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-palmitoylamino-1,2-dideoxy-sn-glycero-3-phosphorylethanolamine (thioether amide-PE), was developed. Other analogues studied included 1-palmitylthio-2-palmitoylamino-1,2-dideox-sn-glycero-3-phosphorycholine, 1-palmitoyl-2-palmitoylamino-2-deoxy-sn-glycero-3-phosphorylcholine, 1-palmitoyl-2-palmitoylamino-2-deoxy-sn-glycero-3-phosphorylcholine, 1-palmitoyl-2-palmitylamino-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 phosphatidylcholine hydrolysis. This systematic study of phospholipase A₂ inhibition led to the following conclusions about phospholipid-phospholipase 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 phosphatidylcholine 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 A₂, 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 dibromophenacylbromine, an irreversible inhibitor that specifically modifies the active site histidine residue (8, 9). Other covalent inhibitors are the natural product manoside (10, 11) and its synthetic analogue, manoside analogue (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 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-
cally studied the effect of substituting an amide group for the sn-2 ester, of changing the polar head group, of varying the hydrophilicity of the phospholipid molecule (13-16). The effect of various phospholipid analogues on cobra venom phospholipase A₂ 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.

**Experimental procedures**

**Materials**

Phospholipase A₂ was purified to homogeneity from cobra venom (Naja naja atra) as described previously (18). 4,4'-Dithiodipyridine, 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-palmitoylamino-2-deoxy-sn-glycero-3-phosphorylcholine (ether amide-PC), 1-palmitoyl-2-palmitoylamino-2-deoxy-sn-glycero-3-phosphorylcholine (ester amide-PC), 1-palmitoylthio-2-[(tetradeoxyxycarbonyl)amino]-1,2-dideoxy-sn-glycero-3-phosphorylcholine (alkylureido-PC) were synthesized as described previously (13-16). 3-Trityl-1,2-dideoxy-1,2-(thiocarbonyldithio)-sn-glycerol was prepared according to the method of Hendrickson et al. (19). Choline toluenesulfonate was prepared as described by Brockerhoff and Ayengar (20). D-Serine methyl ester hydrochloride was prepared as described by Greenstein and Winitz (21). 2-Phthalimidoethylphosphoryl dichloride was prepared by the procedure of Rosenthal (22).

**Synthesis**

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 charring on a hot plate. Trityl compounds were visualized by their bright yellow color after gently warming the sprayed plate. Phosphorus pentoxide was used for deoxygenation. All other solvents were reagent grade or better. Silica Gel 60 (230-400 mesh, Merck) was purchased from Aldrich. The ion exchanger, Rhenex 1-300, was obtained from Fisher.

**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 charring on a hot plate. Trityl compounds were visualized by their bright yellow color after gently warming the sprayed plate. Phosphorus pentoxide was used for deoxygenation. All other solvents were reagent grade or better. Silica Gel 60 (230-400 mesh, Merck).

**Enzyme Assay**

The phospholipase A₂ 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, phospholipid analogue, and Triton X-100 were prepared fresh daily by one of the following two methods: (i) A mixture of the desired amounts of phospholipid and lipid analogue in a chloroform solution was dried under a stream of nitrogen and then in vacuo. Triton X-100, in standard assay buffer, was then added and the mixture was sonicated until a clear solution was obtained. (ii) Alternatively, the micelles of phospholipid and Triton X-100, and the micelles of the phospholipid analogue and Triton X-100, were prepared separately. Aliquots of these solutions were combined to obtain the proper concentration of the inhibitor in the micelles. The mixture was again sonicated prior to assay.

**Inhibition Studies**

The phospholipase A₂ 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, phospholipid analogue, and Triton X-100 were prepared fresh daily by one of the following two methods: (i) A mixture of the desired amounts of phospholipid and lipid analogue in a chloroform solution was dried under a stream of nitrogen and then in vacuo. Triton X-100, in standard assay buffer, was then added and the mixture was sonicated until a clear solution was obtained. (ii) Alternatively, the micelles of phospholipid and Triton X-100, and the micelles of the phospholipid analogue and Triton X-100, were prepared separately. Aliquots of these solutions were combined to obtain the proper concentration of the inhibitor in the micelles. The mixture was again sonicated prior to assay.

**FAD Mass Spectra**

FAD mass spectra were obtained at the University of California at Berkeley. The FAD standard was prepared and purified as described by Still et al. (24) using Silica Gel 60 (230-400 mesh, Merck). Trimethyl phosphine was added to a solution of 1-trityl-2,3-dimercapto-1-propanol 2 (3.64 g, 81% yield), which gave a single spot on TLC in hexanes/acetone (50:1). 1H NMR (CDCl₃) δ 2.91 (d, 2H), 3.03 (m, 2H), 3.24 (m, 2H), 3.872 (m, 1H), 7.294 and 7.417 ppm (m, 15H).

**Results and Discussion**

The phospholipase A₂ 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, phospholipid analogue, and Triton X-100 were prepared fresh daily by one of the following two methods: (i) A mixture of the desired amounts of phospholipid and lipid analogue in a chloroform solution was dried under a stream of nitrogen and then in vacuo. Triton X-100, in standard assay buffer, was then added and the mixture was sonicated until a clear solution was obtained. (ii) Alternatively, the micelles of phospholipid and Triton X-100, and the micelles of the phospholipid analogue and Triton X-100, were prepared separately. Aliquots of these solutions were combined to obtain the proper concentration of the inhibitor in the micelles. The mixture was again sonicated prior to assay.

**Conclusion**

The phospholipase A₂ 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, phospholipid analogue, and Triton X-100 were prepared fresh daily by one of the following two methods: (i) A mixture of the desired amounts of phospholipid and lipid analogue in a chloroform solution was dried under a stream of nitrogen and then in vacuo. Triton X-100, in standard assay buffer, was then added and the mixture was sonicated until a clear solution was obtained. (ii) Alternatively, the micelles of phospholipid and Triton X-100, and the micelles of the phospholipid analogue and Triton X-100, were prepared separately. Aliquots of these solutions were combined to obtain the proper concentration of the inhibitor in the micelles. The mixture was again sonicated prior to assay.

**Thio-TEC Substrate**

Thio-TEC was prepared by the procedure of Rosenthal (22). Chloroform and benzene were dried by distillation over phosphorus pentoxide. Trityl compounds were visualized by their bright yellow color after gently warming the sprayed plate. Phosphorus pentoxide was used for deoxygenation. All other solvents were reagent grade or better. Silica Gel 60 (230-400 mesh, Merck) was purchased from Aldrich. The ion exchanger, Rhenex 1-300, was obtained from Fisher.

**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 charring on a hot plate. Trityl compounds were visualized by their bright yellow color after gently warming the sprayed plate. Phospholipid analogues were separated by charring on a hot plate. Trityl compounds were visualized by their bright yellow color after gently warming the sprayed plate. Phosphorus pentoxide was used for deoxygenation. All other solvents were reagent grade or better. Silica Gel 60 (230-400 mesh, Merck) was purchased from Aldrich. The ion exchanger, Rhenex 1-300, was obtained from Fisher.

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**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 tetramethylsilane as an internal standard. Carbon 13 NMR and phosphorus 31 NMR spectra were obtained at 50 and 81 MHz, respectively, on a 200-MHz Nicolet NMC-NB-200 spectrometer. Trimethyl phosphine (5%) was used as an external standard for the 13C 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.
by the addition of p-toluenesulfonyl chloride (12.8 g, 67.0 mmol) in hexanes/acetonitrile (7:1) (R_f = 0.45). After the reaction was complete, the mixture was cooled to room temperature. The reaction mixture was washed with two portions of water; methanol was added to break the emulsion. The chloroform was purified on a silica gel column (200 g) using the same solvent mixture.

Choline p-toluenesulfonate (3.64 g, 9.73 mmol) 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 hexanes/acetone (7:1) (R_f = 0.45). After the reaction was complete, the mixture was cooled in an ice bath. Pyridine (7.0 ml, 86 mmol) was then added, followed by cooling, and the solution was then refluxed for an additional 30 min. The mixture 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 was crystallized from acetone/hexanes to yield (5.3 g, 41%) of compound 9. 1H NMR (CDCl3) δ 0.880 (t, 6H), 1.255 (s, 50H), 1.549 (m, 2H), 1.649 (m, 2H), 2.246 (t, 2H), 2.492 (t, 2H), 2.986 (m, 2H), 3.766 (m, 3H), 4.830 (m, 1H), 6.293 (d, 1H).

1-Palmityloxy-2-palmitylamino-1,2-dIDEOXY-S-N-Glycero-3-Phosphorylethanolamine [12]—A mixture of compound 10 (0.5 g, 0.577 mmol), 2-phthalimidoethylphosphoryl dichloride (3.2 g, 10 mmol), and pyridine (1.0 g, 20 mmol) was stirred at room temperature for 24 h. The solvent was evaporated and the residue was dissolved in 100 ml of methanol, and the mixture was filtered for an additional 30 min. The mixture 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 was dissolved in 100 ml of chloroform/methanol (21) and passed through an ion exchange column of R-A (Whatman, 200 cm). The product was dissolved in 150 ml of chloroform/methanol (21). The product was purified by flash chromatography on silica gel with chloroform/methanol/water (65:25:1) as eluting solvent. The yield of the original method was 22% (95%:78). The yield of compound 9 was 182 mg (30%). 1H NMR (CDCl3, CD3OD): δ 0.879 (t, 6H), 1.255 (s, 50H), 1.583 (br, 2H), 2.246 (t, 2H), 2.492 (t, 2H), 2.986 (br, 2H), 3.133 (br, 2H), 3.895 (br, 1H), 4.076 (br, 2H), 6.129 (br, 2H).

C20H41O2N3P (M + H+)  Calculated: 369.3398 Found: 369.3392

Synthesis of 1,2-Bis(decanoylthio)-1,2-dIDEOXY-S-N-GLyCER0-3-Phosphorylethanolamine [12]—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 advantages 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...
Phospholipase $A_2$ Inhibitors

The phenomenon is observed when the enzymatic activity depends on active $\alpha$-carbon in D-serine provided the asymmetric center substrate; any molecule which disturbs the structure, i.e., the physisorbed first and then acylated with decanoyl chloride. This modified procedure afforded pure compound $3$ in quantitative yields. Because of 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, phoshpatidyleholamine 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 serine methyl ester and the subsequent selective reduction, with LiBH$_4$, of the ester group. The chirality of the optically active $\alpha$-carbon in D-derivided provided the asymmetric center in thioether amide-PE. Reaction of one equivalent of palmityl 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, LiBH$_4$, led to the formation of the cysteine derivative $10$ in essentially quantitative yield. Finally, the phosphorylethanolamine 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 observed inhibition of phospholipase $A_2$ activity by phospholipid analogs is presumably due to the direct interaction of the enzyme with the inhibitors and not due to indirect effects of the inhibitors on the substrate micelles.

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 $A_2$ 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 $A_2$ 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 produce a dilute 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 to not 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 $A_2$ hydrolysis of thio-PC. In group B (compounds $12, 13$), the effect of the head group was studied by replacing the phosphocholine with phosphophylethanolamine. 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, can not react with the chromogenic

![Fig. 1](http://www.jbc.org/)  
**Fig. 1** Effect of the substitution of the sn-2 ester by an amide on the interaction of the phospholipase $A_2$ and 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.
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 thio 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 IC₅₀ of 0.156 mM.

Replacing the phosphorylcholine polar head group by phosphoryl ethanolamine, group B, also affects the potency of phospholipase A₂ inhibition. As shown in Fig. 2, the half-maximal inhibition of thio-PC hydrolysis by thioether amide-PE was 0.45 mM, 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 IC₅₀ of thioether amide-PE was about 6.5 μM. The thioether amide-PC did not follow a simple inhibition curve; thus, an IC₅₀ 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.

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 18 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 IC₅₀ 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 A₂ hydrolysis of phosphatidylcholine 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 A₂ 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 ω position of the sn-2 fatty chain, the former does not affect the activity of phospholipase A₂, 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 IC₅₀ 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 A₂. 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
Phospholipase A\textsubscript{2} Inhibitors

Inhibition of phospholipase A\textsubscript{2} 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>
<thead>
<tr>
<th>Group</th>
<th>Compound</th>
<th>No.</th>
<th>Structure</th>
<th>IC\textsubscript{50}</th>
</tr>
</thead>
</table>
| A     | Dipalmitoyl-PC |    | \begin{align*} & O \quad CH_3OOCR \\
                    \quad RCO \quad H \\
                    \quad CH_2OPC \end{align*} | ND |
| B     | Thioether amide-PE | 12 | \begin{align*} & O \quad CH_3SR \\
                    \quad RCHN \quad H \\
                    \quad CH_2OPE \end{align*} | 0.00045 |
| B, C  | Thioether amide-PC | 13 | \begin{align*} & O \quad CH_3SR \\
                    \quad RCHN \quad H \\
                    \quad CH_2OPC \end{align*} | 0.002 |
| C     | Ether amide-PC | 14 | \begin{align*} & O \quad CH_3OR \\
                    \quad RCHN \quad H \\
                    \quad CH_2OPC \end{align*} | 0.038 |
| A, C  | Ester amide-PC | 15 | \begin{align*} & O \quad HOCHCH=CHR \\
                    \quad RCHN \quad H \\
                    \quad CH_2OPC \end{align*} | 0.156 |
| C     | Sphingomyelin |    | \begin{align*} & O \quad CH_3OR \\
                    \quad RCHN \quad H \\
                    \quad CH_2OPC \end{align*} | ND |
| D     | Carbamoyl-PC | 16 | \begin{align*} & O \quad CH_3SR \\
                    \quad ROCHN \quad H \\
                    \quad CH_2OPC \end{align*} | ND |
| D     | Alkylurido-PC | 17 | \begin{align*} & O \quad CH_3OOCR \\
                    \quad RHNCHN \quad H \\
                    \quad CH_2OPC \end{align*} | ND |

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\textsubscript{2}. 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\textsubscript{2} 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\textsubscript{2} 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\textsubscript{2} (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 \(\mu\text{M} \) IC\textsubscript{50} of thioether amide-PE 12 is one of the tightest phospholipase A\textsubscript{2} 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 \( \alpha \)-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\textsubscript{2}. (iv) The ethanolamine-
containing inhibitor is more potent than the choline-containing analogues.

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 A₂ (35-37) have demonstrated the importance of a catalytic histidine residue. In addition, the three-dimensional structure of bovine pancreatic phospholipase A₂ 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 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 A₂ 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 α-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 A₂ 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 phospholipids with either a phosphorylcholine or phosphorylethanamine 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 activator site than to the catalytic site. The replacement of the α-methylene group, compounds 16 and 17, yields analogues that are very poor inhibitors but good activators. Apparently the α-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 sphingomyelin is a more efficacious activator than PC. PC activates PE hydrolysis by 7-10-fold; sphingomyelin activates it by 20-fold (42). If PC hydrolysis is activatable, the addition of sphingomyelin should produce an additional activation over and above that of PC itself. When surface dilution effects are taken into account, thi-PC hydrolysis is clearly activatable 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 sphingomyelin. This would seem to indicate that sphingomyelin’s binding to the catalytic site is much weaker than the substrate’s. These results clearly demonstrate that PC not only activates PE 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 A₂ and its substrate. First, the amide analogues bind tighter than natural phospholipids.
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 w-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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