The Interaction of Phospholipase A2 with Phospholipid Analogues and Inhibitors*

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A series of structurally modified phospholipids have been used to delineate the structural features involved in the interaction between cobra venom (Naja naja naja) phospholipase A2 and its substrate. Special emphasis has been placed on sn-2 amide analogues of the phospholipids. These studies have led to a very potent, reversible phospholipase A2 inhibitor. A six-step synthesis of this compound, 1-palmitylthio-2-palmitoyl-amino-1,2-dideoxy-sn-glycero-3-phosphorylcholine, (thioether amide-PE), was developed. Other analogues studied included 1-palmitylthio-2-palmitoyl-amino-1,2-dideoxy-sn-glycero-3-phosphorylcholine, 1-palmitoyl-2-palmitoylamino-2-deoxy-sn-glycero-3-phosphorylcholine, 1-palmitoyl-2-palmitoylamino-2-deoxy-sn-glycero-3-phosphorylcholine, 1-palmitoyl-2-palmitoylamino-2-deoxy-sn-glycero-3-phosphorylcholine, and sphingomyelin. Inhibition studies used the well defined Triton X-100 mixed micelle system and the spectroscopic thio assay. The phospholipid analogues showed varying degrees of inhibition. The best inhibitor was the thioether amide-PE which had an IC50 of 0.45 μM. In contrast, sphingomyelin, a natural phospholipid that resembles the amide analogues, did not inhibit but rather activated phosphatidylycholine hydrolysis. This systematic study of phospholipase A2 inhibition led to the following conclusions about phosphidiphospholipase A2 interactions: (i) sn-2 amide analogues bind tighter than natural phospholipids, presumably because the amide forms a hydrogen bond with the water molecule in the enzyme active site, stabilizing its binding. (ii) Inhibitor analogues containing the ethanolamine polar head group appear to be more potent inhibitors than those containing the choline group. This difference in potency may be due solely to the fact that the cobra venom phospholipase A2 is activated by choline-containing phospholipids. Thus, choline-containing non-hydrolyzable analogues both inhibit and activate this enzyme. Both of these effects must be taken into account when studying phosphatidylycholine inhibitors of the cobra venom enzyme. (iii) The potency of inhibition of these analogues is significantly enhanced by increasing the hydrophobicity of the sn-1 functional group. (iv) The α-methyl-

Phospholipase A2 is a lipolytic enzyme that specifically hydrolyzes the sn-2 ester bond of phospholipids (1). The enzyme is widespread in nature and exists in both extracellular and intracellular forms (1-3). In addition to the obvious role it plays in phospholipid catabolism, the enzyme is also believed to be involved in a series of vital regulatory processes via its ability to release arachidonic acid for the subsequent biosynthesis of eicosanoids (4-6). Eicosanoids are implicated in the pathophysiology of many diseases, especially those involving inflammation and allergy (5-7). Due to the biological importance of phospholipase A2, there is considerable current interest in understanding the enzyme's mechanism and how its activity is controlled in vivo.

To date the most extensively studied phospholipase A2s are the extracellular ones obtained from mammalian pancreas and snake venom. The studies of these enzymes have served as models for the studies of the less abundant, more difficult to obtain intracellular enzymes. An important aspect of such studies has been the use of inhibitors to probe substrate/ enzyme interactions. Inhibitors can provide valuable information about how phospholipids interact with phospholipase A2s, about which enzyme residues are involved, and about the enzyme's general mechanism of action.

A growing number of compounds have been found that inhibit these phospholipase A2s. The best characterized inhibitor is p-bromophenacylbromine, an irreversible inhibitor that specifically modifies the active site histidine residue (8, 9). Other covalent inhibitors are the natural product manoolide (10, 11) and its synthetic analogue, manoolane (12), which have been shown to covalently modify lysine residues. While helping to identify active site residues, irreversible inhibitors, especially those that do not resemble the substrate, offer little information about the nature of substrate binding. Information about binding is more readily obtained from reversible inhibitors. By comparing the binding affinities of a closely related series of inhibitors, one can draw inferences about what structural features in the phospholipid are important for binding. This information can be correlated with sequence and x-ray crystal data to help delineate the active site interactions of the enzyme and its mechanistic details.

To this end, we have previously synthesized several phospholipid analogues containing a sn-2 amide (13-16) and, in a preliminary communication (17), have reported on an ether amide phospholipid analogue which is a reversible inhibitor. We have now extended these earlier studies (17) to include a series of substrate analogues that allow us to correlate phospholipid structure to inhibitory potency. We have systemati-
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Inhibition Studies

The inhibitory potency of the phospholipid analogues was evaluated by determining the analogue concentration required to decrease the hydrolytic activity of phospholipase \( A_2 \) by 50% (IC\(_{50}\)). The effect of various phospholipid analogues on cobra venom phospholipase \( A_2 \) was determined using the well defined Triton X-100/phospholipid mixed micelle system (25). In this study, thio-PC and thio-PE were used as substrate.

Enzyme Assay

The phospholipase \( A_2 \) activity toward thio-PC and thio-PE was determined spectrophotometrically at 324 nm by measuring the change in absorbance due to the reaction of the product’s free thiol group with 4,4’-dithiobispyridine. The mixed micelles of phospholipid, thio-PC, and Triton X-100 were prepared fresh daily. The standard assay mixture consisted of 0.5 mM of either thio-PC or thio-PE substrate, 1.75 mM Triton X-100, 25 mM Tris buffer (pH 8.5), 0.1 M KCl, and 10 mM CaCl\(_2\). The enzyme solution (10 ng/ml) contained 4 mM Triton X-100 in the Tris buffer. The cuvette, containing 0.3 ml of assay mixture and 5 \( \mu \)l of 4,4’-dithiobispyridine (50 mM in ethanol), was placed in the compartment of a Perkin-Elmer model 592 spectrophotometer thermostated at 30 °C and balanced against a blank containing water only. After incubation of the substrate for 2 min at 30 °C, phospholipase \( A_2 \) (5 \( \mu \)l containing 50 ng of protein) was added to initiate the reaction. The average of duplicates is reported.

Synthesis of 1,9-Bis(decanoylthio)-1,9-dideoxy-sn-glycero-3-phosphorylcholine (Thio-PC)

1-Trimyl-2,3-dimercaptol-1-propanol (2) — A tetrabutylammonium salt of 3-trimyl-1,2-dioxyn-1,2-dideoxy-sn-glycero-3-phosphorylcholine 1 (4.98 g, 12.2 mmol) was reduced by LiAlH\(_4\), according to the procedure of Hendrickson et al. (19). The crude mercaptan was dissolved in 25 ml of acetone and applied to a 250-ml column of silica gel (50:1). Prior to packing the column, the silica gel was dehydrated by vacuum and stored under argon. The column was eluted with 1200 ml of hexanes/acetone (50:1). The effluent was monitored by TLC developed with hexanes/acetone (7:1). A clear oil was obtained (3.64 g, 81% yield), which showed a single spot on TLC (RF = 0.35 for detritylated product). The reaction went to completion in 12 h. The crude detritylated compound 4 was dissolved in 130 ml of dry pyridine, and the mixture was sonicated until a clear solution was obtained. One-dimensional proton NMR spectra and two-dimensional homonuclear J-correlation (1H COSY) spectra were taken on an extensively modified Varian 360 MHz NMR spectrometer, using tetrachloromethane as an internal standard. Carbon-13 NMR and phosphorus 31 NMR spectra were obtained at 50 and 61 MHz, respectively, on a 200-MHz Nicolet NMC-NB-200 spectrometer. Trimethyl phosphate (0.5%) was used as an external standard for the 31P spectra. Optical rotation was measured with a Perkin-Elmer model 141 polarimeter. FAD mass spectra were obtained at the University of California Riverside mass spectrometry facility.

General Methods

Thin-layer chromatography (TLC) was carried out on Analtech Silica Gel G-250 glass plates. General detection was by exposing to iodine vapor or spraying the plates with 2 M sulfuric acid, followed by heating on a hot plate. Trityl compounds were visualized by their bright yellow color after gently warming the sprayed plate. Phospholipid was detected with molybdate spray (23). Flash chromatography (silica gel 60-400 mesh, Merck) was purchased from Aldrich. The ion exchanger, Rexyn I-300, was obtained from Fisher.

EXPERIMENTAL PROCEDURES

Materials

Phospholipase \( A_2 \) was purified to homogeneity from cobra venom (\( Naja \) naja naja) as described previously (18). 4,4’-Dithiobispyridine, palmitoyl chloride, and decanoyl chloride were purchased from Aldrich. Triton X-100 was obtained from Sigma. 1,2-Dipalmitoyl-sn-glycero-3-phosphorylcholine (DPPC) and sphingomyelin were obtained from Avanti Polar Lipids, Inc. 1-Palmitylthio-2-palmitoylaminol-1,2-dideoxy-sn-glycero-3-phosphorylcholine (thioether amide-PC), 1-palmityl-2-palmitoylaminol-2-deoxy-sn-glycero-3-phosphorylcholine (ether amide-PC), 1-palmityl-2-palmitoylaminol-2-deoxy-sn-glycero-3-phosphorylcholine (ester amide-PC), 1-palmitylthio-2-[[tetradeccyloxylcarbonyl]amino]-1,2-dideoxy-sn-glycero-3-phosphorylcholine (alkylamide-PC) and 1-palmitylthio-1,2-bis(decaprylthio)-1,2-dideoxy-sn-glycero-3-phosphorylcholine (alkylureido-PC) were synthesized as described previously (13-16). 3-Chloroperphoryl dichloride was prepared by the procedure of Rosenthal (22). Phosphorylcholine (carbamoyl-PC), and 1-palmityl-2-l[(octadecyloxyl)carbonyl]amino]-3-deoxy-sn-glycero-3-phosphorylcholine (alkylamide-PC) were synthesized as described previously (13-16). Chloroform and benzene were dried by distillation over phosphorus pentoxide. Phosphorus oxychloride was freshly distilled prior to use.

All other solvents were reagent grade or better. Silica Gel 60 (230-400 mesh, Merck) was purchased from Aldrich. The ion exchanger, Rexyn I-300, was obtained from Fisher.
CHCl₃ containing 1.0 ml of dry pyridine (12.5 mmol). A solution of freshly distilled POCl₃ (1.8 g, 11.7 mmol) in 30 ml of dry CHCl₃ was added to the solution of compound 4. The reaction was stirred under argon at 50 °C for 1 h. The disappearance of the starting material was followed by TLC in hexane/acetone (7:1) (Rf = 0.45). After the reaction was complete, the mixture was cooled to room temperature. Choline p-toluenesulfonate (0.84 g, 9.73 mmol) and 3.0 ml of dry pyridine were added. The mixture was stirred for 3 h at room temperature. The reaction mixture was washed with two portions of water; methanol was added to break the emulsion. The chloroform solution was evaporated in vacuo and lyophilized. The solid was dissolved in 30 ml of chloroform/methanol/water (65:25:4) and purified by flash chromatography using chloroform/ethyl acetate (9:1) to give pure methyl-aminopyridine (3.92 g, 32.1 mmol). To this mixture was added hydrochloride (5.0 g, 32.1 mmol) was suspended in 250 ml of chloroform containing triethylamine (3.25 g, 32.1 mmol) and N,N-di-ethanolamine (1.24 g, 15 mmol). The mixture was stirred for 2 h at room temperature. The combined extracts were washed over anhydrous sodium sulfate, and the chloroform was evaporated under vacuum. The product showed only one spot on TLC (RF 0.5 in hexanes/acetone 7:3).

Synthesis of 1,2-Bis(decanoyloxy)-1,2-dideoxy-sn-glycerol-3-phosphorylcholine (Thio-PE)

Thio-PC (150 mg, 0.25 mmol) was transformed into thio-PE by the action of phosphodiester D according to the procedure of Comforti and Zwaal (26). The crude product was purified by flash chromatography using chloroform/methanol/water (65:25:1) as eluting solvent. The product was dissolved in 100 ml of absolute ethanol. To this solution was added 15 ml of 10% hydrazine in ethanol over 10 min and refluxed for an additional 30 min. The solution was cooled in an ice bath and 1 N HCl was added to quench the reaction. The mixture was diluted with water and extracted with chloroform. The combined extracts were dried over anhydrous sodium sulfate, and the chloroform was evaporated under vacuum. The product showed only one spot on TLC (RF 0.5 in hexanes/acetone 7:3). Compound 10 was obtained in 99% yield (0.52 g).

\[ \text{C}_5H_{12}O_2NPS}_2 \quad (M + H^+) \]
Calculated: 598.3364
Found: 598.3364

RESULTS

Chemical Synthesis—The thio-PC was synthesized by a modified version of the procedure of Hendrickson et al. (19). As shown in Scheme 1, the basic synthetic strategy of this modified method is the same as that of the original paper. The disadvantages of the previous procedure is (a) the difficulty of purifying the intermediate, compound 3, and the product, thio-PC, and (b) the acyl migration and ester hydrolysis of compound 4 that occur during its purification by silica gel column chromatography. The low yield of the original method can be overcome by the following modifications. Originally, compound 3 was made through the direct acylation of the crude reduction product of compound 1. Instead of the laborious purification of compound 3, compound 2 was purified
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The phenomenon is observed when the enzymatic activity depends on the potential acyl migration and hydrolysis of compound 4, it was directly phosphorylated without purification. Purification of compound 5 does not involve acyl migration or hydrolysis. Finally, phosphatidylethanolamine was purified by ion exchange and silica gel column chromatography rather than by high pressure liquid chromatography as in the original paper (27). The overall yield of the modified method was twice that of the original one (19 versus 9%).

The synthesis of the thioether amide-PE is outlined in Scheme 2. This approach provides an alternate synthetic pathway to that employed by Bhatia et al. (16). The synthesis is based on the selective acylation of the amino group of D-serine methyl ester and the subsequent selective reduction, with LiBH₄, of the ester group. The chirality of the optically active a-carbon in D-serine provided the asymmetric center in thioether amide-PE. Reaction of one equivalent of palmitoyl chloride with D-serine methyl ester at room temperature afforded compound 7 in quantitative yield after workup. By activating the hydroxyl group of compound 7 with tosyl chloride, the thioether was introduced by refluxing the methanol solution of tosylate and hexadecyl mercaptan. The reduction of compound 9 with a mild reducing reagent, LiBH₄, led to the formation of the cysteine derivative 10 in essentially quantitative yield. Finally, the phosphorylthanolamine moiety was introduced by treating compound 10 with 2-phthalimidoethylphosphoryl dichloride (22).

Kinetic Studies—There are two important factors that must be taken into account when studying phospholipases. The first is that the activity of these enzymes critically depends on the physicochemical structure of the phospholipid substrate; any molecule which disturbs the structure, i.e., the lipid-water interface, could influence phospholipase activity. It is important to choose an assay system that minimizes this secondary effect. We, therefore, used the well-defined Triton X-100/phospholipid mixed micelle system (25, 28). These mixed micelles are composed predominately of the nonionic detergent Triton X-100. The presence of small amounts of substrate and phospholipid analogue have negligible effect on the surface environment and micelle structure (29). Thus, the method was modified by replacing the phospholipid with phosphorylthanolamine. In group C (compounds 13, 14, 15, and sphingomyelin), the analogues were modified systematically at the sn-1 position with an ester, an ether, or a thioether group, and in addition, the natural phospholipid, sphingomyelin, was also studied. In group D (compounds 16, 17), the molecules were modified by replacing the ester group at the sn-2 position with either carbamoyl or alkylureido groups.

The second factor is that any enzyme acting on an aggregated substrate can exhibit surface dilution kinetics. This phenomenon is observed when the enzymatic activity depends on both the bulk and the surface concentration of substrate (28, 30, 31). While the addition of enzymatically inert compounds to the interface does not dilute the substrate’s bulk concentration, it does dilute its surface concentration. The effect of this surface dilution on the phospholipase A₂ activity is shown in Fig. 1. Triton X-100 acts as an inert non-hydrolyzable diluent for the cobra venom enzyme (32). Increasing the Triton X-100 concentration dilutes the substrate’s surface concentration resulting in a decrease in phospholipase A₂ activity. Plotting enzyme activity versus Triton X-100 concentration would yield a curve that is very similar to the one that would be produced if Triton X-100 were only a competitive inhibitor. Thus, this Triton X-100 curve is the critical control for these studies. If the compound being tested is truly inert and does not interact with the enzyme at all, it would still surface dilute the substrate and produce a curve identical to the Triton X-100 curve. Therefore, it is the deviation from this curve that determines whether a compound is an activator, an inhibitor, or neither. Surface dilution ceases to be a problem if the inhibitor is very potent and its concentration in the interface is low enough not to dilute the substrate’s surface concentration significantly.

For the structure-activity analysis, the compounds were grouped into four categories according to their structural relationships. In group A, the importance of the amide group at the sn-2 position of a phospholipid was studied by comparing the effect of dipalmitoyl-PC and the amide analogue 15 on phospholipase A₂ hydrolysis of thio-PC. In group B (compounds 12, 13), the effect of the head group was studied by replacing the phosphocholine with phosphorylthanolamine. In group C (compounds 13, 14, 15, and sphingomyelin), the analogues were modified systematically at the sn-1 position with an ester, an ether, or a thioether group; in addition, the natural phospholipid, sphingomyelin, was also studied. In group D (compounds 16, 17), the molecules were modified by replacing the ester group at the sn-2 position with either carbamoyl or alkylureido groups.

The replacement of the sn-2 ester by an amide has a significant effect on the interaction of the enzyme with its substrate. This effect was evaluated by comparing the inhibition of thio-PC hydrolysis by the compounds in group A, DPPC (which should theoretically be a competing substrate) and compound 15. Compound 15 differs from DPPC only in the sn-2 functional group. While DPPC is hydrolyzed by the enzyme, its hydrolysis is not detected in the thio assay because the lysophospholipid produced does not contain a free sulfhydryl group and, thus, cannot react with the chromogenic

![Fig 1 Effect of the substitution of the sn-2 ester by an amide on the inhibition of the phospholipase A₂ substrate analogues. Dipalmitoyl-PC (●); ester amide-PC (○). Triton X-100 (△) is used to illustrate the surface dilution effect. Thio-PC concentration is 0.5 mM and Triton X-100 is 4.0 mM.](http://www.jbc.org/fig1)
reagent. Since DPPC binds to the enzyme but its hydrolysis is not detected, one would expect it to look like a competitive inhibitor in the thioc assay. However, DPPC did not show any inhibition at concentrations up to 10 mM. By comparing it to the Triton X-100 control, DPPC clearly activates the hydrolysis of thio-PC. On the other hand, compound 15 clearly shows a significant inhibition. Due to the small amount of compound 15 used in the assay, the surface dilution effect caused by it is negligible. Therefore, compound 15 is a potent inhibitor having an IC$_{50}$ of 0.156 mM.

Replacing the phosphorylcholine polar head group by phosphorylthanolamine, group B, also affects the potency of phospholipase A$_2$ inhibition. As shown in Fig. 2, the half-maximal inhibition of thio-PC hydrolysis by thioether amide-PE was 0.45 μM, which is 4 times lower than the thioether amide-PC (2 μM). The inhibition of PE hydrolysis was also measured as shown in Fig. 3. The IC$_{50}$ of thioether amide-PE was about 6.5 μM. The thioether amide-PC did not follow a simple inhibition curve; thus, an IC$_{50}$ could not be determined. In both of these experiments, the concentration of inhibitor was negligible compared to the concentration of surfactant and the total surface, and, thus, the surface dilution effect was also negligible.

Since the thioether amide PE was the most potent inhibitor, we chose it to test whether the inhibition was reversible. The enzyme and inhibitor were incubated together and then diluted 200-fold into an assay. We found that the effect of the inhibitor could be overcome by dilution or by a high substrate concentration. We also measured a time course of inhibition and found that it was not time dependent. Therefore, we conclude that the inhibition is reversible for thioether amide-PE. Since the other compounds are closely related to the thioether amide-PE, we assume that their inhibition is also reversible.

![Fig. 2. Inhibition of phospholipase A$_2$ hydrolysis of thio-PC/Triton X-100 mixed micelles by thioether amide-PE (C) and thioether amide-PC (A). Substrate concentration is 0.5 mM.](image)

In group C, the hydrophobicity of the sn-1 functional group was varied. As shown in Table I, the potency of inhibition increased with increasing hydrophobicity. The most potent inhibitor in this series was the thioether amide-PC 13 which has the most hydrophobic group, the thioether, at the sn-1 position. Its inhibition of thio-PC hydrolysis is shown in Fig. 2, and the IC$_{50}$ was 2 μM. On the other hand, sphingomyelin, which has the most hydrophilic group at the sn-1 position, affects thio-PC hydrolysis in a complex manner, Fig. 4. Apparently, two competing phenomena are involved. Sphingomyelin activates phospholipase A$_2$ hydrolysis of phosphatidylethanolamine and, at high concentrations, dilutes the substrate surface concentration. In Fig. 4, the effect of surface dilution on the enzymatic activity is illustrated by substituting Triton X-100 for sphingomyelin. Taking into account surface dilution, by comparing the sphingomyelin curve to the Triton X-100 curve, it is clear that sphingomyelin activates the hydrolysis of thio-PC but the observed inhibition is due solely to surface dilution. Sphingomyelin does not appear to inhibit the enzyme directly.

In group D, the binding specificity of the substrate analogues was altered by replacing the α-methylene group in the sn-2 chain. As shown in Table I, the analogues in group D are not inhibitors of phospholipase A$_2$ at all. Taking into account surface dilution, compound 16 shows no inhibition of the thio-PC hydrolysis. Although compound 16 differs from compound 13 only in the α position of the sn-2 fatty chain, the former does not affect the activity of phospholipase A$_2$, while the latter is an excellent inhibitor. Compound 17 did not show any inhibition, even at the highest concentration (5 mM) used, while compound 15 is a potent inhibitor having an IC$_{50}$ of 0.156 mM. These results imply that the α-methylene group of the sn-2 acyl chain is required for phospholipid binding to the catalytic site of phospholipase A$_2$. Although the compounds in group D did not inhibit PC hydrolysis, they were excellent activators of PE hydrolysis. Fig. 5 shows that compound 17 has no effect on the hydrolysis of thio-PC but activates the hydrolysis of thio-PE. In other experiments, we have also shown that all of the other PC analogues were also activators of PE hydrolysis, even though they inhibit the hydrolysis of PC.

**DISCUSSION**

"Surface Dilution Kinetics" and Inhibition—All enzymes that act on lipid interfaces can potentially be affected by the phenomenon of surface dilution. This effect is particularly troublesome when studying reversible inhibition because many of these inhibitors are amphipathic molecules that will intercalate into the interface and dilute the surface concentration of the substrate. This dilution can be mistaken for competitive inhibition and must be taken into account if the interaction of these molecules with the enzyme is to be detected. There are several ways to deal with this phenomenon.

The first method applies if the inhibitor is very potent. In this case, its surface concentration will be negligible, and thus, the surface dilution that it causes will also be negligible. This was found to be the case with several of the inhibitors in this study. A second method of decreasing the inhibitor’s surface dilution effect is to dilute both the substrate and the inhibitor with a large amount of some inert non-hydrolysable compound. Under these conditions the change in surface area caused by changing the inhibitor concentration is reduced simply because the total surface area is increased. In some cases, the concentration of substrate can be kept constant by balancing changes in the inhibitor concentration with changes in the inert diluent. This is difficult to do when working with
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**TABLE I**

Inhibition of phospholipase A<sub>2</sub> by phospholipid analogues

All inhibition studies were carried out in mixed micelles of thio-PC (0.5 mM) and Triton X-100 (4.0 mM). ND, no inhibition was detected even at millimolar concentrations.

<table>
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<tr>
<th>Group</th>
<th>Compound</th>
<th>No.</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (mM)</th>
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<td>A</td>
<td>Dipalmitoyl-PC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Thioether amide-PE</td>
<td>12</td>
<td>O CH₂OPC</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>B, C</td>
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<td>13</td>
<td>O CH₂OPC</td>
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<td>O CH₂OR</td>
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<tr>
<td>A, C</td>
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<td>15</td>
<td>O CH₂OOCR</td>
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<tr>
<td>C</td>
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<td>O CH₂SR</td>
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<td>D</td>
<td>Alkylurido-PC</td>
<td>17</td>
<td>O CH₂OOCR</td>
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</table>

vesicles but is more tractable in a mixed micelle system. The Triton X-100/phospholipid mixed micelle system is one such system that works for the cobra venom phospholipase A<sub>2</sub>. A second advantage of the mixed micelle system is that the effect of the inhibitor on interface and the surrounding lipids is also reduced.

The third strategy is to actually measure the dilution effect directly. We have employed this technique by using Triton X-100 as a diluent and measuring activity as a function of surface concentration of substrate. All of our inhibition profiles were compared to this curve. One could also calculate its effect if a suitable kinetic model were available. In any case, surface dilution must be taken into account if false inhibition results are to be avoided.

**Potent Phospholipase A<sub>2</sub> Inhibitors**—The design of inhibitors based on non-hydrolyzable phospholipid analogues has proven to be an effective means by which tight binding inhibitors of phospholipase A<sub>2</sub> can be obtained. The replacement of the ester moiety by the corresponding amide function at the sn-2 position abolishes the enzymatic activity of phospholipase A<sub>2</sub> (13, 17, 33, 34) and also increases the affinity of the analogues for the enzyme. The judicious substitution of other groups on the amide analogues has increased their potency. The 0.45 μM IC<sub>50</sub> of thioether amide-PE 12 is one of the tightest phospholipase A<sub>2</sub> inhibitors found to date. In addition to finding this potent inhibitor, these studies have pinpointed four aspects of enzyme/lipid interaction that are important for substrate binding: (i) The introduction of an amide moiety in the sn-2 position significantly increased the binding of the phospholipid derivative to the catalytic site. (ii) Increasing the hydrophobicity of the functional group in the sn-1 position increases the affinity between the phospholipid molecule and the enzyme. (iii) The α-methylene group of the sn-2 fatty chain plays an important role in differentiating the binding of phospholipid to the two functional sites of the cobra venom phospholipase A<sub>2</sub>. (iv) The ethanolamine-
Regarding point i, the high affinity of the nonhydrolyzable isosteric phospholipid derivatives can be explained on the basis of hydrogen bonding. Chemical modification (8, 9) and x-ray crystallographic studies of related phospholipase A$_2$s (35–37) have demonstrated the importance of a catalytic histidine residue. In addition, the three-dimensional structure of bovine pancreatic phospholipase A$_2$ indicates that there is a tightly bound water molecule lying in the plane of the imidazole ring. The amide proton could form a hydrogen bond with this water molecule inside the active site. Formation of such a hydrogen bond would increase the binding affinity of the analogue to the enzyme. The hydrogen binding characteristics and the orientation of the carbonyl oxygen of the normal substrate could preclude such an effect. A more definitive analysis of this hypothesis awaits the x-ray structure of the enzyme with substrate analogue bound in the catalytic site.

The correlation between hydrophobicity of the sn-1 functional group and the binding affinity, point ii, is consistent with the observation that the substrate binding pocket of the catalytic site of phospholipase A$_2$ is hydrophobic. X-ray crystallographic studies (38–40) have revealed that the interior wall of the catalytic site is covered with highly conserved, hydrophobic residues: Phe-5, Ile-9, Phe-22, Ala-102 and -103, Phe-106, and the disulfide bridge between Cys-20 and -45. These residues form a hydrophobic binding pocket at the catalytic site. By increasing the hydrophobicity of the sn-1 functional group, the hydrophobic interaction between the enzyme and substrate analogue appears to be enhanced.

The "Dual Phospholipid Model" and Inhibition—Points iii and iv, the effects of the $\alpha$-methylene group and the head group, can be explained by the Dual Phospholipid Model. The Dual Phospholipid Model was developed for the cobra venom phospholipase A$_2$ which has two distinct functional sites: an activator site and a catalytic site. The activator site specifically requires a phosphorylcholine group to activate the enzyme while the catalytic site hydrolyzes phospholipid with either a phosphorylcholine or phosphorylethanolamine head group (29, 41, 42). We have previously shown that this effect is due to a direct interaction between the activator and the enzyme and is not due to some general effect on the interface. Thus, this enzyme interacts with two phospholipid molecules, whether this takes place at two distinct physical sites or in one large site that binds two phospholipids is not known.

A manifestation of this phenomenon was found in the inhibition of phosphatidylethanolamine hydrolysis by thioether amide-PC as shown in Fig. 3. This unusual inhibition curve is produced by the two competing phenomena: activation and inhibition. Because of the phosphorylcholine moiety, the thioether amide-PC can bind to the activator site and activate thio-PE hydrolysis. This effect predominates at low inhibitor concentration and is responsible for the increase in activity in this region. As the thioether amide-PC concentration increases, it begins to compete with the substrate for the catalytic site. At high concentrations, the inhibition predominates and significant overall inhibition is observed. This implies that the binding of the thioether-PC is tighter to the catalytic site than to the activator site. The replacement of the $\alpha$-methylene group, compounds 16 and 17, yields analogues that are very poor inhibitors but good activators. Apparently the $\alpha$-methylene group is required for the binding to the catalytic site but not to the activator site. These two results indicate that the two functional sites have different environments and are probably two physically different sites.

An aspect of the activation process yet to be investigated is whether PC activates its own hydrolysis? While the activation of PE hydrolysis was easily demonstrated, the activation of PC hydrolysis was much more difficult to test. Because PC acts as both substrate and activator, it was not possible to ascertain the non-activated rate of PC hydrolysis. We have now made use of the fact that sphingomyeline is a more efficacious activator than PC. PC activates PE hydrolysis by 7-10-fold: sphingomyeline activates it by 20-fold (42). If PC hydrolysis is activatable, the addition of sphingomyeline should produce an additional activation over and above that of PC itself. When surface dilution effects are taken into account, thio-PC hydrolysis is clearly activated as shown in Fig. 3. Thus, PC hydrolysis can be activated by a more potent activator. In addition, there appears to be no inhibition by sphingomyeline. This would seem to indicate that sphingomyeline's binding to the catalytic site is much weaker than the substrate's. These results clearly demonstrate that PC not only activates PC hydrolysis but also its own.

In conclusion, the studies presented in this article demonstrate the importance of taking into account surface dilution when studying enzymes that act on lipid interfaces. They have also pinpointed four structural features involved in the interaction of phospholipase A$_2$ and its substrate. First, the amide analogues bind tighter than natural phospholipids.

![Fig. 4. Effect of sphingomyeline (\(\bullet\)) on the hydrolysis of thio-PC catalyzed by phospholipase A$_2$. The surface dilution effects is also shown by substituting Triton X-100 (\(\triangle\)) for sphingomyeline. Thio-PC concentration is 0.5 mM.](image1)

![Fig. 5. Effect of alkylyurido-PC 16 on the phospholipase A$_2$ activity using thio-PC (\(\triangle\)) or thio-PE (\(\bullet\)) as substrate. Substrate concentration is 0.5 mM and Triton X-100 is 4.0 mM.](image2)
Second, the phosphorylethanolamine-containing analogues appear to bind to the phospholipase A₂ tighter than those containing phosphorylcholine head groups but the activation caused by the PC compounds must be taken into account. Third, the hydrophobicity of the sn-1 group has a significant effect on the binding of phospholipid to the enzyme. The more hydrophobic the sn-1 functional group, the tighter the binding of phospholipid. Fourth, the α-methylene group of the sn-2 fatty chain is essential for the binding of the analogue to the catalytic site but not to the activator site. Further studies are in progress to develop a kinetic model for this inhibition.

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