Effect of Polar Head Groups on the Interactions of Phospholipase A_2 with Phosphonate Transition-State Analogues†

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ABSTRACT: Several new phosphonate-containing phospholipid analogues were synthesized as inhibitors of cobra venom (*Naja naja naja*) phospholipase A_2. These phospholipid analogues contained a novel thioether at the *sn*–1 position, a tetrahedral phosphonate moiety in place of the scissile ester bond at the *sn*–2 position, and several different polar head groups, including phosphocholine, phospho(*N*,*N*-dimethylethanolamine), phospho(*N*-methylethanolamine), and phosphoethanolamine. The affinities of these analogues for the enzyme were evaluated in the well-defined Triton X-100 mixed micelle system using thio-PC and thio-PE substrates. These phosphonates inhibited thio-PC hydrolysis with very similar potencies. Inhibition of phospholipase A_2 by phosphonates is known to be pH-dependent [Yu, L., & Dennis, E. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9325–9329]. At pH 5.5, all of the new analogues had IC_{50}s of about 2×10^{-5} mol fraction. At this pH, these inhibitors are the most potent reversible inhibitors of phospholipase A_2 reported to date. In contrast, at pH 8.5, the PE analogue was a potent inhibitor of thio-PC hydrolysis (IC_{50} 1.8×10^{-3} mol fraction) but was a very poor inhibitor of thio-PE hydrolysis (IC_{50} is not detectable). However, the inhibition of thio-PE hydrolysis was dramatically enhanced when the enzyme was activated by sphingomyelin, suggesting that the phosphonate inhibitors bind much more tightly to the activated enzyme than to the nonactivated enzyme. The activation and inhibition of the enzyme have different pH dependencies; the enzyme activation is not pH-dependent, whereas the enzyme inhibition is pH-dependent. These results confirm the presence of a functionally distinct activator site on this enzyme.

Phospholipase A_2 (PLA_2)[1] is a ubiquitous enzyme which specifically catalyzes the hydrolysis of fatty acyl ester bonds at the *sn*-2 position of phospholipids (Dennis, 1983). The enzymes from different sources show variations in substrate specificity for different polar head groups (Roberts *et al.*, 1979; Plückthun & Dennis, 1985; Volwerk *et al.*, 1986). Cobra venom (*Naja naja naja*) PLA_2, one of the most extensively studied enzymes, hydrolyzes phosphatidylcholine (PC) 10–20 times faster than phosphatidylethanolamine (PE) (Roberts *et al.*, 1978). If both phospholipids are present, however, PE becomes the preferred substrate. The effect of phosphocholine-containing lipids on PE hydrolysis apparently results from a direct interaction of the activating molecule with the enzyme (Plückthun & Dennis, 1982), rather than from a secondary effect on the interface of the PE substrate in mixed micelles as occurs for some additives when the substrate phospholipid consists of sonicated vesicles (Jain *et al.*, 1991; Jain & Berg, 1989; Ghomashchi *et al.*, 1991). This polar head group effect was also seen recently in the inhibition of PLA_2 with amide phospholipid analogues (Yu *et al.*, 1990). The PE amide analogue bound to the enzyme about 5 times more tightly than the PC analogue.

In the present study, we have used a series of new phosphonate transition-state analogues to further investigate the role that the polar head group plays in enzyme–substrate binding. Phosphonate transition-state analogues are potent PLA_2 inhibitors first described by Yuan and Gelb (Yuan & Gelb, 1988) which contain a phosphonate function in place of the scissile *sn*-2 ester group. The phosphonate analogues used in this study were designed with a thioether at the *sn*-1 position to increase potency and a series of different polar head groups, including phosphocholine, phospho(*N*,*N*-dimethylethanolamine), phospho(*N*-methylethanolamine), and phosphoethanolamine. We studied the effect of the head group on the enzyme–substrate interactions using both PC and PE substrates, the effect of the activator sphingomyelin on the interaction of the enzyme with the PE phosphonate analogues, and the effect of pH on enzyme activation and inhibition.

EXPERIMENTAL PROCEDURES

Materials. Phospholipase A_2 (*Naja naja naja*) was purified from lyophilized cobra venom as described previously (Hazlett & Dennis, 1985; Reynolds & Dennis, 1991). 4,4′-Dithiodipyridine and *p*-bromophenacyl bromide (*p*-BPB) were obtained from Aldrich. Triton X-100 was obtained from Sigma. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and sphingomyelin were obtained from Avanti Polar Lipids, Inc. 1-Hexadecyl mercaptan, (*S*)-2,2-dimethyl-4-[[(toluene-4-sulfonyl)oxy]methyl]-1,3-dioxolane were obtained from Fluka. 2-Bromoethyl dichlorophosphate was prepared by the reaction of 2-bromoethanol with phosphorus oxychloride as adapted from Hansen *et al.* (1982). Methyl hexadecyl-

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[1] Abbreviations: *p*-BPB, *p*-bromophenacyl bromide; DM-SPPE, 1-deoxy-1-(hexadecylthio)-2-(hexadecylphosphonyl)-*sn*-glycero-3-phospho(*N*,*N*-dimethylethanolamine); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; MM-SPPE, 1-deoxy-1-(hexadecylthio)-2-(hexadecylphosphonyl)-*sn*-glycero-3-phospho(*N*-methylethanolamine); PLA_2, phospholipase A_2; P-Me SPPEa + P-Me SPPEb, 1-deoxy-1-(hexadecylthio)-2-(methyl hexadecylphosphonyl)-*sn*-glycero-3-phosphoethanolamine; SPM, sphingomyelin; SPPC, 1-deoxy-1-(hexadecylthio)-2-(hexadecylphosphonyl)-*sn*-glycero-3-phosphocholine; SPPE, 1-deoxy-1-(hexadecylthio)-2-(hexadecylphosphonyl)-*sn*-glycero-3-phosphoethanolamine; thio-PC, 1,2-dideoxy-1,2-bis(decanoylthio)-*sn*-glycero-3-phosphocholine; thio-PE, 1,2-dideoxy-1,2-bis(decanoylthio)-*sn*-glycero-3-phosphoethanolamine; SAPC, 1,2-dideoxy-1-(hexadecylthio)-2-(hexadecanoylamino)-*sn*-glycero-3-phosphocholine.

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phosphonochloridate was prepared as reported in the literature (Yuan & Gelb, 1988). 1-(Hexadecylthio)-2-(hexadecanoylamino)-1,2-dideoxy-*sn*-glycero-3-phosphocholine (SAPC) was synthesized previously (Yu & Dennis, 1992). 1,2-Bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine (thio-PC) and 1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphoethanolamine (thio-PE) were prepared as described previously (Yu & Dennis, 1991b). Chloroform was distilled from phosphorus pentoxide. An aminopropyl solid-phase extraction column was purchased from Varian Associates, Inc. All other chemicals were reagent grade or better.

General Methods. Flash chromatography was performed with silica gel 60 (230–400 mesh, Merck). Thin-layer chromatography (TLC) was carried out on Analtech Silica Gel G-250 glass plates. The compounds were detected by viewing the plate under ultraviolet light for UV-active compounds or by charring the plate after spraying with 2 M sulfuric acid. Trityl compounds were detected by their bright yellow color after gentle warming of the sprayed plate. Phosphorus compounds were detected by their blue color after spraying with a molybdate solution (Ryn & BacCoss, 1979). ^{1}H NMR spectra were recorded on an extensively modified Varian 360-MHz NMR spectrometer, using tetramethylsilane as an internal standard. Coupling constants are reported in hertz. ^{31}P NMR spectra were obtained at 81 MHz on a 200-MHz Nicolet NMC-NB-200 spectrometer, using 5% trimethyl phosphate in $CDCl_3$ as an external standard (δ = +3.02). High-resolution mass spectrometric analyses were conducted at the University of California, Riverside, mass spectrometry facility.

Phospholipase A_2 Assay. PLA_2 activity was determined spectrophotometrically at 324 nm using the thio assay as described previously (Yu *et al.*, 1990). The assay was carried out at 30 °C in a standard 25 mM Tris-HCl buffer (pH 8.5), containing 100 mM KCl, 10 mM $CaCl_2$, 4.25 mM Triton X-100, and 0.5 mM either thio-PC or thio-PE. When assays were done at pH 6.5 and 7.5, 25 mM 2-(*N*-morpholino)-ethanesulfonic acid was used. When assays were carried out at pH 5.5, 25 mM acetate buffer was used. The enzyme activity was followed by monitoring the production of the free thiol product by its reaction with the reagent 4,4′-dithiodipyridine. Enzymatic activity was calculated by using an extinction coefficient of 13 400 M^{-1} cm^{-1} for pH 8.5 and 19 500 M^{-1} cm^{-1} for pH 5.5 (Yu & Dennis, 1991b). Mixed micelles of Triton X-100/phospholipid substrate and Triton X-100/phosphonate analogue were prepared fresh daily. Aliquots of the two solutions were combined to obtain the proper concentration of substrate and inhibitor in the assays. The reaction was initiated either by the addition of the enzyme (50 ng) in assay buffer or by the addition of substrate. The initial steady-state velocity in the presence of an inhibitor was calculated from the linear portion of each progress curve. The depletion of substrate was less than 5% in all assays. The enzyme assays were conducted in duplicate, interspersing controls without inhibitor after every 5–6 runs with inhibitor.

p-Bromophenacyl Bromide Inactivation. The studies of PLA_2 inactivation by *p*-BPB were carried out by incubating the enzyme (20 mg/mL) in 25 mM Tris-HCl (pH 8.0), 0.1 M KCl, 10 mM $CaCl_2$, and 4.25 mM Triton X-100, with 0.2 mM *p*-bromophenacyl bromide at 25 °C. The reagent was added from a 20 mM stock solution in acetone. Aliquots were removed at various time points and tested at 40 °C in a pH-stat assay (Deems & Dennis, 1981) for the residual activity. The substrate used for the pH-stat assay was 5 mM 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine in 40 mM Triton X-100 solution containing 0.1 M KCl and 10 mM $CaCl_2$. The pseudo-first-order rate constant for the alkylation of PLA_2 was calculated from a least-squares fit of an inactivation time course, which included at least four data points.

Preparation of (S)-4-[(Hexadecylthio)methyl]-2,2-dimethyl-1,3-dioxolane (**2**). A mixture of (*S*)-2,2-dimethyl-4-[[(toluene-4-sulfonyl)oxy]methyl]-1,3-dioxolane **1** (5.0 g, 17.5 mmol), 1-hexadecyl mercaptan (6.7 g, 25.9 mmol), and sodium methoxide (1.4 g, 25.9 mmol) in 100 mL of methanol was refluxed under nitrogen overnight. The methanol solution was then extracted three times with hexanes. After removal of the hexanes under vacuum, a colorless syrup was obtained. The product gave a major spot on TLC (R_f 0.9 in hexanes/acetone 7:3). The crude product was used directly in the next step without further purification.

Preparation of 1-Deoxy-1-(hexadecylthio)-sn-glycerol (**3**). Hydrogen chloride was bubbled through a solution of the crude compound **2** from the previous step in 100 mL of methanol and 5 mL of water for 0.5 h. The reaction mixture was allowed to stand at room temperature for 2 h. Water (200 mL) was added and the mixture was extracted three times with methylene chloride. After the combined extracts were dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography on silica gel with hexanes/ethyl acetate (8:2) as the eluting solvent to give 1-(hexadecylthio)-1-deoxy-*sn*-glycerol **3** (4.83 g, 83% from **1**) as a white solid: R_f 0.42 (hexanes/acetone 7:3); ^{1}H NMR ($CDCl_3$) δ 0.879 (t, 3 H), 1.255 (s, 26 H), 1.569 (br s, 2 H), 2.553 (t, 2 H), 2.637 (quartet, 1 H), 2.694 (quartet, 1 H), 3.563 (quartet, 1 H), 3.737 (quartet, 1 H), 3.777 (br s, 1 H).

Preparation of 1-Deoxy-1-(hexadecylthio)-3-trityl-sn-glycerol (**4**). Triethylamine (3 mL) was added slowly at room temperature to a solution of 1-(hexadecylthio)-1-deoxy-*sn*-glycerol **3** (4.8 g, 14.4 mmol) and trityl chloride (6.0 g, 21.6 mmol) in methylene chloride (100 mL). After the solution was stirred at room temperature overnight, 100 mL of methylene chloride was added and the solution was washed with water, with 0.5 M HCl, and then with water. The solution was dried over anhydrous sodium sulfate and the solvent was removed under vacuum. The crude product was purified on silica gel with hexanes/diethyl ether (9:1) as eluting solvents to yield a white solid (7.5 g, 90%): R_f 0.73 (hexanes/acetone 7:3); ^{1}H NMR ($CDCl_3$) δ 0.878 (t, 3 H), 1.253 (s, 26 H), 1.561 (m, 2 H), 2.480 (t, 2 H), 2.620 (quartet, 1 H), 2.747 (quartet, 1 H), 3.248 (m, 2 H), 3.833 (br s, 1 H), 7.301–7.443 (m, 15 H).

Preparation of 1-Deoxy-1-(hexadecylthio)-2-(methylhexadecylphosphonyl)-3-trityl-sn-glycerol (**5a,b**). A solution of compound **4** (4.0 g, 6.97 mmol) in 30 mL of chloroform and 12 mL of pyridine was added dropwise over a period of 0.5 h to a solution of methyl hexadecylphosphonochloridate (4.0 g, 11.2 mmol) in 20 mL of dry chloroform with stirring in an ice bath. After the addition was complete, the solution was stirred overnight at room temperature. The reaction mixture was washed twice with 0.5 N HCl, once with water, and then with 5% $NaHCO_3$. The organic phase was dried over anhydrous sodium sulfate. The residue obtained after removal of the solvent in vacuo was purified on silica to give a mixture of two diastereomers, **5a** and **5b** (3.16 g, 52%) as a white solid: R_f 0.54 (hexanes/acetone 7:3); ^{1}H NMR ($CDCl_3$) δ 0.887 (t, 6 H), 1.262 (s, 52 H), 1.521–1.620 (m, 4 H), 1.750 (m, 2 H), 2.461 (t, 2 H), 2.619 (m, 2 H), 3.269 (m, 1 H), 3.387 (m, 1 H), 3.564 (d, 1.5 H), 3.721 (d, 1.5 H), 4.679 (br s, 1 H), 7.322–7.486 (m, 15 H).

Preparation of 1-Deoxy-1-(hexadecylthio)-2-(methyl hexadecylphosphonyl)-sn-glycerol (**6a,b**). A solution of a mixture of diastereomers **5a** and **5b** (3.0 g, 3.42 mmol) in methylene chloride (50 mL) containing a 14% methanol solution of boron trifluoride (2 mL, 4.13 mmol) was stirred at 0 °C. The reaction was monitored by TLC in hexanes/acetone (7:3) by following the disappearance of the starting material. After the reaction was complete (about 0.5 h), the reaction mixture was washed twice with ice water. The organic layer was dried over sodium sulfate and the methylene chloride was evaporated under reduced pressure. The two diastereomers were purified and separated by flash chromatography using hexanes and acetone (9:1) as eluents to give two white solids: diastereomers **6a** (0.90 g, 41%) and **6b** (1.03 g, 47%). Diastereomer **6a**: R_f 0.44 in hexanes/acetone (7:3); ^{1}H NMR ($CDCl_3$) δ 0.880 (t, 6 H), 1.256 (s, 52 H), 1.599 (m, 4 H), 1.829 (m, 2 H), 2.561 (t, 2 H), 2.470 (m, 2 H), 3.739 (d, 3 H), 3.726–3.827 (m, 2 H), 4.499 (br s, 1 H). Diastereomer **6b**: R_f 0.40 in hexanes/acetone (7:3); ^{1}H NMR ($CDCl_3$) δ 0.884 (t, 6 H), 1.253 (s, 52 H), 1.575 (m, 4 H), 1.830 (m, 2 H), 2.563 (t, 2 H), 2.472 (m, 2 H), 3.783 (d, 3 H), 3.726–3.827 (m, 2 H), 4.500 (br s, 1 H).

Preparation of 1-Deoxy-1-(hexadecylthio)-2-(methyl hexadecylphosphonyl)-sn-glycero-3-phospho(2-bromoethanol) (**7a**). A solution of dry chloroform (2 mL) and 2-bromoethyl dichlorophosphate (200 mg, 0.826 mmol) was placed in a flask equipped with a magnetic stirrer and a pressure-equalizing dropping funnel containing a solution of diastereomer **6a** (200 mg, 0.315 mmol) and dry pyridine (0.2 mL) in 2 mL of dry chloroform. The solution of diastereomer **6a** was added slowly over a period of 1 h to the 2-bromoethyl dichlorophosphate solution, which was stirring at 0 °C. After the addition, the reaction mixture was allowed to warm to room temperature, and stirring was continued for an additional 2 h. The mixture was washed twice with 0.5 M HCl and then with water. The reaction mixture was dried over sodium sulfate and solvent was removed under vacuum. The crude product was used directly in the next step without further purification.

Preparation of 1-Deoxy-1-(hexadecylthio)-2-(methyl hexadecylphosphonyl)-sn-glycero-3-phosphoethanolamine (P-Me SPPEa) (**8a**). The crude diastereomer **7a** from the previous step was dissolved in 19 mL of chloroform/2-propanol/dimethylformamide (1:4:4) and 9.5 mL of 25% ammonia solution. After the reaction mixture was stirred at 40 °C for 9 h, the ammonia and the volatile solvent were removed under vacuum, and the remaining solvent was removed by lyophilization. Flash chromatography on silica (chloroform/methanol 8:2) yielded 114 mg (48% from compound **6a**) of a white solid: ^{1}H NMR ($CDCl_3$) δ 0.879 (t, 6 H), 1.254 (s, 52 H), 1.562 (m, 4 H), 1.819 (m, 2 H), 2.521 (t, 2 H), 2.788 (m, 2 H), 3.216 (br s, 2 H), 3.788 (d, 3 H, J = 10.8 Hz), 3.989 (br s, 1 H), 4.117 (br s, 1 H), 4.188 (br s, 2 H), 4.712 (br s, 1 H); ^{31}P NMR ($CDCl_3$) δ –0.845 (br s, 1 P), 34.584 (s, 1 P). HRMS (FAB, MH^+) Calcd for $C_{38}H_{82}NO_7P_2S$: 758.5287. Found: 758.5317.

Preparation of 1-Deoxy-1-(hexadecylthio)-2-(methyl hexadecylphosphonyl)-sn-glycero-3-phosphoethanolamine (P-Me SPPEb) (**8b**). Diastereomer **8b** was prepared from diastereomer **6b** following the procedure for synthesis of diastereomer **8a**: yield, 56%; ^{1}H NMR ($CDCl_3$) δ 0.880 (t, 6 H), 1.256 (s, 52 H), 1.566 (m, 4 H), 1.811 (m, 2 H), 2.558 (t, 2 H), 2.692 (quartet, 1 H), 2.790 (quartet, 1 H), 3.165 (br s, 2 H), 3.750 (d, 3 H, J = 12.96 Hz), 3.979 (br s, 1 H), 4.059 (br s, 1 H), 4.194 (br s, 2 H), 4.642 (br s, 1 H); ^{31}P NMR ($CDCl_3$) δ 0.103 (br s, 1 P), 34.678 (s, 1 P). HRMS (FAB, MH^+) Calcd for $C_{38}H_{82}NO_7P_2S$: 758.5287. Found: 758.5269.

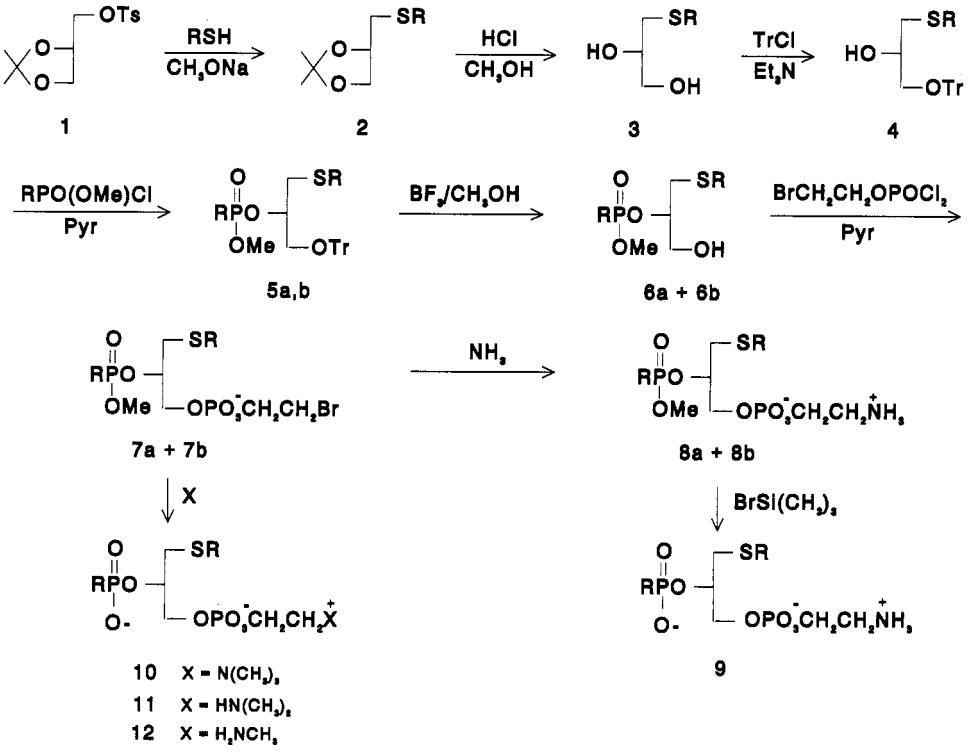
Preparation of 1-Deoxy-1-(hexadecylthio)-2-(hexadecylphosphonyl)-sn-glycero-3-phosphoethanolamine (SPPE) (**9**). Bromotrimethylsilane (0.5 mL) was added to a stirring solution of a mixture of diastereomers **8a** and **8b** (120 mg, 0.158 mmol) in 5 mL of dry chloroform at room temperature. The reaction mixture was stirred for 30 min and then evaporated under reduced pressure. The residue was treated with 5 mL of water and extracted twice with chloroform. Methanol was added to break up the emulsion. The chloroform solution was dried over sodium sulfate and the solvent was removed under vacuum. The residue obtained was purified by an aminopropyl solid-phase extraction column. The column was first eluted with chloroform, then with chloroform/methanol with an increase stepwise in the amount of methanol, and finally with 85:15 methanol/ammonia (20%). After evaporation of the solvents under vacuum, a white solid was obtained (89.6 mg, 76%): ^{1}H NMR ($CDCl_3/CD_3OD$ 8:2) δ 0.886 (t, 6 H), 1.259 (s, 52 H), 1.570 (m, 4 H), 1.814 (m, 2 H), 2.564 (t, 2 H), 2.708 (quartet, 1 H), 2.796 (quartet, 1 H), 3.142 (br s, 2 H), 3.984 (br s, 1 H), 4.062 (br s, 1 H), 4.201 (br s, 2 H), 4.586 (br s, 1 H); ^{31}P NMR ($CDCl_3/CD_3OD$ 8:2) δ 0.730 (br s, 1 P), 28.701 (s, 1 P). HRMS (FAB, MH_2^+) Calcd for $C_{37}H_{80}NO_7P_2S$: 744.5131. Found: 744.5156.

Preparation of 1-Deoxy-1-(hexadecylthio)-2-(hexadecylphosphonyl)-sn-glycero-3-phosphocholine (SPPC) (**10**). The crude mixture of diastereomers **7a** and **7b**, which was prepared from the diastereomeric mixture of **6a** and **6b** (150 mg, 0.236 mmol), was dissolved in 3.0 mL of chloroform/2-propanol/dimethylformide (1:4:4) and 1.5 mL of 40% trimethylamine. After the reaction mixture was stirred at 50 °C for 6 h, the trimethylamine and the volatile solvents were removed under vacuum, and the remaining solvent was removed by lyophilization. Flash chromatography on silica (chloroform/methanol/water 65:25:4) yielded 72.5 mg (39% from **6**) of a white solid: ^{1}H NMR ($CDCl_3$) δ 0.879 (t, 6 H), 1.255 (s, 52 H), 1.578 (m, 4 H), 1.749 (m, 2 H), 2.548 (t, 2 H), 2.762 (m, 2 H), 3.416 (s, 9 H), 3.992 (br s, 2 H), 4.112 (br s, 1 H), 4.205 (br s, 1 H), 4.428 (br s, 2 H), 4.610 (br s, 1 H); ^{31}P NMR ($CDCl_3$) δ –1.304 (s, 1 P), 30.361 (s, 1 P). HRMS (FAB, MH_2^+) Calcd for $C_{40}H_{86}NO_7P_2S$: 786.5600. Found: 786.5594.

Preparation of 1-Deoxy-1-(hexadecylthio)-2-(hexadecylphosphonyl)-sn-glycero-3-phospho(N,N-dimethylethanolamine) (DM-SPPE) (**11**). The crude mixture of **7a** and **7b**, which was prepared from the mixture of diastereomers **6a** and **6b** (200 mg, 0.315 mmol), was reacted with *N,N*-dimethylamine by the same procedure used in the preparation of **10**. Instead of trimethylamine, 40% *N,N*-dimethylamine solution was used. Flash chromatography on silica gel with chloroform/methanol/water 65:25:3 as the eluting solvent yielded 89 mg of **11** (37% from **6**) as a white solid: ^{1}H NMR ($CDCl_3/CD_3OD$ 8:2) δ 0.889 (t, 6 H), 1.266 (s, 52 H), 1.609 (m, 4 H), 1.822 (m, 2 H), 2.600 (t, 2 H), 2.706 (quartet, 1 H), 2.810 (quartet, 1 H), 2.906 (s, 6 H), 3.317 (br s, 2 H), 4.112 (br s, 1 H), 4.172 (br s, 2 H), 4.205 (br s, 1 H), 4.622 (br s, 1 H). HRMS (FAB, MH_2^+) Calcd for $C_{39}H_{84}NO_7P_2S$: 772.5444. Found: 772.5404.

Preparation of 1-Deoxy-1-(hexadecylthio)-2-(hexadecylphosphonyl)-sn-glycero-3-phospho(N-methylethanolamine) (MM-SPPE) (**12**). The procedure used in the synthesis of **10** was adapted for the synthesis of **12**. However, 40% *N*-methylamine solution was used to replace the trimethylamine solution. A mixture of diastereomers **7a** and **7b**, which

Scheme I: Synthesis of Phosphonate Analogues



were prepared from the mixture of **6a** and **6b** (200 mg, 0.315 mmol) reacted with *N*-methylamine to afford 126 mg (53% from compound **6**) as a white solid, after flash chromatography on silica gel with chloroform/methanol/water (65:25:1) as the eluting solvent: ^{1}H NMR ($CDCl_3/CD_3OD$ 8:2) δ 0.892 (t, 6 H), 1.272 (s, 52 H), 1.617 (m, 4 H), 1.842 (m, 2 H), 2.615 (t, 2 H), 2.694 (quartet, 1 H), 2.721 (s, 3 H), 2.814 (quartet, 1 H), 3.189 (br s, 2 H), 4.021 (br s, 1 H), 4.148 (br s, 3 H), 4.618 (br s, 1 H). HRMS (FAB, MH_2^+) Calcd for $C_{38}H_{82}NO_7P_2S$: 758.5287. Found: 758.5307.

RESULTS

Chemical Synthesis. The phosphonate transition-state analogues were synthesized from optically active isopropylideneglycerol tosylate **1** as shown in Scheme I. The chirality of **1** provided the asymmetric center in the phosphonate phospholipid analogues. The substitution reaction of isopropylideneglycerol tosylate **1** with 1-hexadecyl mercaptan, followed by removal of acetone with methanolic hydrogen chloride, afforded thioether glycerol **3** in 80% yield. After selective alkylation of the primary hydroxyl group with trityl chloride, the protected thioether glycerol **4** was phosphonylated by reaction with methyl hexadecylphosphonochloridate to give a mixture of two diastereomers, **5a** and **5b**, due to the introduction of the second chiral center at the phosphorus atom. After removal of the trityl group with boron trifluoride in methanol, the two diastereomers, **6a** and **6b**, were separated by silica gel chromatography. The polar head group moiety of the phosphonate analogues was then introduced by phosphorylation of the phosphonate glycerol **6a** and **6b** with 2-bromoethyl dichlorophosphate, followed by amination with the desired amine (Eibl & Nicksch, 1978). The use of 2-bromomethyl dichlorophosphate made the synthetic route more versatile, in that the various head groups were incorporated later in the synthesis by substituting the bromine with different amine groups. The synthesis of SPPC (**10**), DM-SPPE (**11**), and MM-SPPE (**12**) was achieved through a single step by the reaction of a mixture of two diastereomers **7a** and **7b** with the corresponding methylated amines, which act as demethylation reagents in addition to being incorporated into the head group. SPPE (**9**) was synthesized by treating a mixture of **7a** and **7b** with ammonium hydroxide, followed by demethylation with bromotrimethylsilane. The methylated phosphonate analogues, **8a** and **8b**, were prepared by treating diastereomers **7a** and **7b**, respectively, with ammonia.

Inhibition Studies. The inhibition of PLA_2 by the phosphonate inhibitors was studied in the well-defined Triton X-100/phospholipid mixed micelle system. As discussed previously (Yu *et al.*, 1990), the advantage of the mixed micellar system is that the enzyme inhibition detected in this system is most likely due to a direct interaction of the inhibitor with the enzyme rather than to an effect of the inhibitor on the substrate. The inhibitory potencies of these analogues were evaluated by their IC_{50}s, which were determined using either thio-PC or thio-PE substrates. Since all the analogues have long alkyl groups at both the *sn*-1 and *sn*-2 positions, all of the inhibitors are presumed to be incorporated into micelles. Thus, inhibition most likely occurs at the water–lipid interface and the IC_{50}s are given in terms of surface concentration units (mol fraction).

The inhibition of the PLA_2-catalyzed hydrolysis of thio-PC by the phosphonate analogues is shown in Table I. All of the analogues followed simple dose–response inhibition curves. They all were potent PLA_2 inhibitors and their inhibition was also found to be pH-dependent. Each inhibitor bound more tightly at the more acidic pH, as demonstrated previously (Yu & Dennis, 1991a). All the phosphonate analogues except the methylated phosphonates, P-Me SPPEa (**8a**) and P-Me SPPEb (**8b**), had very similar inhibitory potencies. Thus, the effect of the polar head group on the inhibition of thio-PC hydrolysis by these analogues is negligible.

The effect of the phosphonate analogues on the hydrolysis of thio-PE is much more complicated. As shown in Figure 1, none of the phosphonate analogues were potent inhibitors

Table I: Inhibition of Phospholipase A_2 Hydrolysis of Thio-PC

compound	no.	IC_{50}[a] (mol fraction × 10^{-4}) pH = 8.5	pH = 5.5
SPPC	**10**	18	0.24
DM-SPPE	**11**	9.5	0.15
M-SPPE	**12**	9.1	0.14
SPPE	**9**	9.1	0.35
P-Me SPPEa	**8a**	44	1.6
P-Me SPPEb	**8b**	120	3.6

[a] The inhibition studies were performed in mixed micelles of thio-PC (0.5 mM) and Triton X-100 (4.25 mM).

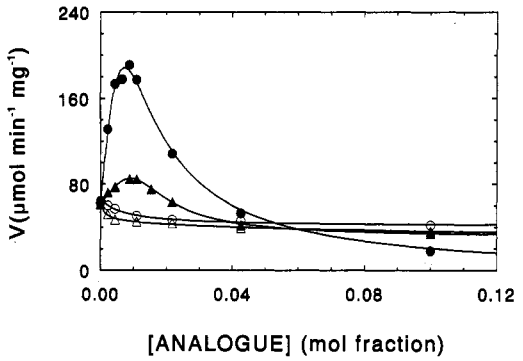


FIGURE 1: Effect of phosphonate analogues on phospholipase A_2 activity toward thio-PE. The enzyme activity was determined using the thio assay at pH 8.5 with 0.5 mM thio-PE, 4.25 mM Triton X-100, and either SPPC (●), DM-SPPE (▲), MM-SPPE (○), or SPPE (△). Smooth curves were drawn through the data; for all points, the error bars were smaller than the size of the symbols.

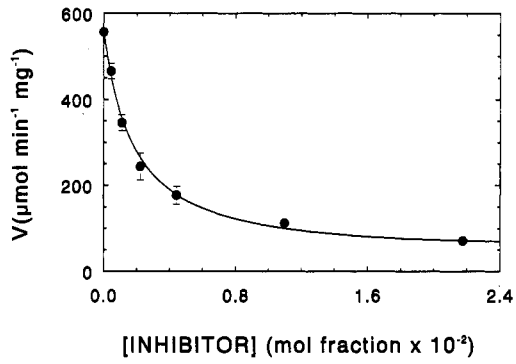


FIGURE 2: Phospholipase A_2 inhibition by SPPE in the presence of sphingomyelin. The enzyme activity was determined at pH 8.5 using the thio assay with 0.5 mM thio-PE, 4.25 mM Triton X-100, and 0.1 mM sphingomyelin. A smooth curve was drawn through the data.

of thio-PE hydrolysis. At pH 8.5, SPPC (**10**) and DM-SPPE (**11**) gave complex inhibition curves, while MM-SPPE (**12**) and SPPE (**9**) showed little inhibition of any sort. At pH 5.5, however, SPPE was a very potent inhibitor with an IC_{50} of 6.7×10^{-4} mol fraction. This inhibition was, however, still 20 times weaker than that measured in the presence of thio-PC at pH 5.5. The magnitude of this effect is very similar to that seen in phospholipase A_2 activation. To further explore the possibility that this activation is affecting the binding of these inhibitors, we determined the effect that sphingomyelin, a potent phospholipase A_2 activator, has on the inhibitory potency of SPPE. Even though SPPE was a very poor inhibitor of the nonactivated PLA_2 hydrolysis of thio PE, it was a potent inhibitor of the activated PLA_2 (Figure 2). Incorporating sphingomyelin into thio-PE micelles increased the enzyme activity about 10-fold, and SPPE then displayed a significant inhibition of the PE hydrolysis. In the presence of 0.10 mM

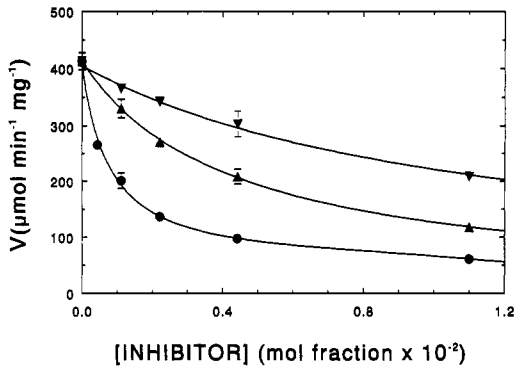


FIGURE 3: Inhibition of thio-PC hydrolysis by PE phosphonate analogues. Enzymatic activity was determined at pH 8.5 using the thio assay with 0.5 mM thio-PC, 4.25 mM Triton X-100, and either SPPE (●), P-Me SPPEa (▲), or P-Me SPPEb (▼). Smooth curves were drawn through the data.

Table II: Effect of Phospholipid Analogues on the Inactivation of Phospholipase A_2 by *p*-Bromophenacyl Bromide[a]

ligand	$k_{app} \times 10^3$ (min^{-1})
none	18.2
SPM (0.1 mM)	19.6
SPPE (0.1 mM)	12.6
SPPE (0.1 mM) + SPM (0.1 mM)	2.7

[a] Enzyme inactivation was performed at pH 8.0 in standard buffer solution containing 20 mg/mL enzyme, 0.2 mM *p*-bromophenacyl bromide, 4.25 mM Triton X-100, and 10 mM $CaCl_2$.

sphingomyelin, SPPE had an IC_{50} of 1.9×10^{-3} mol fraction at pH 8.5.

The importance of the negative charge on the phosphonate group of the transition-state analogues was studied by comparing the inhibitory potency of P-Me SPPEa (**8a**) and P-Me SPPEb (**8b**) to SPPE (**9**). P-Me SPPEa and P-Me SPPEb are diastereomers which are generated by masking the negative charge on the phosphonate group of SPPE by esterifying it with a methyl group. This removes the negative charge on the phosphonate group. The absolute stereochemistry at phosphorus was not determined. At both pH 5.5 and 8.5, SPPE is a more effective inhibitor of PC hydrolysis, binding 5 times more tightly than P-Me SPPEa and 10 times more tightly than P-MeSPPEb (Table I and Figure 3). In addition to the removal of the negative charge, the decrease in inhibition potencies of these methylated phosphonate analogues could be due to steric changes around the tetrahedral phosphorus. Interestingly, P-Me SPPEa binds to the enzyme twice as tightly as its diastereomer P-Me SPPEb regardless of the pH (Table I). In contrast to their effect of PC hydrolysis, these three analogues were very poor inhibitors of PE hydrolysis at pH 8.5.

Effect of Phospholipid Analogues on the Inactivation of PLA_2 by p-Bromophenacyl Bromide. *p*-Bromophenacyl bromide is a potent irreversible inhibitor of cobra venom PLA_2 (Roberts *et al.*, 1977). The treatment of enzyme with *p*-BPB results in complete loss of enzymatic activity due to the alkylation of the catalytic histidine residue. Binding of a molecule to the catalytic site of the enzyme protects the enzyme from *p*-BPB inactivation (Roberts *et al.*, 1977). PLA_2 was incubated with *p*-BPB in standard assay buffer containing 4.25 mM Triton X-100, 10 mM $CaCl_2$, and either SPPE, sphingomyelin, or SPPE plus sphingomyelin. As shown in Table II, sphingomyelin, an activator of PE hydrolysis, does not protect the enzyme any better than Triton X-100 and calcium ion, even though it binds to the enzyme. SPPE alone protects the enzyme only slightly from *p*-BPB inactivation.

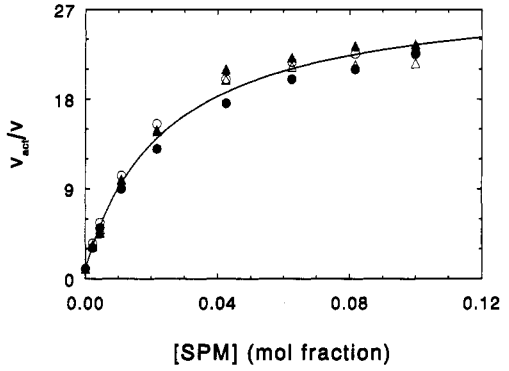


FIGURE 4: Effect of pH on the activation of thio-PE hydrolysis by sphingomyelin (SPM). Thio-PE concentration is 0.5 mM and Triton X-100 is 4.25 mM. The enzyme activity is determined at four different pHs: 5.5 (●), 6.5 (▲), 7.5 (○), and 8.5 (△). A smooth curve was drawn through the data; for all points, the error bars were smaller than the size of the symbols.

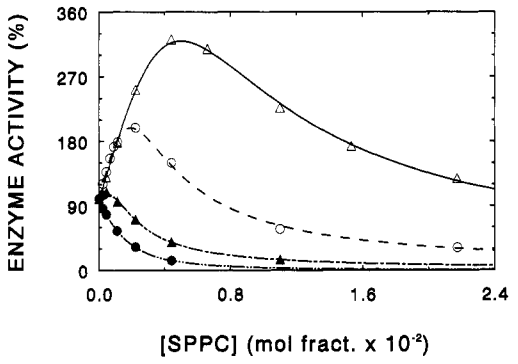


FIGURE 5: Effect of pH on the activation and inhibition of thio-PE hydrolysis by PC phosphonate analogue (SPPC). Activity of the enzyme was measured by following hydrolysis of 0.5 mM thio-PE in 4.25 mM Triton X-100 mixed micelles at 5.5 (●), 6.5 (▲), 7.5 (○), and 8.5 (△). Smooth curves were drawn through the data; for all points, the error bars were smaller than the size of the symbols.

However, SPPE significantly protected the enzyme in the presence of sphingomyelin.

Effects of pH on the Activation and Inhibition of Thio-PE Hydrolysis. The pH dependence of the activation of PE hydrolysis with sphingomyelin was studied over the pH range of 5.5–8.5. The ratio of the enzymatic activity in the presence of sphingomyelin *versus* the activity in the absence of sphingomyelin was plotted as a function of the concentration of sphingomyelin (Figure 4). Although the enzymatic activity decreases as the pH decreases, little pH effect was observed on the sphingomyelin activation of the PE hydrolysis. The magnitude of activation of thio-PE hydrolysis at the four different pHs was very similar. A sphingomylin concentration of 0.1 mol fraction produced a 20-fold activation at all pHs. The pH dependence of activation and inhibition by SPPC as well as by a thioether amide substrate analogue, SAPC, was much more complex, as shown in Figures 5 and 6. As the pH decreases, the maximum in the phosphonate profile decreases and shifts to a lower inhibition concentration. A similar phenomenon occurs for the amide analogue except the decrease and shift of the maximum occur as the pH increases.

DISCUSSION

A well-established strategy for the design of enzyme inhibitors is to replace the scissile bond of a substrate with other moieties to give a nonhydrolyzable compound which otherwise resembles the substrate. The scissile bond to target

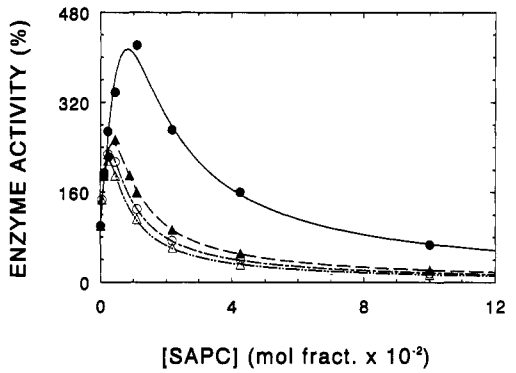


FIGURE 6: Effect of pH on the activation and inhibition of thio-PE hydrolysis by PC amide analogue (SAPC). Activity of the enzyme was measured by following hydrolysis of 0.5 mM thio-PE in 4.25 mM Triton X-100 mixed micelles at 5.5 (●), 6.5 (▲), 7.5 (○), and 8.5 (△). Smooth curves were drawn through the data; for all points, the error bars were smaller than the size of the symbols.

in the design of PLA_2 inhibitors is the *sn*-2 ester bond of the phospholipid substrate. Two of the most successful PLA_2 inhibitors to date have replaced this ester with an amide functional group (Yu *et al.*, 1990; Davidson *et al.*, 1986), which hydrogen-bonds with His 48 (Yu & Dennis, 1991a; Thunnissen *et al.*, 1990), or with a phosphonate group (Yuan & Gelb, 1988), which mimics the tetrahedral transition state and hydrogen-bonds to the acidic form of His 48 (Yu & Dennis, 1991a). Previously, we have shown, using amide phospholipid analogues, that inhibitory potency is significantly enhanced by increasing the hydrophobicity of the *sn*-1 functional group (Yu *et al.*, 1990). On the basis of these observations, we have designed a new series of phosphonate transition-state analogues which contain the more hydrophobic thioether functional group at the *sn*-1 position as well as a tetrahedral phosphonate group at the *sn*-2 position. The resulting compounds, SPPC (**10**), DM-SPPE (**11**), MM-SPPE (**12**), and SPPE (**9**), are some of the most effective PLA_2 inhibitors reported to date. Under optimal conditions (pH 5.5), their IC_{50}s were in the range of 2×10^{-5} mol fraction.

In addition to the design of these potent inhibitors, three aspects of PLA_2 inhibition by these phosphonate analogues were considered in the present study: (i) the effect of the head group on the inhibitory potency of the phosphonate analogue; (ii) the effect of the enzyme activator, sphingomyelin, on the interaction of the enzyme with the PE phosphonate analogue; and (iii) the effect of pH on the inhibition and activation of PLA_2.

Polar Head Group Effect. The effect of the polar head group of the phosphonate analogues on PLA_2 inhibition is complicated by PC activation of the enzyme. We have shown that cobra venom PLA_2 hydrolysis of PE can be activated by phosphocholine-containing lipids (Roberts *et al.*, 1979; Plückthun & Dennis, 1985; Hendrickson & Dennis, 1984). These studies have strongly suggested that the enzyme has two binding sites: an activator site and a catalytic site (Roberts *et al.*, 1979; Plückthun & Dennis, 1985; Adamich *et al.*, 1979). These two sites have distinct structural specificities and binding affinities for substrate. The activator site requires an *N*-methylated phosphoethanolamine head group, either phospho(*N*-methylethanolamine), phospho(*N*,*N*-dimethylethanolamine), or phosphocholine (Plückthun & Dennis, 1985). The ability to activate the enzyme increases with the number of methyl groups on the amine of the head group. Phosphocholine activates the enzyme most efficiently. In contrast to the activator site, the catalytic site does not distinguish between PC and PE polar head groups. The enzyme can bind both

phosphatidylcholine and phosphatidylethanolamine and catalyze their hydrolysis.

We have found that the polar head group of the inhibitor has negligible effect on the inhibition of PC hydrolysis. All four compounds showed nearly the same inhibitory potency with IC_{50}s around 1×10^{-3} mol fraction at pH 8.5 and about 2×10^{-5} mol fraction at pH 5.5. Although little difference in binding affinity was observed in the inhibition of PC hydrolysis, a dramatic effect on PE hydrolysis was observed. The anomalous inhibitory curves (Figure 1) produced by SPPC (**10**) and DM-SPPE (**11**) resulted from the two competing processes: activation and inhibition. SPPC and DM-SPPE bind to both the activator and catalytic sites. Thus, both activation and inhibition occur simultaneously. The binding of these two compounds to the activator site appears to be tighter than that to the catalytic site. Thus, the activation effect predominates at low inhibitor concentration while the inhibitory effect predominates at higher concentrations, resulting in the observed profiles. MM-SPPE (**12**) and SPPE (**9**) showed no activation and only very poor inhibition.

Effect of Enzyme Activation. It is interesting that SPPE inhibits the activated enzyme at least 50 times more potently than the nonactivated enzyme. Two possible mechanisms can be proposed to explain this difference: (i) SPPE inhibits the enzyme by simply binding to the activator site and blocking activation or (ii) SPPE inhibits the enzyme by binding to the catalytic site and binds more tightly to the activated enzyme than to the nonactivated enzyme. To distinguish between these two mechanisms, the *p*-BPB inactivation experiment was carried out. *p*-BPB has been shown to alkylate the His residue in the catalytic site (Roberts et al., 1977). The observation that sphingomyelin, the best activator of cobra venom PLA_2, does not protect the enzyme from *p*-BPB inactivation any better than Triton X-100 and Ca^{2+} suggests that the two sites are physically distinct. If SPPE binds only to the activator site, then SPPE plus sphingomyelin should not protect the enzyme any better than SPPE alone. But our results indicate that SPPE protects the enzyme from the *p*-BPB inactivation much more effectively in the presence of the enzyme activator than in its absence. Thus, the *p*-BPB inactivation experiment strongly argues for the second mechanism. SPPE must bind to the catalytic site of the enzyme and its affinity to the enzyme is dramatically increased by the activation of the enzyme with sphingomyelin.

The low affinity of a PE phosphonate analogue to the enzyme in the absence of phosphocholine-containing lipid has also been observed recently by Yuan *et al.* (1990). They noticed a dramatic improvement in binding of a short-chain inhibitor to cobra venom PLA_2 in the presence of a PC substrate analogue using equilibrium dialysis techniques. Their experiemtnal results are consistent with ours. They attribute the poor binding of the PE phosphonate analogue to the enzyme as being due to the absence of a lipid–enzyme microaggregate. In the experiments reported here, microaggregation is probably not operative. Since both the thio-PE substrate and SPPE were solubilized in Triton X-100, they should all exist in a mixed micellar state. Even though SPPE is in an aggregate with the substrate, it still binds to the enzyme very poorly in the absence of PC. The second mechanism proposed here can explain these results. The binding of PE phosphonate analogue can be significantly enhanced by activating the enzyme with phosphocholine-containing lipid.

pH Effect on Enzyme Activation and Inhibition. To further understand the enzyme activation phenomenon, we have characterized the pH effect on the enzyme activation by sphingomyelin and the pH effect on the enzyme activation and inhibition by the PC phosphonate and PC amide analogues. Enzyme activation does not appear to be a function of pH. This result is consistent with the model suggested by Ortiz *et al.* (1992) that cobra venom phospholipase A_2s have separate activator and catalytic sites for phosphocholine-containing lipids. Their proposed activator site is composed of residues Glu 55, Trp 61, Tyr 63, Phe 64, and Lys 65. The positive charge on the polar head group is stabilized through cation–π interactions with Trp 61, Tyr 63, and Phe 64 and through long-range electrostatic interactions with Glu 55. The negatively charged phosphate interacts with Lys 65. Since the two ionizable residues, Glu 55 and Lys 65, have pK_as of around 4.5 and 10.0, respectively, no pH dependence would be predicted for enzyme activation within the pH range used in this study.

The activation and inhibition of thio-PE hydrolysis by SPPC at different pHs is shown in Figure 5. As the pH decreased, the maxima of the curves decreased in height and shifted to the left until at pH 5.5 there was no maximum. This behavior can be explained on the basis of the previous observations that the binding affinity of the PC phosphonate analogue for the catalytic site increases as the pH decreases (Yu & Dennis, 1991a) while enzyme activation is pH-independent. Therefore, as the pH decreases, inhibition increases while activation remains constant, producing the net effect shown in Figure 5. To further illustrate this pH effect, the effect of pH on the activation and inhibition of the enzyme by a PC amide analogue was also studied (Figure 6). In contrast to the phosphonate analogue, the amide analogue binds to the enzyme tighter at high pHs. Thus, the apparent activation of the enzyme is increased as the pH decreases. These results indicate that the activator site and catalytic site have different pH dependencies.

Our previous work has shown that cobra venom phospholipase A_2 has two distinct functional phospholipid binding sites, an activator site and a catalytic site. The data presented here indicate that these two sites are also physically distinct. Phospholipid binding to these sites has very different characteristics. We have previously shown that these sites do not have the same specificities, and we have now shown that the pH dependencies of phospholipid binding to these sites is also different. This indicates that the physical/chemical makeup of the sites is not the same and that they are probably physically separated.

Conclusion. It now appears that cobra venom phospholipase A_2 binding of PE phospholipids, be they substrates or inhibitors, whether they have an oxyester, an amide, or a phosphonate moiety in the *sn*-2 position, can be enhanced by PC-containing phospholipids. In marked contrast to the binding of amide and phosphonate inhibitors to the catalytic site, which is strongly pH-dependent, the binding of sphingomyelin, an amide-containing PC phospholipid, is pH-independent. These various factors combine to produce the complex inhibition patterns seen for this enzyme.

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