To better understand the principles underlying the substrate specificity of A-type phospholipases (PLAs), a high throughput mass spectrometric assay was employed to study the effect of acyl chain length and unsaturation of phospholipids on their rate of hydrolysis by three different secretory PLAs in micelles and vesicle bilayers. With micelles, each enzyme responded differently to substrate acyl chain unsaturation and double bond position, probably reflecting differences in the accommodative properties of their substrate binding sites. Experiments with saturated acyl positional isomers indicated that the length of the sn2 chain was more critical than that of the sn1 chain, suggesting tighter association of the former with the enzyme. Only the first 9–10 carbons of the sn2 acyl chain seem to interact intimately with the active site. Strikingly, no discrimination between positional isomers was observed with vesicles, and the rate of hydrolysis decreased far more with increasing chain length than with micelles, suggesting that translocation of the phospholipid substrate to the active site is rate-limiting with bilayers. Supporting this conclusion, acyl chain structure affected hydrolysis and spontaneous intervesicle transfer, which correlates with lipid efflux propensity, analogously. We conclude that substrate efflux propensity plays a more important role in the specificity of secretory PLA2s than commonly thought and could also be a key attribute in phospholipid homeostasis in which (unknown) PLA2s are key players.

Several PLAs display significant specificity toward the phospholipid acyl chain(s) in vitro (6, 7). However, despite numerous studies, the factors underlying such specificity have remained largely elusive. In principle, two factors contribute to selective hydrolysis of phospholipids by PLA: 1) accommodation of the acyl chains in the protein lipid binding site and 2) the ease of efflux of the phospholipid substrate from the bilayer (8, 9). The understanding of acyl chain accommodation has been greatly hampered by the lack of crystal structures of PLAs complexed with a physiological substrate. Crystal structures have been obtained for some PLAs with a bound noncleavable short-chain substrate analogue (10–13), but because these analogues have truncated acyl chains, they only provide very limited information on the factors underlying acyl chain specificity. Furthermore, the information could also be biased, since studies with other lipid-binding proteins have shown that the mode of accommodation of an alkyl chain can be markedly influenced by the other alkyl chain of the bound lipid (14, 15). Another uncertainty with non-cleavable lipid analogues is that the “unnatural” moieties may greatly increase their affinity for the enzyme (16), thus potentially masking effects of acyl chain structure.

Notably, even if crystal structures with bound natural substrates could be obtained, they would not reveal how the efflux of the phospholipid substrate from the bilayer contributes to PLA specificity. This contribution is likely to be a major one, because lipid-lipid interactions influence the vertical movement of the phospholipid (or efflux propensity), which is thought to be necessary for its binding to the active site of the enzyme (8, 17–19). Such translocation should be easier for phospholipids that interact less tightly with their lipid neighbors (i.e. for those with short, polyunsaturated, or oxidized acyl chains). In conclusion, the relative contributions of the PLA active site accommodation and substrate efflux propensity to phospholipid hydrolysis are poorly understood at present. Such information is necessary to fully understand the mode of action of PLAs as well as to establish why homeostatic PLAs preferentially hydrolyze certain phospholipid species (20), thus helping to maintain the characteristic phospholipid composition of cellular membranes.

To obtain information on acyl chain specificity of PLA2s, early studies typically compared the rates of hydrolysis obtained with vesicles consisting only of a single phospholipid species. This, however, is not a good approach, because the observed rate often reflects more the affinity of the PLA for the vesicles (which can vary greatly with the phospholipid used) than the true substrate specificity of the PLA under study (21).
Although incorporation of phospholipids in detergent micelles partially alleviates this problem, others are introduced (22). Much more reliable information can be obtained by comparing the hydrolysis of a \({\text{\textsuperscript{3}}H}\)-labeled species with a \({\text{\textsuperscript{14}}}C\)-labeled one incorporated together in vesicles (21). However, this approach is not optimal if a large number of species are to be studied. One solution is to use biological membranes or liposomes made of natural lipids as the macrosubstrate and to determine the rates of hydrolysis of the multiple species present by gas or liquid chromatography or mass spectrometry (23–25). However, the species that can be studied are limited to those present in a particular cell or tissue, and thus systematic information on the effects on acyl chain length and unsaturation and sn position cannot be readily obtained.

Here, we studied factors underlying the specificity of PLAs by determining the kinetics of hydrolysis of a large variety of phospholipid molecular species by three different secretory PLA\(_2\)s with detergent micelles or vesicle bilayers as the macrosubstrate. We used electrospray ionization mass spectrometry, which allows facile analysis of a multitude of phospholipid species (26–28), to monitor the hydrolysis of each species simultaneously, thus avoiding bias due to substrate-dependent variations of the interfacial properties. With micelles, significant differences between the three PLA\(_2\)s were found regarding the effect of double bond number and position. In addition, the length of the sn2 acyl chain was found to be more critical for hydrolysis than that of the sn1 chain, suggesting tighter interaction of the former with the lipid binding cavity of the enzyme. With vesicles, the effect of chain length was much more pronounced than with micelles, and, strikingly, no significant difference in the effect of double bond number and position was observed.

These data suggest that with bilayers, substrate efflux propensity is the key determinant of PLA\(_2\)-mediated hydrolysis and tends to mask differences in the accommodative properties of lipid binding cavity of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Lipids and Other Chemicals**—Cholesterol, unlabeled phosphatidylycholines (PCs), and lysophosphatidylycholines were from Avanti Polar Lipids (Alabaster, AL). D\(_{3}\)-methyl iodide was purchased from Cambridge Isotope Laboratories (Andover, MA). Ethanolamine, phospholipase D (Streptomyces sp.), and phospholipases A\(_2\) from cobra venom (\textit{Naja mossambica mossambica} (P7778), bee venom (P9279), and porcine pancreas (P6534) were obtained from Sigma, and the solvents were from Merck. The sn1-16:0/sn2-2-P-C and sn1-C\(_{16}/\text{sn}2-16:0-\text{PC} (\text{where } n \text{ indicates the number of acyl carbons}) \text{ species were synthesized by acylation of sn1-16:0-lysophosphatidylcholine with an anhydride of a C\(_{n}\)-fatty acid or an sn1-C\(_{n}\)-lysophosphatidylcholine with 16:0-fatty acid, respectively (29). D\(_{3}\)-labeled PC species were synthesized from the corresponding phosphatidylethanolamine species by methylation with D\(_{3}\)-methyl iodide essentially as described (30) except that benzene was replaced with toluene. The phosphatidylethanolamine species were synthesized from corresponding phosphatidylcholines using phospholipase D-mediated transphosphatidylolation (31). The lipids were purified using normal-phase high pressure liquid chromatography on a diol-modified silica column (32). The purity of phospholipids was confirmed by TLC and mass spectrometry, and their concentrations were determined based on phosphate content (33). The concentration of the phospholipases was determined with a fluorometric protein assay (34).

**Assay of PLA\(_2\) Specificity with Detergent Micelles**—A mixture of phosphatidylcholine species (see “Results”), 1-palmitoyl-2-oleyl-phosphatidic acid, and 17:0, 21:0, or 25:0 sphingomyelin were mixed in chloroform, and the solvent was evaporated under a nitrogen stream and further under high vacuum for 1 h. Then 2 ml of 0.1 M Tris, 0.1 mM CaCl\(_2\) buffer (pH 7.4) containing 30 mM octyl glucoside was added, and the lipids were dispersed by vortexing for 2 min at room temperature. The mixed micelles (0.25 mM phospholipid) were incubated in the presence of a PLA\(_2\) (typically 2 ng for bee, 1 ng for cobra, and 1.5 \(\mu\)g for porcine enzyme) at 37 °C, aliquots were removed at intervals and mixed with 1 ml of chloroform/methanol (1:2 (v/v)), and the lipids were extracted according to Folch et al. (35). The lower phase was washed three times with the theoretical upper phase and taken to dryness under an N\(_2\) stream, and the residue was reconstituted in 300 \(\mu\)l of chloroform/methanol (1:2) and stored at −20 °C.

**Assay of PLA\(_2\) Specificity with Unilamellar Vesicles**—The lipids were mixed and dried as above, and 0.1 M Tris buffer (pH 7.4) containing 0.1 mM CaCl\(_2\) was added. Small unilamellar vesicles (SUVs) were prepared by sonication with a probe for argon for 5 × 2 min. Butylated hydroxytoluene (1 mol %) was included as an antioxidant when the lipid mixture contained unsaturated species. Any undispersed lipid and probe particles were removed by centrifugation for 5 min at 6000 \(\times\) g. To prepare large unilamellar vesicles (LUVs), the dried lipids were covered with buffer, and the tubes were placed in a 4 °C water bath for 1 min and then in a 50 °C bath for 1 min. This cooling-heating cycle was repeated four times. The lipids were then dispersed by vortexing for 1 min at room temperature, and the resulting multilamellar vesicles were extruded 10 times through a 1.2-\(\mu\)m polycarbonate membrane and then 10 times through two 0.4-\(\mu\)m polycarbonate membranes using a Lipex extruder (Lipex, Vancouver, Canada). SUVs or LUVs (0.2 mm) were incubated with a PLA\(_2\) in 0.1 M Tris, 0.1 mM CaCl\(_2\) buffer (pH 7.4) at 37 °C. The amount of PLA\(_2\) used per nmol of phospholipid was typically 1 ng for bee, 1 ng for cobra, and 20 \(\mu\)g for porcine PLA\(_2\). Otherwise, the assay was carried as outlined above for micelles.

**Measurement of Spontaneous Intervesicle Translocation Rates**—The spontaneous intervesicle translocation rates of PC species were determined as described previously (36) Briefly, negatively charged donor vesicles consisting of 1-stearyol-2-oleyl-PC, D\(_{3}\)-labeled PC-mix27, and tetraoleyl-cardiolipin (45:45:10 (mol/mol/mol)) and uncharged acceptor vesicles consisting of dioleoyl-PC and di-22:1-D\(_{3}\)-PC (99:1 mol/mol) were prepared in 10 mM HEPES, 25 mM KCl, 0.5 mM EDTA (pH 7.0) by probe sonication. Donor and acceptor vesicles (1.5 and 7.5 \(\mu\)mol of total phospholipid, respectively) were coincubated at 37 °C, and aliquots were withdrawn at intervals and applied to a DEAE-Sephal (Amersham Biosciences) minicolumn to trap the negatively charged donors. The D\(_{3}\)-PC species transferred to the eluted acceptor vesicles were then quantified by mass
spectrometry using di-22:1-D$_9$-PC present in the acceptor vesicles as an internal standard.

**Mass Spectrometry and Data Analysis**—After the addition of NH$_4$OH (4%), the sample was infused at 6 μl/min to a Micromass Quattro Micro triple-quadrupole mass spectrometer operated as described previously (37). The D$_9$-labeled and unlabeled PC species were detected by scanning for precursors of m/z +193 and m/z +184, respectively. Spectra were acquired for 5 min over a mass range of m/z 400–950 at a frequency of 4 scans/min. The spectra were exported to Microsoft Excel, and the PC species were quantified by using the LIMSA software (38). The concentrations of the individual lipid species were plotted against time, and the rate constants of hydrolysis were obtained by fitting a first order exponential decay model to the data. Based on the theoretical fraction of phospholipid accessible to PLA$_2$s, the maximal hydrolyzable fraction at $t_\infty$ was constrained to 1.0, 0.67, or 0.5 with micelles, SUVs, and LUVs, respectively.

**RESULTS**

**High Throughput Assay of Acyl Chain Specificity of PLA$_2$s**—To obtain information on the factors underlying the acyl chain specificity of PLA$_2$s, we devised a mass spectrometry-based assay that allows the rates of hydrolysis of a multitude of phospholipid species to be determined simultaneously. We used three different mixtures of PC species. One of them, PC-mix27, consisted of 1) 10 disaturated species with the length of the acyl chains varying from 13 to 22 carbons; 2) three monounsaturated species (14:0/18:1, 16:0/18:1, and 18:0/18:1); 3) six species with two double bonds, including 16:0/18:2, and five dimonounsaturated species with acyl chain lengths varying from 14 to 22 carbons; 4) three species with four double bonds (14:0/20:4, 16:0/20:4 and 18:0/20:4); 5) three species with six double bonds (18:3/18:3, 16:0/22:6, and 18:0/22:6); 6) one with eight (20:4/20:4); and 7) one with 12 double bonds (22:6/22:6). The second mixture (16:0/C$_n$-PCs; $n$ = 6–24) consisted of 18 saturated PC species with a 16:0 sn1 chain and a sn2 chain of a variable number of carbons, whereas the third mixture (C$_n$/16:0-PCs) consisted of the positional isomers of the second one. Hydrolysis of PC species was studied both in octyl glucoside micelles and small and large unilamellar vesicles to see how lipid packing influences the apparent specificity of the PLA$_2$s. A sphingomyelin species (10 mol %) was included as a nonhydrolyzable internal standard.

**Micelles**—Fig. 1A shows the spectra obtained for a mixture of PC species in octyl glucoside micelles before (top) and after (bottom) incubation with bee PLA$_2$s for 3 min. Clearly, a major decrease in the amount of several PC species has taken place during the 3-min incubation, whereas some others have changed only slightly. These differences are more clearly

![FIGURE 1](https://example.com/figure1.png)
revealed by Fig. 1B, showing the kinetics of hydrolysis of six select species.

Fig. 2A displays the relative rates of hydrolysis of PC species with acyl chains of variable length and unsaturation by bee PLA₂. Significant and systematic differences were observed. Increasing acyl chain length resulted in a modest decrease of hydrolysis rate of the saturated and diunsaturated species, whereas no significant effect was observed for the mono- and polyunsaturated species. The hydrolysis of species with same total acyl chain carbons generally increased with increasing unsaturation. Interestingly, however, the arachidonic acid-containing 14:0/20:4, 16:0/20:4, 18:0/20:4, and 20:4/20:4 species were hydrolyzed significantly faster than predicted by this general trend.

In striking contrast to the bee enzyme, substrate unsaturation had hardly any effect on hydrolysis by cobra PLA₂ (Fig. 2B). Thus, all species of equal chain length were hydrolyzed at a comparable rate apart from those containing 18:3 or 22:6 fatty acids, which were hydrolyzed somewhat faster. A modest decrease of hydrolysis with increasing acyl chain length was observed.

Yet a different pattern was obtained for the porcine PLA₂ (Fig. 2C). This enzyme, unlike bee and cobra PLA₂s, preferred the short diunsaturated species as well as the 16:0/18:2 and di-18:3 species. Increasing acyl chain length inhibited hydrolysis, particularly in the case of the diunsaturated species.

Vesicles—We next studied the specificity of the different PLAs with SUVs. We found that in SUVs, the hydrolysis of most species leveled off when ~67% of them had been hydrolyzed. Because this percentage corresponds to the theoretical fraction of phospholipids in the outer leaflet in SUVs, and because secretory PLA₂s have access only to phospholipid molecules in the outer leaflet (39), the composition of the outer leaflet should be similar to the total composition. However, a somewhat higher fraction (70–80%) of species with short chains (e.g. 13:0/13:0-PC) was hydrolyzed, thus indicating that they, unlike others, were somewhat enriched in the outer leaflet. Such species are more cone-shaped than those with longer chains, which may favor their enrichment in the outer leaflet, which has a positive curvature. Nevertheless, such differences in the outer leaflet concentrations do not bias the specificity data, because the rate of hydrolysis of a species was determined based on its normalized concentration, rather than the absolute one, at different time points.

The specificity profile obtained with bee PLA₂ in SUVs differed markedly from that obtained with micelles (Fig. 3A). First of all, the differences in the rate of hydrolysis were far more pronounced with SUVs. The most rapidly hydrolyzed species (i.e. 14:1/14:1) was hydrolyzed ~100-fold faster than the most slowly hydrolyzed one (i.e. 22:0/22:0), whereas in micelles, the difference between the fastest (di-20:4) and slowest (di-22:1) species was only ~10-fold. With vesicles, the rate of hydrolysis of both saturated and diunsaturated PC species decreased strongly and monotonously with increasing acyl chain length. In addition, the hydrolysis increased with increasing degree of unsaturation (i.e. among the species of equal chain length, the lowest rate was always observed for the saturated species, and the rate generally increased with the number of double bonds).
However, the hydrolysis of arachidonoyl (20:4) species was again faster than expected from the general trend. Notably, although the relative rates varied somewhat between experiments, probably due to slightly different amounts of enzyme used, the specificity profile obtained was essentially identical (supplemental Fig. 1). Analogous results were obtained with large unilamellar vesicles (supplemental Fig. 2A).

Also, with cobra PLA₂ the length of the acyl chain(s) had a much stronger effect on the rate hydrolysis of both saturated and diunsaturated PC species as compared with micelles (Fig. 3B). Hydrolysis of the arachidonic acid-containing species was again relatively slow, and that of the 18:3- or 22:6-containing species was relatively fast as compared with the bee enzyme. Parallel results were obtained with LUVs (supplemental Fig. 2B).

With porcine PLA₂ the inhibitory effect of increasing chain length was also much stronger in vesicles versus micelles (Fig. 3C). However, differently from the other two enzymes, the relative rate of hydrolysis of the shortest saturated and diunsaturated species was exceptionally high (note the break in the y axis). As with micelles, the hydrolysis of the arachidonoyl species was slow as compared with bee PLA₂. In contrast to the other enzymes, the di-22:6 species was hydrolyzed only somewhat faster than other species of equal total chain length.

**Effect of sn1 Versus sn2 Acyl Chain Length in Micelles** —To obtain more detailed information on the contribution of the individual acyl chains, we synthesized two sets of saturated PC species in which the length of either the sn₁ or sn₂ acyl chain was varied from 6 to 24 carbons, whereas the length of the chain in the other sn-position was kept constant at 16 carbons. The Cₙ/16:0-PC species contained a D₉-labeled choline headgroup, whereas the 16:0/Cₙ-PC species were unlabeled. Both sets were present together in the same vesicles, which allowed us to study their hydrolysis under identical conditions. The relative rates of hydrolysis were determined by comparing peak areas with that of the SM internal standard. In addition, relative rates of hydrolysis of positional isomers could be determined accurately by plotting the ratio of the isomer peak areas versus time.

In micelles, variation of the sn₁ versus sn₂ chain length distinctly affected the rate of hydrolysis with each PLA₂. With bee PLA₂, increasing the length of the sn₂ chain from 6 to 8 carbons increased hydrolysis, which then decreased sharply at C9-C11 and finally leveled off (Fig. 4A). In contrast, the rate decreased monotonously with increasing length of the sn₁ chain. The ratio of the rate for sn₂ to that of sn₁ isomer is plotted in Fig. 4D. The plot shows a peak for sn₂ preference at C8-C9 but little if any preference for either isomer at longer chain lengths.

With cobra PLA₂, hydrolysis increased when the length of the sn₂ chain increased from 6 to 8 carbons and then decreased sharply and finally leveled off. As observed with the bee enzyme, only a modest and monotonous decrease took place with increasing length of the sn₁ chain. The ratio of the rate for sn₂ to that of sn₁ isomer is plotted in Fig. 4D. The plot shows a peak for sn₂ preference at C8-C9 but little if any preference for either isomer at longer chain lengths.

With porcine PLA₂, hydrolysis increased when the length of the sn₂ chain increased from 6 to 8 carbons and then decreased sharply and finally leveled off. As observed with the bee enzyme, only a modest and monotonous decrease took place with increasing length of the sn₁ chain (Fig. 4B). The isomer ratio plot (Fig. 4D) shows ~2-fold preference for the isomers with a C8-C9 sn₂ chain and a lesser one for those with a C13-C14 sn₂ chain. On the other hand, the species with a C6-C7 or C10–11 chain in the sn₁-position seem to be preferred over their positional isomers. Identical results were obtained independently of which isomeric set was D₉-labeled (data not shown), thus excluding an isotope effect.

**FIGURE 3. Hydrolysis of saturated and unsaturated PC species of varying chain length in SUVs.** Sonicated vesicles consisting of PC-mix27, POPA, and 21:0-SM (8:1:1, mol/mol) were incubated together with bee PLA₂ (A), cobra PLA₂ (B), or porcine PLA₂ (C) at 37 °C, and the relative rate of hydrolysis was determined as described under “Experimental Procedures.” See legend of Fig. 2 for other details. The data shown are mean ± S.D. of three independent experiments.

| Total acyl chain carbons | Bee | Cobra | Porcine |
|--------------------------|-----|-------|---------|
| 24                       |     |       |         |
| 26                       |     |       |         |
| 28                       |     |       |         |
| 30                       |     |       |         |
| 32                       |     |       |         |
| 34                       |     |       |         |
| 36                       |     |       |         |
| 38                       |     |       |         |
| 40                       |     |       |         |
| 42                       |     |       |         |
| 44                       |     |       |         |
| 46                       |     |       |         |

However, the hydrolysis of arachidonoyl (20:4) species was again faster than expected from the general trend. Notably, although the relative rates varied somewhat between experi-
The porcine PLA₂ discriminated between positional isomers even more than the other two enzymes. The sn2 chain length versus rate plot displays a major peak at C8-C9 and a minor peak at C13-C14 (Fig. 4C). Again, the rate varied only modestly and smoothly with the length of the sn1 chain. The isomer ratio plot shows that the 16:0/C9 species was hydrolyzed 9-fold faster than its positional isomer (i.e. C9/16:0) (Fig. 4D).

**Effect of sn1 Versus sn2 Acyl Chain Length in Vesicles**—With bee PLA₂, the rate of hydrolysis diminished markedly with increasing sn2 chain length (Fig. 5A), but the effect was more pronounced than in micelles. A precipitous decrease was observed from 6 to 10–11 carbons, whereas there was only a slight decrease beyond. Remarkably, unlike with micelles, a practically identical curve was obtained for the sn1 chain, thus signifying the absence of significant sn isomer preference for any chain length (Fig. 5A, inset). No significant isomer preference was observed for the cobra enzyme either (Fig. 5B). Practically identical results were obtained for both bee and cobra PLAs with large unilamellar vesicles (data not shown).
As shown in Fig. 6B, the rate of hydrolysis by bee PLA₂ also decreases logarithmically with chain length but much less per methylene unit than efflux (note the different y-scale in A versus B). Some species, like di-20:4 and 18:0/20:4 in the case of bee and di-22:6 in case of cobra PLA₂, were hydrolyzed much faster than predicted from their rate of efflux, presumably due to their favorable interactions with the substrate binding site of the enzymes.

**DISCUSSION**

Hydrolysis of phospholipids by secretory PLAs has been studied extensively for more than 4 decades. A commonly accepted model emerging from those studies is that 1) the enzyme associates peripherally with the macromembrane surface, 2) the phospholipid substrate moves upward and engages with the active site cavity of the enzyme, and 3) hydrolysis of the sn2 ester bond takes place, and the products are released from the enzyme (8, 18, 19, 45–50). Based on this model, the key factors determining the substrate specificity can be considered to be 1) accommodation of phospholipid acyl chain(s) in the active site cavity of the enzyme and 2) propensity of the phospholipid substrate to efflux from the bilayer. However, the relative contributions of these processes to the substrate specificity of secretory PLA₂s have remained obscure. In the present study, we provide evidence that although substrate accommodation is the key factor in micelles, substrate efflux propensity seems to be more important with bilayers.

**Phospholipid Acyl Chain-Protein Active Site Interactions**—The importance of accommodation of phospholipid acyl chains at the lipid binding site of secretory PLA₂s has been suggested previously based on poor hydrolysis by cobra PLA₂ of phospholipids with a bulky group in the acyl chains close to the carbonyl moiety (51, 52). In the present study, the importance of acyl chain accommodation is best revealed by the micelle data obtained for the two isomeric PC series, in which the length of either the sn1 or the sn2 chain was varied from 6 to 24 carbons. The length of a saturated sn1 chain had only a modest effect on the rate of hydrolysis by all three PLA₂s, and no abrupt changes at particular chain lengths were observed (Fig. 4). In stark contrast, the rate versus sn2 chain length plots displayed abrupt changes (i.e. peaks and kinks with each PLA₂). These findings strongly suggest that the sn2 chain associates more intimately with the enzyme than the sn1 one, consistent with the crystallographic data obtained for cobra PLA₂ complexed with a non-hydrolyzable 1-octyl, 2-heptyl analogue (53). NMR studies carried out with a cobra PLA₂ complexed with a truncated nonhydrolyzable PC analogue also support tighter interaction of the sn2 chain (54). The fact that the activity peaks when the length of sn2 chain is ~9 carbons (Fig. 4) indicates that this equals the length of the sn2 chain binding site/cavity in the enzyme, in agreement with crystallographic data obtained for bee PLA₁-inhibitor complex (11). The studies of Yu and Dennis (16) are also in good agreement with our data. These authors showed that the affinity of a truncated monomeric phospholipid analogue for the active site of cobra PLA₂ increased steadily when the length of the sn2 chain reached 9 carbons and then leveled off abruptly (16). When the inhibitor was incorporated in short-chain PC or Triton X-100 micelles, a more com-
plex chain length dependence was observed, but again the highest affinity was observed when the sn2 chain was 9–10 carbons long. Also, otherwise the chain length dependence patterns obtained by Yu and Dennis (16) were strikingly similar to that observed here for the cobra enzyme (Fig. 4C).

Also, previous activity measurements, albeit not discriminating between the effects of sn1 versus sn2 chain length and potentially biased by lipid-dependent variations in macromolecule properties, are consistent with our data. Thus, Pattus and co-workers found that porcine pancreatic PLA2 prefers di-9:0-PC over di-8:0, di-10:0, and di-12:0 in monolayers (55), whereas Roberts et al. (56) found that among di-Cn−PCs (n = 6, 8, 12, 14, or 16), the di-C8 species was best hydrolyzed by cobra PLA2 in Triton X-100 micelles.

Assuming that with micelles the rate of hydrolysis is mainly determined by substrate accommodation rather than lipid efflux propensity (see below), the data obtained with PC-mix27 indicate that there are significant differences between the three PLA2s studied in the ability of their substrate binding cavities to accommodate acyl chain double bonds (Fig. 2). For instance, the PC species containing arachidonic acid (Δ5,8,11,14) in the sn2-position were much better substrates for the bee enzyme than for the cobra or porcine enzymes, thus indicating that the lipid binding cavity of the bee enzymes accommodates Δ5 and/or Δ8 double bonds well, whereas the interactions with two other enzymes are more restrictive. On the other hand, the porcine enzyme does not seem to accommodate an sn2 22:6 (Δ4,7,10,13,16,19) chain as well as the other two PLA2s. The fact that differences were observed mainly for the species with polyunsaturated chains in the sn2-position is not unexpected because only these chains have double bonds above carbon 9 (i.e. in the region that associates closely with the protein lipid binding cavity) (see above). In principle, faster hydrolysis of a species with a particular acyl chain could be due to more facile release of the corresponding fatty acid product from the active as compared with other chains. This possibility is, however, inconsistent with previous mechanistic studies (57).

Efflux Propensity—Previous studies with cross-linkable or polymerizable lipids have provided evidence that upward movement with the phospholipid substrate is necessary for hydrolysis by venom PLA2s (49, 58), consistent with the concept that these enzymes do not penetrate significantly into the macromolecule, and thus the active site remains well above the interface. As discussed above, with micelles, each PLA2 studied discriminated between positional isomers, thus strongly suggesting that interaction of the acyl chains with the substrate binding cavity of the enzyme significantly affects the rate of hydrolysis in this system. Consistently, the number and position of double bonds affect the rate of hydrolysis differently with each enzyme. However, with vesicles, such differences were significantly diminished or even abolished, and most importantly, no obvious differences in the rate of hydrolysis were observed between positional isomers (Fig. 5). These findings strongly suggest that with phospholipid bilayers, substrate efflux propensity largely determines the rate of hydrolysis, whereas the contribution of acyl chain-protein interactions is less critical. This conclusion is supported by the qualitative similarities of the effects of acyl chain structure on the rate of hydrolysis versus spontaneous intervesicle translocation, because efflux from the bilayer is the rate-limiting step in the latter process (36, 40, 41). The effect of acyl chain structure on the rate of hydrolysis was far less than that on spontaneous transfer (Fig. 6). This finding is, however, consistent with the fact that 1) only about half of the phospholipid acyl chain methylenes translocate from the bilayer to the enzyme active site, and 2) the translocation does not require entropically costly reorganization of bulk water, unlike in the case of spontaneous intervesicle translocation. It is thought that association of the enzyme with the macromolecule, which occurs via so-called i-face containing hydrophobic amino acid residues, results in significant dehydration of the bilayer surface (8, 47). Consequently, when a phospholipid molecule moves upward in the bilayer (or micelle) to the active site of the enzyme, its acyl chains should not interact with bulk water.

Although it is not obvious why PLA2-mediated hydrolysis with vesicles decreases so strongly with increasing acyl chain length, we speculate that this could be due to unfavorable change in acyl chain tail entropy. In liquid-crystalline phospholipid bilayers, the upper 8–9 acyl chain methylenes are conformationally highly ordered due to tight lateral packing of the chains, but this order rapidly decays when moving toward the center of the bilayer (59). When a phospholipid substrate moves up in the bilayer to reach the active site of the enzyme, the part of the acyl chain that initially resided in the ordered region (i.e. above C9) should experience little change in its conformational freedom because the active site cavity is also conformationally restrictive. In contrast, the chain segments that initially resided in the disordered region of the bilayer (i.e. below carbons 8–9) experience a major change in their conformational freedom as they are placed in the upper, ordered region of the bilayer. Accordingly, the change in entropy (and free energy) of a phospholipid molecule becomes increasingly unfavorable when the length of its acyl chains increases beyond 8–9 carbons. This line of reasoning is supported by recent molecular dynamics simulations providing transversal distribution of the entropy and free energy of hexane (which mimics an acyl chain segment) in phospholipid bilayers (60). It is also supported by the fact that acyl chain length has much less influence with octyl glucoside micelles, since these are far more disordered than lipid bilayers (61), and the orientational order of acyl chains of incorporated phospholipids (when minor components, as in this study) also changes little with depth as indicated by data obtained with similar systems (62).

Substrate Replenishment Bias Is Unlikely—Previous studies have indicated that when there is less than one enzyme molecule per macromolecule particle and the enzyme is tightly associated with the particles, hydrolysis can be limited by interparticle exchange of phospholipid molecules (21, 22). Because the predicted rate of intervesicle translocation of the phospholipids used in this study varies greatly (Fig. 6), it is important to exclude the possibility that the measured relative rates of hydrolysis are not biased by differences in the substrate replenishment rate. The following findings strongly suggest that such bias is not significant here. First, the effect of acyl chain length/unsaturation on the rate of hydrolysis of PC species in vesicles was more than 2 orders of magnitude less than expected based
Specificity of Secretory PLA₂s

Specificity of Secretory PLA₂s

—The key finding of this study is that efflux propensity seems to be a far more important factor in the hydrolysis of membrane-bound phospholipid molecules by secretory PLA₂s than commonly appreciated. Efflux propensity may depend on the entropy change experienced by phospholipid acyl chain tails when the phospholipid moves upward in the bilayer in order to reach the active site of PLA₂. Accommodation of the phospholipid acyl chains at the substrate binding site of the enzyme also seems to contribute to the specificity of the secretory PLA₂s studied here, as indicated by the data obtained with micelles. However, differences in binding site structure seem to be largely masked with bilayer-bound substrates, probably because the much tighter lipid-lipid interactions make substrate efflux rate-limiting in this system.

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