The molecular basis of phosphatidylcholine preference of human group-V phospholipase A_2

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Human group-V phospholipase A_2 ($hVPLA_2$) is a secretory phospholipase A_2 (PLA_2) that is involved in eicosanoid formation in such inflammatory cells as macrophages and mast cells. We showed that $hVPLA_2$ can bind phosphatidylcholine membranes and hydrolyse phosphatidylcholine molecules much more efficiently than human group-IIa PLA_2, which accounts for its high activity on the outer plasma membrane of mammalian cells. To understand the molecular basis of the high phosphatidylcholine specificity of $hVPLA_2$, we mutated several residues (Gly-53, Glu-56 and Glu-57) that might be involved in interaction with an active-site-bound phospholipid molecule. Phospholipid head-group specificities of mutants determined using polymerized mixed-liposome substrates indicate that a small glycine residue in position 53 is important for accommodating a bulky choline head group. Also, results indicated that two anionic residues, Glu-56 and Glu-57, favourably interact with cationic head groups of phosphatidylcholine and phosphatidylethanolamine. Together, these steric and electrostatic properties of the active site of $hVPLA_2$ allow for effective binding and hydrolysis of a bulky cationic choline head group of phosphatidylcholine, which is unique among mammalian secretory PLA_2s.

Key words: head-group specificity, interfacial catalysis, pro-inflammatory enzyme, secretory PLA_2, substrate specificity.

INTRODUCTION

Phospholipases A_2 (PLA_2) are a large family of ubiquitous enzymes that are found both intra- and extracellularly in mammalian tissues. Mammalian secretory PLA_2s ($sPLA_2$s) are homologous proteins that are divided in a number of groups, Ib, IIa, IIc, IId, IIe, IIf, V and X, on the basis of minor structural differences [1]. All these $sPLA_2$s share the same catalytic mechanism in which Ca^{2+} plays an essential catalytic role [2,3]. Intracellular PLA_2s, including group-IV cytosolic PLA_2 [4] and group-VI Ca^{2+}-independent PLA_2 [5], share no structural homology with $sPLA_2$s and have distinct catalytic mechanisms. Recent cell studies have indicated that both $sPLA_2$s and cytosolic PLA_2 are involved in eicosanoid production [6,7]. The critical involvement of cytosolic PLA_2 was demonstrated by recent gene-knock-out studies [8,9]. However, the nature of pro-inflammatory $sPLA_2$ 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 [10,11]. Also, group-V $sPLA_2$ has been shown to be involved in eicosanoid formation from murine macrophages and mast cells [12,13]. Recently, we showed that human group-V PLA_2 ($hVPLA_2$) can bind zwitterionic phosphatidylcholine (PC) membranes and hydrolyse PC molecules much more efficiently than human group-IIa PLA_2 ($hIIaPLA_2$). This suggested that $hVPLA_2$ is better suited than $hIIaPLA_2$ for acting on the outer plasma membranes of mammalian cells that are composed largely of zwitterionic PC and sphingomyelin [14]. Our subsequent cell studies demonstrated that exogenous $hVPLA_2$ has much greater activity than $hIIaPLA_2$ with respect to releasing fatty acids and eliciting eicosanoid formation from various mammalian cells [15]. We also showed that a single tryptophan residue (Trp-31) located on the putative interfacial binding surface is essential for its high affinity for PC membranes [15]. In this study, we performed a structure–function analysis on the putative substrate-binding site of $hVPLA_2$ to identify the residues essential for its unique ability to bind and hydrolyse PC molecules.

EXPERIMENTAL

Materials

1-Hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC), -ethanolamine (pyrene-PE) and -glycerol (pyrene-PG) were purchased from Molecular Probes (Eugene, OR, U.S.A.). 1-Hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphoserine (pyrene-PS) was prepared by the phospholipase D-catalysed transphosphatidylation of pyrene-PC and purified as described by Comfurius and Zwaal [16]. 1,2-*bis*[12-(Lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphoglycerol (BLPG) was prepared as described elsewhere [17,18]. Polymerized mixed liposomes were prepared by polymerizing large unilamellar liposomes (100 nm in diameter) prepared by extrusion as described in [17,18]. Phospholipid concentrations were determined by phosphate analysis [19]. Fatty acid-free BSA was from Bayer (Kankakee, IL, U.S.A.). All restriction enzymes, T4 ligase, T4 polynucleotide kinase and isopropyl β-D-thiogalactoside were obtained from Boehringer Mannheim. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, U.S.A.) and used without further purification.

Mutagenesis and protein expression

The mutagenesis of $hVPLA_2$ was performed using a Sculptor *in vitro* mutagenesis kit from Amersham Pharmacia Biotech and a phagemid DNA prepared from the pSK vector in the presence of

Abbreviations used: BLPG, 1,2-*bis*[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphoglycerol; PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; $hIIaPLA_2$, human group-IIa PLA_2; $hVPLA_2$, human group-V PLA_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; pyrene-PC, 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine; pyrene-PE, 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphoethanolamine; pyrene-PG, 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol; pyrene-PS, 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphoserine.

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helper phage R408 as described previously [20]. Proteins were expressed in *Escherichia coli*, refolded and purified to near homogeneity (> 90% pure) as described previously [15] and stored as lyophilized powder at −20 °C.

Kinetic measurements

The PLA_2-catalysed 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-labelled phospholipids (1 mol%) in 9.9 μM BLPG, 2 μM BSA, 0.16 M NaCl and 10 mM $CaCl_2$ [17,21]. 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 band width 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].

RESULTS

Molecular cloning of group-V PLA_2s from different species showed that these enzymes, although homologous to group-IIa PLA_2s, have some unique variations in amino acid sequence [23]. Structure–function studies on several $sPLA_2$s have shown that residues 53–58, which are located in the C-terminal end of a long α-helix, are involved in interaction with the phospholipid head group [24–28]. As illustrated in Figure 1, $hVPLA_2$ has two noticeable amino acid substitutions in this substrate-binding site when compared with $hIIaPLA_2$; a small Gly-53 and an anionic Glu-57 in place of Lys residues. Mouse group-V PLA_2 also contains Gly-53 and Glu-57. We reasoned that these residues are responsible for the unique activity of $hVPLA_2$ to effectively hydrolyse a bulky cationic PC head group. To test this notion, we mutated Gly-53 and Glu-57 of $hVPLA_2$ to Lys (G53K and E57K respectively). We also mutated Gly-53 to Glu (G53D) because some $sPLA_2$s have Glu in position 53 [29]. Finally, Glu-56 was mutated to Lys (E56K) to see if this residue is involved in interaction with cationic PC and phosphatidylethanolamine (PE) head groups, as seen with $hIIaPLA_2$ [26]. Initial attempts to prepare these mutants were hampered by extremely low refolding efficiency of their solubilized inclusion bodies. To overcome this difficulty, we used W79A of $hVPLA_2$, which was shown to be as active as and much more stable than the wild type [15], as a template for mutant preparation (e.g. G53K/W79A). All the double mutants produced were expressed in high yields as inclusion bodies and their refolding yields were uniformly high (i.e. > 2 mg/l of culture after purification).

	51									60		69
$hIbPLA_2$	C	Y	D	Q	A	K	K	L	D	S	C	Y
$hIIaPLA_2$	C	Y	K	R	L	E	K	R	-	G	C	K
$hVPLA_2$	C	Y	**G**	R	L	**E**	**E**	K	-	G	C	**R**
$mVPLA_2$	C	Y	G	Q	L	E	E	K	-	G	C	R
$hXPLA_2$	C	Y	T	R	A	E	E	A	-	G	C	K
AppD49	C	Y	G	-	-	-	K	V	T	G	C	K

Figure 1 Partial amino acid sequences of selected $sPLA_2$s, including human group-Ib pancreatic PLA_2 ($hIbPLA_2$), $hIIaPLA_2$, $hVPLA_2$, mouse group-V PLA_2 ($mVPLA_2$) and human group-X PLA_2 ($hXPLA_2$), and an Asp-49 PLA_2 from *Agkistrodon piscivorus piscivorus* (App-D49)

Amino acid sequences between two conserved cysteines (Cys-51 and Cys-61) and in position 69 are shown. Mutated residues of $hVPLA_2$ are shown in bold type.

Table 1 Phospholipid head-group specificity of $hVPLA_2$ and mutants

See the Experimental section for experimental conditions and methods for calculating rate constants. Values of k_{cat}^*/K_m^* represent means ± S.D. from triplicate determinations. PG, phosphatidylglycerol.

	$k_{cat}^*/K_m^* \times 10^6$ ($M^{-1} \cdot s^{-1}$)					
Enzyme	Pyrene-PC	Pyrene-PE	Pyrene-PG	Pyrene-PS	PC/PG	PC/PE
$hVPLA_2$ (W79A)	6.5 ± 1.5	4.8 ± 1.0	9.5 ± 1.5	2.4 ± 0.6	0.7	1.4
G53K/W79A	1.2 ± 0.5	4.9 ± 1.5	11.5 ± 2.5	1.8 ± 0.6	0.1	0.2
G53D/W79A	3.6 ± 1.0	4.6 ± 1.5	9.1 ± 1.0	1.2 ± 0.5	0.4	0.8
E56K/W79A	2.6 ± 0.8	1.5 ± 0.3	1.7 ± 0.8	1.1 ± 0.4	1.5	1.7
E57K/W79A	2.0 ± 1.0	1.8 ± 1.0	10.0 ± 3.0	1.7 ± 0.3	0.2	1.1
R69K/W79A	5.0 ± 2.0	5.0 ± 1.2	4.2 ± 2.0	0.5 ± 0.2	1.2	1.0
R69Y/W79A	7.5 ± 4.0	7.5 ± 3.0	6.5 ± 4.0	0.6 ± 0.3	1.2	1.0
$hIIaPLA_2$*	0.2 ± 0.1	2.0 ± 0.3	28.0 ± 4.0	1.9 ± 0.4	< 0.01	0.09

* From [26].

We then measured the phospholipid head-group specificities of the mutants with polymerized mixed liposomes. In the polymerized mixed-liposome system, it is possible to accurately determine the head-group specificity of PLA_2 by varying the head-group structure of hydrolysable pyrene-phospholipids in an inert polymerized matrix [17,21]. Two zwitterionic phospholipids, pyrene-PC and pyrene-PE, and two anionic phospholipids, pyrene-PG and pyrene-PS, were used as inserts in the BLPG polymerized matrix. The anionic BLPG was used as a polymerized matrix because $hVPLA_2$, albeit active on zwitterionic membranes, still prefers anionic membranes to zwitterionic ones [15]. Values of k_{cat}^*/K_m^* determined for the mutants and various polymerized mixed liposomes are summarized in Table 1. Note that the differences in k_{cat}^*/K_m^* values between wild-type and mutant enzymes were in general modest and in some cases fell within the range of experimental error. Thus a direct comparison was made between wild type and mutants for a particular substrate only when the difference was large enough to be statistically significant. First of all, the phospholipid head-group specificity of W79A was essentially identical with that of wild type [14], i.e. pyrene-PG > pyrene-PC > pyrene-PE > pyrene-PS, thereby validating the use of this mutant as a wild-type substitute. When compared with W79A, G53K/W79A showed a 5.4-fold lower activity on pyrene-PC but comparable activities on all other pyrene lipids, including zwitterionic pyrene-PE. This suggested that the effect of mutation is largely steric and not electrostatic. This notion is supported by the similar activities of G53D/W79A, i.e. 1.8-fold lower activity on pyrene-PC and no significant effects on other pyrene lipids. A smaller decrease in activity on pyrene-PC for this mutation might simply reflect the size difference between Asp and Lys. The mutation of Glu-57 to Lys reduced the activity of W79A towards pyrene-PC and pyrene-PE by factors of 3.3 and 2.7, respectively, supporting the notion that this residue interacts favourably with a cationic head group. On the other hand, the activities on pyrene-PG and pyrene-PS were changed less significantly. A slight decrease in pyrene-PS activity might indicate that Glu-57 also interacts favourably with the ammonium group of pyrene-PS. Similarly, E56K/W79A exhibited lower activities on pyrene-PC, -PE and -

PS. Interestingly, however, this mutant also showed much reduced activity on pyrene-PG. This is in contrast with the effect of the same mutation on hIIaPLA$_2$, which reduced its pyrene-PC and pyrene-PE activities but not pyrene-PG activity. Presumably, the side-chain orientation of Glu-56 and its interaction mode with the phospholipid head group are different in the two enzymes.

As shown in Table 1, hVPLA$_2$ is significantly less active than hIIPLA$_2$ on pyrene-PG. Interestingly, hVPLA$_2$ has Arg in position 69 that is normally occupied by Lys for group-II PLA$_2$s [3] and the K69R mutation of hIIPLA$_2$ resulted in a 5-fold drop in pyrene-PG activity [26]. We thus measured the effects of mutation of Arg-69 of hVPLA$_2$ to either Lys or Tyr (R69K and R69Y) to test if the presence of Arg-69 in place of Lys is responsible for its low activity on pyrene-PG. Unexpectedly, however, both mutations modestly affected the activities of W79A on pyrene-PC, pyrene-PE and pyrene-PG but significantly decreased the activity on pyrene-PS ($\approx$ 4-fold). Thus it appears that Arg-69 is involved in specific interactions with the phosphatidylserine (PS) head group.

DISCUSSION

We showed previously that hVPLA$_2$ can bind zwitterionic PC membrane surfaces and hydrolyse a PC molecule much more effectively than other human sPLA$_2$s [14]. We subsequently demonstrated that Trp-31 in its putative interfacial binding surface is largely responsible for its high affinity for PC membranes [14]. The present structure–function study of hVPLA$_2$ identifies Gly-53 as an important determinant of its unique PC activity. As shown in Figure 1, hVPLA$_2$ is the only known human sPLA$_2$ with Gly in position 53. The model structure of a hVPLA$_2$–PC analogue complex, built on the basis of the structure of a hIIaPLA$_2$–PE analogue complex [30], is shown in Figure 2. X-ray structures of phospholipids show that the polar head group of PC ($\approx$ 350 Å^3) is much larger than that of PE ($\approx$ 250 Å^3) [31]. A model building also indicates that the PC head group is considerably larger than those of phosphatidylglycerol (PG) and PS (results not shown). Figure 2 suggests that the room provided by a small glycine side chain in position 53 of hVPLA$_2$ is essential for the binding of the PC head group to its active site. In fact, this strategy has been widely adapted by snake-venom PLA$_2$s (group IIa) acting on PC membranes. For instance, we showed previously that a PLA$_2$ from the venom of *Agkistrodon piscivorus piscivorus* contains Gly-53 and, consequently, hydrolyses pyrene-PC as well as pyrene-PE and pyrene-PG in polymerized mixed liposomes [20]. Since all human sPLA$_2$s, except for group-Ib pancreatic sPLA$_2$, are released to the extracellular space as fully active enzymes, it might be important for the purpose of regulation and cell protection to keep the activities of

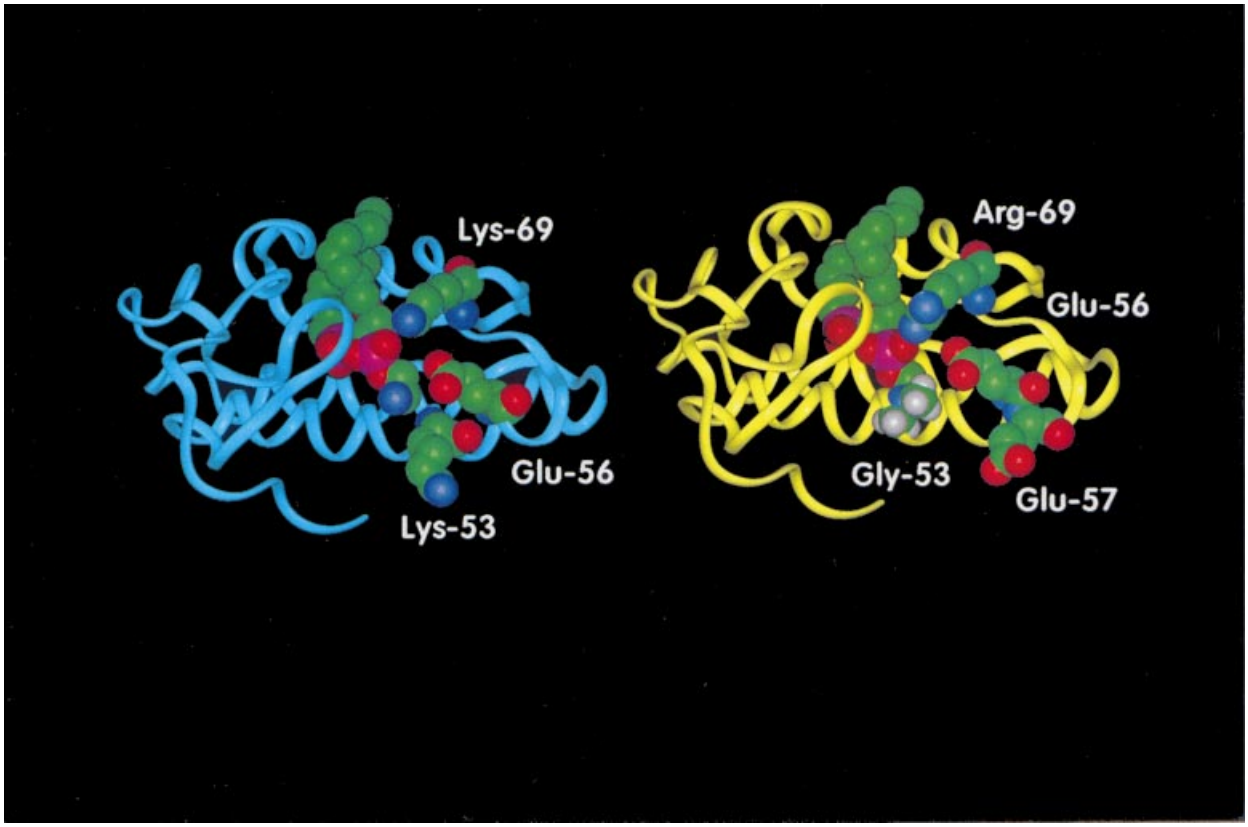

Figure 2 A model structure of a hVPLA$_2$–inhibitor complex

The model structure of hVPLA$_2$ shown in ribbon diagram (yellow) is built on the backbone of hIIaPLA$_2$ (blue) in a complex with the transition-state analogue inhibitor, L-1-*O*-octyl-2-heptylphosphonyl-*sn*-glycero-3-phosphoethanolamine [30]. All side chains were substituted for by hVPLA$_2$ residues using a program, Biopolymer (Molecular Simulation). Then, a PC analogue of the transition-state inhibitor was built on to its backbone using a program, Discover (Molecular Simulation), and docked into the active site of hVPLA$_2$ to a position that corresponds to that of L-1-*O*-octyl-2-heptylphosphonyl-*sn*-glycero-3-phosphoethanolamine in the active site of hIIaPLA$_2$. Energy minimization was not performed. The inhibitors and side chains of mutated residues are shown in space-filling representation. Carbon atoms are shown in green, nitrogen in blue, oxygen in red, phosphorus in pink and hydrogens of the PC head group in white.

$sPLA_2$s on the outer plasma membranes (i.e. PC activity) in check. The fact that $hVPLA_2$ has evolved to possess this activity thus points to its direct involvement in interaction with the outer plasma membranes of inflammatory cells to elicit inflammatory responses.

$hVPLA_2$ and $hIIaPLA_2$ are distinct from group-IIa snake-venom PLA_2s in that the former have an extended sequence (residues 54–58) that is deleted in the latter. The extension forms part of an elongated α-helix in $hIIaPLA_2$ and the side chain of Glu-56 makes direct contact with the ethanolamine head group of an active-site-bound PE analogue (see Figure 2). In the extended sequence, $hVPLA_2$ contains Glu-57 as well as Glu-56. Due to the presence of Gly in position 53 that is known to break the α-helix, it is unlikely that the extension will be part of the α-helix in $hVPLA_2$. Thus, residues 53–58 of $hVPLA_2$ and $hIIaPLA_2$ are expected to have different side-chain orientations with respect to an active site-bound phospholipid. This in turn might account for the fact that neither G53D or G53K showed appreciable effects on the activities of $hVPLA_2$ on any pyrene lipid but pyrene-PC. For $hIIaPLA_2$ [32] and other $sPLA_2$s [25,27], mutations of the residue in position 53 exhibited significant effects on the head-group specificity. Also, differential effects of the E56K mutation on the substrate specificity of $hVPLA_2$ and $hIIaPLA_2$ can be explained in terms of the structural differences. For $hIIaPLA_2$, E56K mutation selectively reduced the enzyme activity on pyrene-PC and pyrene-PE without affecting the activity on pyrene-PG, and enhanced the activity on pyrene-PS [26]. In contrast, the same mutation decreased the activities of $hVPLA_2$ on all pyrene lipids, with the largest activity drop seen with pyrene-PG. However, the effects of E57K mutation on $hVPLA_2$ activities are in line with the effects of K57E mutation on $hIIPLA_2$ activities [26]. A full explanation for these findings would require the tertiary structural information on a $hVPLA_2$–inhibitor complex. Evidently, however, these results indicate that Glu-56 and Glu-57 in the substrate-binding pocket interact favourably with zwitterionic substrates, PC and PE, thereby enhancing its activity on these substrates versus anionic ones.

Mammalian group-V PLA_2s uniquely contain Arg in position 69 that is occupied by Lys in most group-II PLA_2s and invariably by Tyr in group-I PLA_2s [3]. X-ray structures of enzyme–inhibitor complexes showed that either Tyr or Lys in position 69 forms a hydrogen bond with pro-S non-bridging oxygen of *sn*-3 phosphate [30,33]. Our previous study on *A. p. piscivorus* PLA_2 showed that a K69Y mutation selectively reduced the enzyme activity on pyrene-PG without interfering with PC and PE activities, suggesting that Lys-69 of group-II PLA_2s might be important for their anionic head-group specificity [20]. Similar but less pronounced effects were observed with the K69Y mutation of $hIIaPLA_2$ [26]. Interestingly, the K69R mutation of $hIIaPLA_2$ uniformly ($\approx$ 5-fold) reduced the activities on all pyrene lipids, suggesting the specific nature of the Lys-69-*sn*-3 phosphate hydrogen bond. This, in conjunction with the finding that $hVPLA_2$ is about 5-fold less active than $hIIPLA_2$ on pyrene-PG, implies that one might be able to improve the activity of $hVPLA_2$ on anionic phospholipids by R69K mutation. Our results indicate otherwise. The R69K mutation selectively reduced the activities on anionic phospholipids, pyrene-PS in particular. This suggests that Arg-69 of $hVPLA_2$ is involved in specific interactions with PS (and PG) head groups that cannot be fully simulated by Lys. Slight increases in PC and PE activities by R69Y mutation also suggest that Tyr is slightly more effective than Arg in forming a hydrogen bond with the *sn*-3 phosphate in $hVPLA_2$. Again, the lower activities of R69Y on pyrene-PG and pyrene-PS suggest that Tyr cannot replace the role of Arg in interactions with anionic lipid head groups. Together, these results underscore the complex nature of the structural determinants of phospholipid head-group specificity of $sPLA_2$s. Whatever the origin of lower activity of $hVPLA_2$ on PG substrates might be, it would not compromise pro-inflammatory actions of $hVPLA_2$ since PG is only a minor component of mammalian plasma membranes. However, $hVPLA_2$ might have significantly lower activity on PG-rich bacterial cell membranes than $hIIaPLA_2$, which has been shown to have potent bactericidal activities [34].

This work was supported in part by a Biomedical Science Grant from the Arthritis Foundation and a National Institutes of Health grant (GM52598). W. C. is an Established Investigator of the American Heart Association.

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Received 14 December 1999/18 March 2000; accepted 3 April 2000