme. Mutation of Trp31
to Ala reduced the turnover number by 44-fold. Thus, Trp31
seems to be a key residue for promoting high activity of
hsPLA2-V on zwitterionic vesicles.

We also measured the activity of hsPLA2-V and mutants on
anionic polymerized mixed liposomes. In polymerized mixed
liposome system, it is possible to accurately determine the head
group specificity of PLA2 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 previ-
ously (14), hsPLA2-V has comparable activities on pyrene-PC
and pyrene-PG, whereas hsPLA2-IIa has much lower activity
on pyrene-PC. As a result, hsPLA2-V is about 35 times more
active than hsPLA2-IIa on pyrene-PC/BLPG polymerized
mixed liposomes and 5 times less active than hsPLA2-IIa on
pyrene-PG/BLPG polymerized mixed liposomes. Most impor-
tantly, the effects of W31A and W79A mutations on the activ-
ities of hsPLA2-V on the two polymerized mixed liposomes are
comparable, indicating that the mutations have no effect on the
head group specificity of hsPLA2-V. Thus, the effects of W31A
mutation on kinetic activities of hsPLA2-V are solely due to the
reduced interfacial binding. All together, the data in Table I
indicate that the dramatically lower activity of hsPLA2-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 hsPLA2-V has much greater activity on PC than does
hsPLA2-IIa because of its higher affinity for zwitterionic inter-
faces and comparable affinity of its active site for PC and
anionic phospholipids.

Membrane Affinities of hsPLA2-V and Mutants—To further
study how Trp31 promotes the high activity of hsPLA2-V on
zwitterionic PC vesicles, we measured the binding affinity of
wild type and mutants for PC- and PG-coated beads. Phospho-
lipid-coated hydrophobic beads have been shown to be useful in
determining the membrane affinity of PLA2s (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).

We first measured the binding affinity of hsPLA2-V for beads
coated with DHPC, a nonhydrolyzable ether analog of PC. Note
that Kd is expressed in terms of molarity of enzyme binding
sites composed of n phospholipids (Equation 1). Thus, nKd 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/nKd). As shown in Fig. 3, hsPLA2-V had
relatively high affinity for DHPC-coated beads in the presence
and absence of Ca2+. nKd = 1.5 ± 0.3 μM with 10 mM Ca2+
and = 1.8 ± 0.3 μM with 0.1 mM EDTA. Under the same conditions,
hsPLA2-IIa showed much lower affinity (i.e. nKd >
100 μM).

The Ca2+ independence of the interfacial binding of
hsPLA2-V allowed us to measure the binding to PC- and PG-
coated beads in the absence of Ca2+ using natural phospholip-
ids instead of ether analogs. hsPLA2-V showed essentially the
same affinity for DHPC and POPC-coated beads in the absence
of Ca2+ (see Fig. 3). Thus, we measured the relative affinity of
wild type hsPLA2-V and mutants for beads coated with readily
available POPC and POPG in the absence of Ca2+ (i.e. with 0.1
mm EDTA). nKd and relative affinity values are summarized in
Table II. The relative affinity is calculated as the ratio of 1/nKd.

Table I

| Enzyme            | diC6thio-PC | DMPM | DMPC | Pyrene-PC/BLPG | Pyrene-PG/BLPG |
|-------------------|-------------|------|------|----------------|----------------|
| hsPLA2-V          | 0.33 ± 0.02 | 1.5 ± 0.2 | 2.2 ± 0.2 | 7 ± 2          | 7 ± 3          |
| W31A hsPLA2-V     | 0.26 ± 0.02 | 1.1 ± 0.1 | 0.05 ± 0.01 | 2 ± 1          | 1.5 ± 0.3      |
| W79A hsPLA2-V     | 0.34 ± 0.02 | 1.0 ± 0.1 | 2.4 ± 0.2   | 6 ± 2          | 6 ± 3          |
| hsPLA2-IIa        | ND          | 20 ± 6 | ~0.0003 | 35 ± 10        | ND             |
| N. n. naja PLA2   | 0.54 ± 0.03 | ND   | ND   | ND            | ND             |

a Measured after the short lag phase (see Fig. 1).

b ND, not determined.
value for hsPLA₂-V/POPC-coated beads binding to that for other enzyme/lipid combinations. Compared with wild type hsPLA₂-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 Trp₃¹ plays an important role in the binding of hsPLA₂-V to PC membranes, whereas Trp₇⁹ is not directly involved in the process. hsPLA₂-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 hsPLA₂-IIa. hsPLA₂-V had 2-fold higher affinity for PG membranes than did hsPLA₂-IIa. Thus, hsPLA₂-V has high intrinsic affinity for both zwitterionic and anionic membranes, whereas hsPLA₂-IIa has high affinity only for anionic interfaces. Unlike the case with PC-coated beads, the effect of the Trp₃¹ to Ala mutation on the affinity of hsPLA₂-V for PG-coated beads was not pronounced (approximately a 2.3-fold drop) and comparable to the effect of the Trp₇⁹ to Ala mutation (3.8-fold decrease). This indicates that Trp₃¹ plays a less critical role in the binding of hsPLA₂-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 PLA₂s loaded POPG to check the validity of our binding data using phospholipid-coated beads. As shown in Table II, all PLA₂s 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 PLA₂s 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 PLA₂s to biological membranes.

**Monolayer Penetration of hsPLA₂-V and Mutants**—To better understand how Trp₃¹ contributes to interfacial binding of hsPLA₂-V, we measured the penetration of wild type hsPLA₂-V and mutants into the DHPC monolayer at the air-water interface. In these studies, a phospholipid monolayer of a given initial surface pressure (πₒ) was spread at constant area and the change in surface pressure (Δπ) 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 πₒ. The monolayer penetration ability of W31A was similar to that of hsPLA₂-IIa, which has extremely low activity on PC monolayers and bilayers. This data thus suggests that the higher activity of hsPLA₂-V on PC membranes derives from the ability of Trp₃¹ to partially penetrate into zwitterionic PC membranes, thereby making favorable interfacial interactions.

**Activities of hsPLA₂-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 hsPLA₂-V, which has higher affinity and activity for PC membranes than does hsPLA₂-IIa, might show relatively high activity on the outer cell membrane. The activities of hsPLA₂-V and mutants as well as hsPLA₂-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 PLA₂, which is highly active on PC vesicles and intact cells was studied for comparison. As summarized in Table III, PLA₂-V was 20–30 times more active than hsPLA₂-IIa but 15–20-fold less active than N. n. naja PLA₂, on these cells. Also, W31A hsPLA₂-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 hsPLA₂-IIa and hsPLA₂-V and the effects of tryptophan mutations on the activity of hsPLA₂-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 hsPLA₂-V to release fatty acids from mammalian cells derives from its ability to avidly bind PC membranes and that Trp₃¹ plays an important role.

We also measured the release of fatty acids and LTB₄ from human neutrophils by various exogenously added sPLA₂s, including hsPLA₂-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. LTB₄ is a major eicosanoid produced by neutrophils upon activation by inflammatory agonists. All sPLA₂s showed comparable activities on neutrophils, RAW264.7, and CHO-K1 cells. As shown in

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**Table II**

Membrane binding affinities of wild type and mutant hsPLA₂-V and hsPLA₂-IIa

Values represent the mean and S.D. of triplicate determinations. Relative affinity is the ratio of 1/nKₐ value of hsPLA₂-V for POPG-coated beads to that for other enzyme/lipid combinations.

| Enzyme     | POPC-coated beads | POPG-coated beads | POPG vesicles |
|------------|-------------------|-------------------|--------------|
|            | nKₐ             | Relative affinity | nKₐ            | Relative affinity | nKₐ             | Relative affinity |
| hsPLA₂-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            |
| hsPLA₂-IIa | >70              | <0.02             | 0.38 ± 0.06   | 4.2             | 0.10 ± 0.07     | 16             |

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FIG. 3. Binding isotherms of hsPLA₂-V and hsPLA₂-IIa. The binding of hsPLA₂-V to DHPC-coated beads (10 μM of bulk phospholipid concentration) was measured in the presence of 10 mM Ca²⁺ (○) or 0.1 mM EDTA (●). The binding of hsPLA₂-V (□) and hsPLA₂-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 Kₐ values.
**FIG. 4.** Penetration of hsPLA$_2$-V (○), W31A (●), W79A (■) and hsPLA$_2$-IIa (△) into the DOPC 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.

**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 quadruplicate measurements.

**TABLE III**

| Enzyme | Relative fatty acid release activity | Relative LTB$_4$ release activity, neutrophils |
|--------|-------------------------------------|-----------------------------------------------|
| hsPLA$_2$-V | RAW264.7 | CHO-K1 cells | Neutrophils | |
| W31A | 1$^a$ | 1$^b$ | 1$^b$ | 1 |
| W79A | 0.31 | 0.14 | 0.23 | 0.29 |
| hsPLA$_2$-IIa | 0.65 | 0.75 | 0.60 | 0.77 |
| N. n. naja PLA$_2$ | 0.05 | 0.03 | 0.10 | 0.30 |

$^a$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.

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$_2$s) 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$_2$s contain multiple tryptophans (three for mouse and rat enzymes and four for hsPLA$_2$-V) among which Trp$^{31}$ and Trp$^{34}$ 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...
substrate head group specificity of hsPLA2-V is currently under investigation. The origin of unique properties of hsPLA2-V is possibly due to its Thr→Ala mutation at position 69 (13). The unique ability of hsPLA2-V to avidly bind both zwitterionic and anionic phospholipids 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, hsPLA2-V has an additional kinetic advantage over hsPLA2-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 hsPLA2-V is approximately 13 times and 5 times lower than that of hsPLA2-IIa toward DMPM vesicles and pyrene-PC/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 hsPLA2-IIa showed that the mutation of its Lys69, 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 hsPLA2-IIa and hsPLA2-V reveals that the latter has Arg in place of Lys in position 69 (13). The origin of unique substrate head group specificity of hsPLA2-V is currently under investigation.

Our data show good correlation between the in vitro ability of sPLA2s 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 hsPLA2-IIa mutants on vesicles and cell membranes (28). Exogenously added hsPLA2-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 hsPLA2-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.

sPLA2s, including hsPLA2-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 hsPLA2-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 arachidonate. This notion is consistent with a recent finding that exogenous arachidonate is rapidly transported across the neutrophil plasma membrane via a protein-facilitated mechanism (39). Due to the inability of hsPLA2-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. phosphatidylethanolamine) (40), prior activation of cPLA2 (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 hsPLA2-V on CHO-K1, RAW264.7, and human neutrophils, thereby suggesting that under the physiological conditions, hsPLA2-V can effectively act on unprimed mammalian cells. The precise physiological significance of the action of hsPLA2-V on neutrophils was not elucidated in this investigation. Neutrophils are not known to have high sPLA2 activities. However, the close proximity of these cells to mast cells and macrophages, which secrete sPLA2s, including hsPLA2-V during many inflammatory processes (10, 11), and the ability of hsPLA2-V to elicit substantial LTb4 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 PLA2 isofrom in granulocytic inflammation.

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