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Phospholipid Activation of Cobra Venom Phospholipase A_2. 2. Characterization of the Phospholipid–Enzyme Interaction†

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ABSTRACT: Activation of cobra venom phospholipase A_2 toward phosphatidylethanolamine by phosphatidylcholine in mixed micelles has been suggested to be caused by a direct phosphatidylcholine–enzyme interaction. Comparable activation of phospholipase A_2 toward phosphatidylethanolamine also occurs with nonsubstrates sphingomyelin and lysophosphatidylcholine. Phospholipids with anionic head groups and water-soluble phosphorylcholine or glycerol phosphorylcholine alone are nonactivators. Hydrolysis of phosphatidylethanolamine is 0.06 that of phosphatidylcholine, and the rate increases abruptly (0.38) when two methyl groups are present on the nitrogen. The hydrolysis of all phospholipids examined is enhanced dramatically in the presence of activator lipid. The activation profile for phosphatidylethanolamine hydrolysis in the presence of sphingomyelin parallels cosubstrate phosphatidylcholine activation. Phospholipid binding studies reveal a similar apparent K_D (1 mM) for all phospholipids examined. Enzyme inactivation by *p*-bromophenacyl bromide, a reagent which inactivates the enzyme through modification of a histidine in the active site, is significantly protected by lipid substrates and fatty acids, while nonsubstrate activator lipids do not protect. The pK_a controlling phosphatidylethanolamine hydrolysis was found to shift from 6.5 to 5.8 in response to phosphatidylcholine activation of the enzyme, suggesting that the activator lipid causes a conformational change in the enzyme. These results can be explained by the existence of two binding sites: (i) an activator site which requires a lipid molecule containing the phosphorylcholine moiety and a fatty acyl chain and (ii) a head-group nonspecific catalytic site.

In human erythrocyte ghosts and in phosphatidylethanolamine/phosphatidylcholine/Triton X-100 mixed micelles, phosphatidylethanolamine is the preferred substrate of cobra venom phospholipase A_2 (*Naja naja naja*) (EC 3.1.1.4) (Adamich & Dennis, 1978a,b). This specificity for phosphatidylethanolamine is in direct contrast to that found if the individual[1] phospholipids in Triton X-100 mixed micelles are treated with phospholipase A_2 (Roberts et al., 1978b). Such behavior has been examined in a variety of other mixed micellar systems as well (Roberts et al., 1979). The activation of phosphatidylethanolamine hydrolysis with cosubstrate phosphatidylcholine occurs under a variety of micelle sizes, surface charges, and concentrations of phospholipid in the surface. Furthermore, monomeric dibutyrylphosphatidylcholine[2] activates phosphatidylethanolamine hydrolysis. These findings suggest that the activation phenomenon is the result of a direct interaction of the phosphatidylcholine molecule with phospholipase A_2 as discussed in the accompanying manuscript (Roberts et al., 1979).

The nature of the lipid–enzyme activation has now been examined in greater detail. Structural features required for the activator molecule are the phosphorylcholine moiety and at least one fatty acyl chain. Phospholipids with head groups other than choline are comparatively poor substrates for phospholipase A_2, but they are hydrolyzed more efficiently when activator lipid is added. The kinetics of the phosphatidylethanolamine activation by both cosubstrate phospha-

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‡ Postdoctoral Fellow of the National Institutes of Health, Grant GM-06071.
§ Postdoctoral Fellow of the National Institutes of Health, Grant GM-05910.

[1] When one species (i.e., head group) of a phospholipid is inserted into a detergent matrix, the mixed micelles are said to contain individual phospholipids. When two or more phospholipids differing in head group are present in the mixed micelle system, it will be referred to as a binary, ternary, etc. mixture.

[2] Abbreviations used: diacylphosphatidylcholine, 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine; Triton, Triton X-100; CTAB, cetyltrimethylammonium bromide; Mes, 2-(*N*-morpholino)ethanesulfonate.

0006-2960/79/0418-3308$01.00/0

tidylcholine and nonsubstrate sphingomyelin were examined and the results are compared with the ability of various phospholipids to bind to phospholipase A_2. Optimal activation of phosphatidylethanolamine hydrolysis occurs at an activator concentration comparable to the apparent dissociation constant for that lipid. The pH–rate profile for phosphatidylethanolamine and phosphatidylcholine individually in Triton X-100 micelles is compared with that of a binary mixture. Phosphatidylcholine activation is observed to lower the pK_a controlling phospholipase A_2 hydrolysis of phosphatidylethanolamine. A variety of phospholipids were examined for their ability to protect phospholipase A_2 from *p*-bromophenacyl bromide inactivation (Roberts et al., 1977c). Interpretation of these data suggests the possibility that two functionally distinct phospholipid binding sites or subsites exist: an activator site which is specific for the lipid polar headgroup and a catalytic site which is nonspecific.

Experimental Procedure

Materials. Lyophilized cobra venom, *Naja naja naja* (Pakistan), lot no. NNP45-1Z, was obtained from the Miami Serpentarium. The phospholipase A_2 was purified as described elsewhere (Deems & Dennis, 1975; Roberts et al., 1977b). Phospholipase A_2 from *Naja naja kaouthia* (partially fractionated venom kindly supplied by Dr. Richard Ulevitch of Scripps Clinic and Research Foundation) was purified in an analogous manner to that from *Naja naja naja*. The material from the final DEAE column contained two active forms (P_I = 4.4, 4.6) in approximately equal amounts. Purified phospholipase A_2 from *Crotalus atrox* was the generous gift of Dr. John Law, University of Chicago.

Egg phosphatidylcholine was prepared from fresh egg yolks (Singleton et al., 1965). Phosphatidylethanolamine, *N*-methylphosphatidylethanolamine, *N*,*N*-dimethylphosphatidylethanolamine, phosphatidylethanol, and phosphatidylglycerol (all prepared by transesterification of egg phosphatidylcholine) were obtained from Avanti Biochemicals. Lysophosphatidylcholine and D-dipalmitoylphosphatidylcholine were prepared by phospholipase A_2 treatment of either egg phosphatidylcholine or DL-dipalmitoylphosphatidylcholine and purified by silicic acid (Clarkson Chemical Co.) chromatography. β-Dipalmitoylphosphatidylcholine was the gift of Dr. Sunney Chan, California Institute of Technology. Other lipids included egg sphingomyelin, beef brain phosphatidylserine, and dihexanoylphosphatidylcholine (all from Avanti Biochemicals) and dibutyrylphosphatidylcholine (Supelco). L-α-Glycerophosphorylcholine, phosphorylcholine, oleic acid, and cetyltrimethylammonium bromide (CTAB) were obtained from Sigma. All phospholipids gave a single spot on one-dimensional thin-layer chromatography as described in the accompanying manuscript (Roberts et al., 1979). *p*-Bromophenacyl bromide was obtained from Aldrich and recrystallized from ethanol before use. Triton X-100 was obtained from Rohm and Haas.

Enzyme Assays. Phospholipase A_2 activity toward individual phospholipids in mixed micelles with Triton X-100 and with added nonhydrolyzable compounds was determined by pH-stat (Dennis, 1973a; Roberts et al., 1978b). Phospholipid mixtures of hydrolyzable substrates were monitored by thin-layer chromatographic techniques and phosphate assay (Adamich & Dennis, 1978a), or by ^{31}P NMR techniques (Roberts et al., 1979). Standard conditions employed were pH 8.0, 40 °C, 10 mM $CaCl_2$, and the indicated amounts of phospholipids (6 mM for ^{31}P NMR, 4 mM for thin-layer chromatography and either 4 mM or 6 mM for pH-stat as indicated) and Triton X-100. For the thin-layer chromatography assay, 25 mM Tris-HCl was also included; for ^{31}P NMR assays, 50 mM Tris-HCl and 30% D_2O were included. All assays are the average of duplicate or triplicate determinations; average errors were about ±5% for pH-stat and thin-layer chromatography assays and ±10% for the ^{31}P NMR assays.

Gel Filtration Binding Studies. Equilibrium gel filtration was used to determine the affinity of phospholipase A_2 for mixed micelles. A Sephadex G-100 column (1 × 50 cm) was equilibrated with enzyme (0.05–0.06 mg mL^{-1}) in 0.05 M Tris-HCl, pH 8.0, 25 °C. A monomer concentration of Triton (0.35 mM) and EDTA (5 mM) or divalent metal ions (10 mM) was included in the equilibrating buffer. A sample of Triton/phospholipid (1.5 mL of 60 mM Triton/10 mM phospholipid) was applied to the column, and enzyme–phospholipid binding was followed by the increase in enzyme activity across the eluted phospholipid peak and decrease across the subsequent trough. The concentration of phospholipid was determined by phosphorus analysis (Eaton & Dennis, 1976). The apparent dissociation constant in terms of the bulk concentration of phospholipid was calculated from the area of the enzyme peak and trough and the average value determined. The average K_D for duplicate experiments is reported and the average error is ±10%.

p-Bromophenacyl Bromide Inactivation. Enzyme (0.02 mg mL^{-1}) in 0.04 M Tris-HCl, pH 8.0, 40 °C, was incubated with 5×10^{-5} M *p*-bromophenacyl bromide. The reagent was added from a 2×10^{-3} M stock solution in acetone. Aliquots were removed for pH-stat assay of remaining activity as a function of reaction time. The pseudo-first-order rate constant for inactivation was calculated from a least-squares fit of at least four time points. Protection experiments were performed with 30 mM Triton, 10 mM Ba^{2+} (except for oleic acid), and 9 mM lipid in the incubation mixture.

Results

Structural Features of the Substrate and Activator Molecule. Phosphatidylcholine activates phospholipase A_2 hydrolysis of phosphatidylethanolamine (Adamich & Dennis, 1978a). Other phospholipids or analogues were examined for their ability to activate cobra venom phospholipase A_2 toward phosphatidylethanolamine. Table I shows that only compounds with the phosphorylcholine moiety and a fatty acyl chain (i.e., phosphatidylcholine, sphingomyelin, lysophosphatidylcholine) enhance phosphatidylethanolamine hydrolysis. Phospholipids with other head groups (phosphatidylserine, phosphatidylglycerol) do not activate. The cationic detergent CTAB, which possesses a quaternary nitrogen atom, also fails to activate the enzyme, thus demonstrating the importance of the anionic phosphate group. Free fatty acid (oleic acid) does stimulate enzyme activity, although the maximum is only 2-fold compared with the 10–20-fold maximum stimulation observed with phosphatidylcholine or sphingomyelin.

The activity of this phospholipase A_2 toward a variety of zwitterionic and anionic phospholipids is shown in Table II along with the effect of sphingomyelin as activator. The effect on enzyme activity of successively methylating the nitrogen atom of phosphatidylethanolamine is striking. With the addition of two methyl groups, the phospholipid hydrolysis rate increases dramatically (about sixfold). Negatively charged lipids such as phosphatidylethanol and phosphatidylglycerol are very poor substrates. When 5 mol % sphingomyelin is added, the hydrolysis rates of all phospholipids, except phosphatidylcholine, are increased. It should be noted that assay conditions include 10 mM $CaCl_2$ which could interact with the negatively charged phospholipids to form phos-

Table I: Effect of Various Ligands on the Rate of Hydrolysis of Phosphatidylethanolamine by Phospholipase A_2 (*Naja naja naja*)[a]

ligand	rate (μmol min^{-1} mg^{-1})
none	30
phosphorylcholine	30
glyceroylphosphorylcholine	35
dibutyrylphosphatidylcholine[b,c]	126
egg phosphatidylcholine	244
β-dipalmitoylphosphatidylcholine	250
D-dipalmitoylphosphatidylcholine	271
lysophosphatidylcholine	246
sphingomyelin	642
CTAB	34
oleic acid[c]	53
phosphatidylserine	25
phosphatidylglycerol	32

[a] Reaction conditions were 5.4 mM phosphatidylethanolamine, 0.6 mM ligand, and 48 mM Triton X-100. Hydrolysis was monitored by pH-stat for nonhydrolyzable ligands and by ^{31}P NMR for substrate ligands. [b] Dibutyrylphosphatidylcholine is not cosolubilized by Triton X-100/phosphatidylethanolamine mixed micelles (Roberts et al., 1979). [c] 4.3 mM phosphatidylethanolamine and 1.7 mM ligand were used.

Table II: Activity of Phospholipase A_2 toward Phospholipids in Triton X-100 Mixed Micelles and the Effect of Sphingomyelin[a]

phospholipid	rel rate[b]	rel rate (+5 mol % sphingomyelin)
phosphatidylethanolamine	0.06	0.45
N-methylphosphatidylethanolamine	0.08	0.60
N,N-dimethylphosphatidylethanolamine	0.38	0.72
phosphatidylcholine	1.00	0.90
phosphatidylglycerol	0.04	0.23
phosphatidylethanol	0.01	0.10

[a] ^{31}P NMR assay was employed with 6 mM total phospholipid and 48 mM Triton X-100. [b] The activity of phospholipase A_2 (*Naja naja naja*) toward egg phosphatidylcholine was 425 μmol min^{-1} mg^{-1}, and all activities are compared with this value.

pholipid–Ca^{2+} complexes. This may be partially responsible for the extremely low rates of hydrolysis for those phospholipids (Roberts et al., 1978b). However, the addition of as little as 5 mol % sphingomyelin should not appreciably alter the phospholipid–Ca^{2+} distribution; yet, it causes remarkable rate enhancements in all cases.

Phosphatidylcholine activation of phosphatidylethanolamine hydrolysis is not unique to phospholipase A_2 from *Naja naja naja*. The enzyme from *Naja naja kaouthia* shows identical substrate specificity toward individual phospholipids and binary lipid mixtures in Triton (Table III). Phospholipase A_2 from *C. atrox* shows a less distinct specificity pattern toward the two individual phospholipids in Triton. As can be seen in Table III, this enzyme acts on phosphatidylethanolamine alone at half the rate of phosphatidylcholine. The addition of 10 mol % phosphatidylcholine does not cause a large increase in the rate of phosphatidylethanolamine hydrolysis. But in equimolar phosphatidylethanolamine/phosphatidylcholine mixtures, phosphatidylethanolamine becomes the preferred substrate. Thus, for nearly equal phosphatidylethanolamine/phosphatidylcholine mixtures, all of these snake venom phospholipases preferentially attack phosphatidylethanolamine, although the exact specificity toward individual phospholipids varies somewhat with the species.

Activity Profiles for Binary Lipid Mixtures. Activation by phosphatidylcholine of phospholipase A_2 activity toward phosphatidylethanolamine in binary lipid mixtures and the resulting phenomenon of specificity reversal are demonstrated in Figure 1. In the presence of about 35 mol % phosphatidylcholine, phosphatidylethanolamine hydrolysis is maximal. At this mol % phosphatidylcholine, the concentration of this lipid is about 1 mM. At equimolar concentrations of the two lipids, phosphatidylethanolamine is the preferred substrate. A rapid decline in the rate for phosphatidylethanolamine follows maximal activation, and a concomitant rise in the rate for phosphatidylcholine hydrolysis is observed. The combined rate for total phospholipid hydrolysis from 35 to 100 mol % phosphatidylcholine is comparable to phosphatidylcholine alone.

Table III: Phosphatidylcholine Activation of Other Snake Venom Phospholipase A_2 Enzymes toward Phosphatidylethanolamine[a]

enzyme source	phospholipid(s)	rel rates[b] PE	rel rates[b] PC
Naja naja kaouthia	PE	0.06	
	PC		1.00
	PE:PC (9:1)	0.55	nd
	PE:PC (1:1)	1.10	0.40
Crotalus atrox	PE	0.49	
	PC		1.00
	PE:PC (9:1)	0.52	nd
	PE:PC (1:1)	1.58	0.80

[a] Hydrolysis rates for binary mixtures were monitored by ^{31}P NMR and for individual phospholipids by pH-stat; 6 mM total phospholipid (with the indicated mole ratio of each phospholipid) and 48 mM Triton were employed. Abbreviations used: PE, phosphatidylethanolamine; PC, phosphatidylcholine; nd, not determined. [b] Rates are relative to values for enzymatic hydrolysis of egg phosphatidylcholine: 405 μmol min^{-1} mg^{-1} for *Naja naja kaouthia* phospholipase A_2.

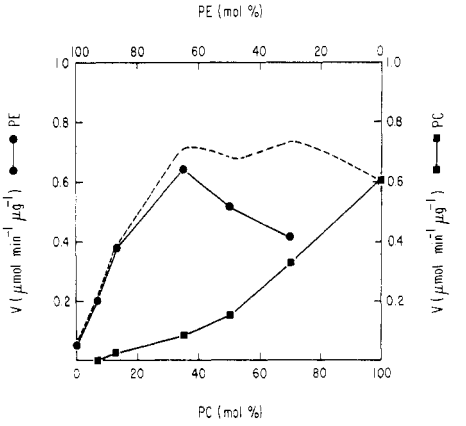

FIGURE 1: Activity of phospholipase A_2 toward phosphatidylethanolamine (●) and phosphatidylcholine (■) in binary mixtures with varying mol % of each phospholipid. Assays were conducted by thin-layer chromatography with 4 mM total phospholipid and 16 mM Triton X-100. Dashed line is the sum of the activities toward the two substrates at each mol %.

The activation profile for sphingomyelin as activator strikingly resembles the phosphatidylcholine activation profile as shown in Figure 2: maximal activation occurs at about 30 mol % or about 1 mM sphingomyelin, and activity falls off sharply following maximal activation. Overall activation by sphingomyelin relative to that observed with phosphatidylcholine is greater as shown in Table I. Note that the experiments in Figure 2 with sphingomyelin were conducted at a molar ratio of Triton to total phospholipid of 8:1 so that the observed rates are comparable to those with phosphatidylcholine at a molar ratio of 4:1 in Figure 1. Sphingomyelin does not accelerate the rate of phosphatidylcholine hydrolysis but, rather, appears to act in a fashion similar to Triton X-100

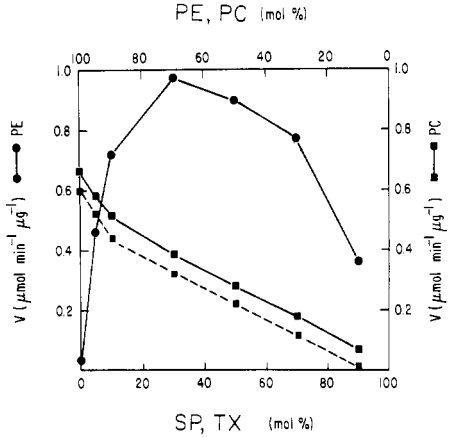

FIGURE 2: Activity of phospholipase A_2 toward binary mixtures of phosphatidylethanolamine/sphingomyelin (●) and phosphatidylcholine/sphingomyelin (■) with varying mol % of each lipid. Assays were conducted by pH-stat with 4 mM total lipid and 32 mM Triton X-100. For comparison (dashed line) is shown the enzymatic activity toward phosphatidylcholine when Triton X-100 is added instead of sphingomyelin. Note that since 32 mM Triton is present initially in all mixtures, at 0 mol % added Triton there is actually 32 mM Triton plus 4 mM phosphatidylcholine and at 90 mol % added Triton there is actually present 35.6 mM Triton plus 0.4 mM phosphatidylcholine.

in decreasing the rate of the reaction as shown in Figure 2 (Dennis, 1973b). Variation of the Triton/phosphatidylethanolamine mole ratio without added phosphatidylcholine shows similar surface dilution behavior as shown in Table I of the accompanying manuscript (Roberts et al., 1979). A similar kinetic profile is observed for the effects of lysophosphatidylcholine on phosphatidylcholine hydrolysis (data not shown).

pH–Rate Profile of Phospholipase A_2 Action. The pK_a for phospholipase A_2 hydrolysis of Triton/phosphatidylcholine mixed micelles is dependent on Ca^{2+} concentration and was found to be 5.6 in 10 mM Ca^{2+} (Roberts et al., 1977b). A similar value (5.9) was found by ^{31}P NMR assay on phosphatidylcholine with 10 mM Ca^{2+} as shown in Figure 3. For phosphatidylethanolamine with 10 mM Ca^{2+}, the pK_a was determined to be 6.5. Note that 30% D_2O was included in the assay mix and this may influence the apparent pK_a of the enzyme as was shown by Wells (1974) for phospholipase A_2 from *C. adamanteus*. Ca^{2+} is also required for enzyme activity toward phosphatidylethanolamine. Furthermore, Ba^{2+} and Zn^{2+} inhibit phosphatidylethanolamine hydrolysis as well as phosphatidylcholine hydrolysis (Roberts et al., 1977b).

Figure 3 shows the pH–rate profiles for the two phospholipids individually in Triton X-100 mixed micelles. The solid lines represent the theoretical curves calculated for phosphatidylethanolamine hydrolysis by assuming one ionizable group with pK_a = 6.5 and maximum rate = 62 μmol min^{-1} mg^{-1} and for phosphatidylcholine hydrolysis by assuming one ionizable group with pK_a = 5.9 and maximum rate = 570 μmol min^{-1} mg^{-1}. While the rate of phosphatidylcholine hydrolysis, either individually or in a binary mixture, does not decline much at high pH, that of phosphatidylethanolamine drops significantly. This drop in activity may indicate that, as phosphatidylethanolamine becomes anionic, it becomes a much poorer substrate. The difference in activity of phospholipase A_2 toward the two phospholipids is not an artifact of pH since, at pH 8.0, the enzyme activity is near its maximum value for both lipids.

In binary mixtures of approximately equal amounts of phosphatidylethanolamine and phosphatidylcholine, where activation is observed, the pK_a for phosphatidylethanolamine

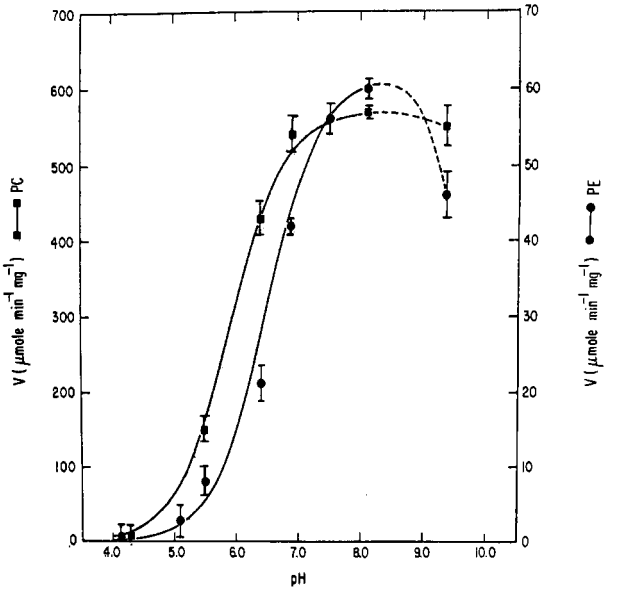

FIGURE 3: The initial rate of hydrolysis by phospholipase A_2 of phosphatidylcholine (■) and phosphatidylethanolamine (●) individually as a function of pH. Assays were conducted by ^{31}P NMR with 6 mM phospholipid and 48 mM Triton X-100. The following buffers (25 mM each) were employed: acetate (pH 4–5.5), Mes (pH 6–7.5), Tris-HCl (pH 7.8–9.4). The average of duplicate determinations is shown with the error ranges.

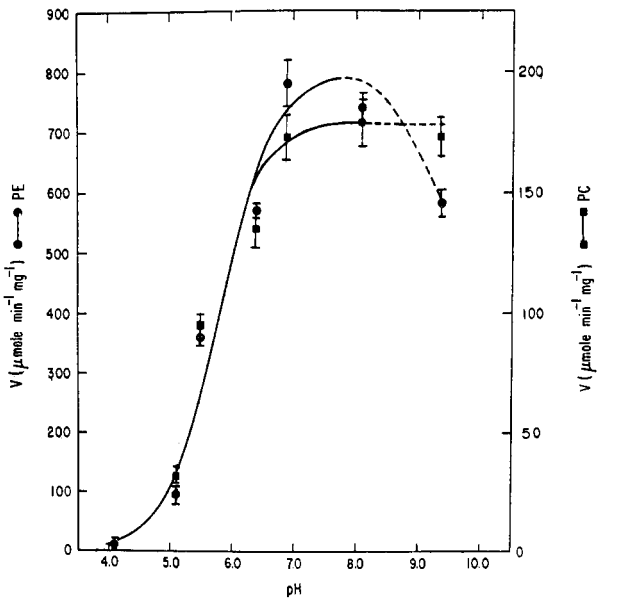

FIGURE 4: The initial rate of hydrolysis by phospholipase A_2 of phosphatidylethanolamine (●) and phosphatidylcholine (■) in a binary mixture in Triton as a function of pH. Experimental conditions were as in Figure 3, except that 6 mM phosphatidylethanolamine, 5.1 mM phosphatidylcholine, and 96 mM Triton were employed.

hydrolysis is lowered to 5.8, equivalent to that for phosphatidylcholine. The titration data are shown in Figure 4: the solid lines represent the theoretical curves for phosphatidylethanolamine hydrolysis by assuming pK_a = 5.8 and maximum rate = 800 μmol min^{-1} mg^{-1} and for phosphatidylcholine hydrolysis by assuming pK_a = 5.8 and maximum rate = 180 μmol min^{-1} mg^{-1}. Thus, binding of the phosphatidylcholine activator to the enzyme lowers the pK_a for phosphatidylethanolamine hydrolysis.

Phospholipid Binding to Cobra Venom Phospholipase A_2. Because Triton/phospholipid mixed micelles with mol wt 100 000 or greater (Robson & Dennis, 1978) are larger structures than the phospholipase A_2 whose molecular weight

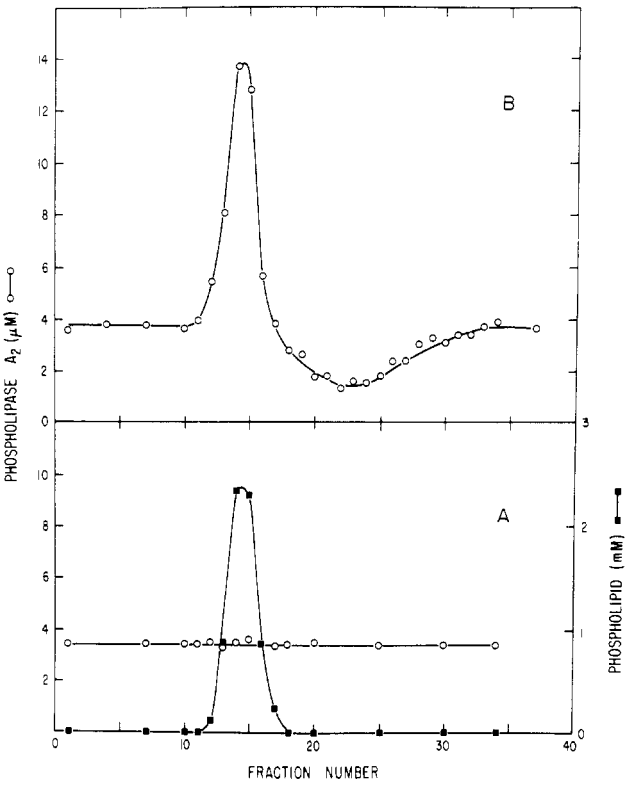

FIGURE 5: Typical elution profiles for phospholipid (■) and enzyme (○) used in determining the affinity of phospholipase A_2 for Triton–phospholipid-mixed micelles. Phosphatidylethanolamine binding in the (A) absence and (B) presence of Ba^{2+} is shown.

Table IV: Binding of Triton/Phospholipid-Mixed Micelles to Phospholipase A_2 as Measured by Gel Filtration

phospholipid	metal ion	app dissociation constant (mM)
phosphatidylethanolamine	EDTA	>100
phosphatidylethanolamine	Ba^{2+}	1.1
phosphatidylcholine	Ba^{2+}	1.0
sphingomyelin	Ba^{2+}	1.0[a]
sphingomyelin	Ca^{2+}	1.0[a]

[a] Single column determination.

is about 13 500,[3] equilibrium gel filtration binding studies were performed with the column equilibrated with enzyme. An aliquot of phospholipid in Triton is then applied and binding is measured by following enzyme activity. Typical Sephadex G-100 gel column profiles are shown in Figure 5. The figure shows that metal ions (in this case Ba^{2+}) are required for phospholipase A_2 to bind phosphatidylethanolamine. The enzyme does not bind to Triton alone or in the presence of metal ions (Roberts et al., 1977a). Interestingly, all phospholipids are bound by the enzyme with the same apparent affinity (about 1 mM). These binding data are summarized in Table IV. In the case of nonsubstrate sphingomyelin, Ca^{2+} can be used instead of Ba^{2+}. The enzyme exhibits a similar apparent K_D for sphingomyelin with both metal ions.

Effect of Substrates and Activators on the Inactivation of Phospholipase A_2 by p-Bromophenacyl Bromide. Phospholipase A_2 can be inactivated by *p*-bromophenacyl bromide through the modification of 0.5 histidine per enzyme molecule (Roberts et al., 1977c). Triton with Ba^{2+} apparently inhibits the inactivation reaction by a combination of sequestering the reagent in the Triton micelles and altering enzyme conformation by metal ion binding. Further inhibition is seen when

[3] A. Jarvis and E. A. Dennis, unpublished results.

Table V: Effect of Ligands on Inactivation of Phospholipase A_2 by *p*-Bromophenacyl Bromide

ligand	Triton X-100	Ba^{2+}	$k_{inact} \times 10^3$ (min^{-1})
	−	−	115
	+	−	21
	+	+	10
egg phosphatidylcholine	+	+	3
egg phosphatidylethanolamine	+	+	1.5
sphingomyelin	+	+	10
dihexanoylphosphatidylcholine	+	+	2
dihexanoylphosphatidylcholine (monomer)[a]	−	+	3
D-dipalmitoylphosphatidylcholine	+	+	3
egg lysophosphatidylcholine	+	+	10
oleic acid	+	−	1.6

[a] Dihexanoylphosphatidylcholine (3 mM) exists as monomers (Tausk et al., 1974a,b).

phosphatidylcholine is incorporated into this micelle system. This was used as evidence of an active site role for the histidine. Protection data with a variety of phospholipids are summarized in Table V. Long-chain phosphatidylcholine, D-dipalmitoylphosphatidylcholine, and phosphatidylethanolamine protect. Monomeric dihexanoylphosphatidylcholine also protects, although not as effectively, probably because the concentration of lipid was decreased from 9 to 3 mM to ensure monomer structures of phospholipid. Dihexanoylphosphatidylcholine, when it forms micelles in the presence of Triton, is as effective as the long-chain lipids. Oleic acid binds to the phospholipase A_2 without metal ion (Roberts et al., 1977a) and also protects against *p*-bromophenacyl bromide modification. Lysophosphatidylcholine and sphingomyelin, which are both capable of binding to phospholipase A_2 and activating it toward phosphatidylethanolamine, do not protect the enzyme any better than Triton and Ba^{2+} alone.

Discussion

Specificity and Structural Properties of the Phospholipid Required for Phospholipase A_2 Activation. The activation by lipids of a variety of membrane-associated enzymes and a few water-soluble enzymes is well known (for reviews, see Coleman, 1973; Farias et al., 1975). Often several different lipids, including detergents, can be used to activate these enzymes and in most cases lipid activation is directed toward nonlipid substrates. Pyruvate oxidase, which is a water-soluble enzyme and is comparable to phospholipase A_2 in this respect, is stimulated by palmitic and oleic acid, lysophosphatidylethanolamine, phosphatidylcholine, triglycerides, and a variety of amphilphiles (Cunningham & Hager, 1971; Blake et al., 1978).

Lipid activation of phospholipase A_2 is unique because one substrate molecule (phosphatidylcholine) can activate the enzyme toward a different cosubstrate (phosphatidylethanolamine). Several other molecules can also activate the enzyme toward phosphatidylethanolamine: β-dipalmitoylphosphatidylcholine, D-dipalmitoylphosphatidylcholine, lysophosphatidylcholine, and sphingomyelin. These molecules have in common the phosphorylcholine group and one or more fatty acyl chains bound by either an ester linkage or by an amide linkage. The activator molecule need not reside in an interface because soluble dibutyrylphosphatidylcholine will enhance interfacial phosphatidylethanolamine hydrolysis (Roberts et al., 1979). Interestingly, the characteristics of the phospholipase A_2 activator lipid are quite reminiscent of the lipid specificity of β-hydroxybutyrate dehydrogenase activation

(Grover et al., 1975; Isaacson et al., 1979). A slight enhancement in phosphatidylethanolamine hydrolysis (at most a factor of two) is observed with added fatty acid (5–28%), but the activation is much less than with the phosphorylcholine-containing lipids. The ability of phosphatidylcholine-containing molecules to serve as activators is generalizable to all substrate phospholipids studied.

Sphingomyelin activates the enzyme to a greater extent than phosphatidylcholine. Binding studies imply that this is not due to differences in the apparent affinity of the two lipids for the enzyme. The cause must be related to the detailed conformation of sphingomyelin and, hence, its interaction with phospholipase A_2. Sphingomyelin differs from phosphatidylcholine most markedly by its double bond and an adjacent hydroxyl group on the equivalent of the *sn*-1 chain, as well as the amide linkage of the equivalent of the *sn*-2 carbonyl. ^1H NMR studies of the fatty acyl α-methylene group in sphingomyelin in various ionic detergents show that sphingomyelin differs from phosphatidylcholine in that region of the molecule (Roberts et al., 1978a). The *sn*-2 α-methylene protons in phosphatidylcholine experience a greater magnetic nonequivalence than the corresponding protons in sphingomyelin. This could reflect subtle packing differences and chain orientations which could be critical for activator interactions with phospholipase A_2.

Nature of the Lipid Activation of Phospholipase A_2. The extent to which different lipids protect against *p*-bromophenacyl bromide inactivation of phospholipase A_2 allows one to divide lipids into two classes: those which bind effectively at the catalytic site and protect against inactivation (phosphatidylcholine (all isomers) and phosphatidylethanolamine) and those which bind to the enzyme, but do not protect (sphingomyelin and lysophosphatidylcholine). The latter lipids may bind at a distinct noncatalytic activator site or they may still bind to the catalytic site, but in a nonproductive fashion. Interestingly, sphingomyelin and lysophosphatidylcholine are activators toward phosphatidylethanolamine and other poor substrates for phospholipase A_2. Thus, the lipid activation of cobra venom phospholipase A_2 suggests that there are two "functionally" distinct binding sites or subsites for phospholipid interacting with the enzyme. One is a phosphorylcholine-specific activator site, while the other is the head-group nonspecific, catalytic site.

The initial phase of phosphatidylethanolamine hydrolysis suggests that phosphatidylcholine–enzyme binding saturates at about 1 mM ($\sim$35 mol %) phosphatidylcholine. Beyond the phosphatidylethanolamine hydrolysis maximum, continual dilution of phosphatidylethanolamine in the interface and competition with phosphatidylcholine is probably responsible for the rate decrease. Similar kinetics are observed for sphingomyelin activation of phosphatidylethanolamine: activation is maximal around 1 mM sphingomyelin followed by a drop in rate probably caused by the decrease in phosphatidylethanolamine concentration and surface dilution. The concentration of phosphatidylcholine required for maximal enzyme activity toward phosphatidylethanolamine is similar to the apparent dissociation constant of the activator for phospholipase A_2. All lipids, phosphatidylethanolamine included, have similar apparent dissociation constants. Therefore, phosphatidylcholine activation of phosphatidylethanolamine must represent primarily an increase in V_{max}, not K_m.

Perhaps a clue to the detailed nature of the activation is given by the pH–rate profiles for individual phospholipids in Triton and for binary phosphatidylethanolamine/phosphatidylcholine mixtures. The hydrolysis of phosphatidylcholine alone in Triton depends on an enzyme residue with an apparent pK_a = 5.6–5.9 (Roberts et al., 1977b). With phosphatidylethanolamine as the sole substrate in Triton, the pK_a controlling the reaction is 6.5, a value considerably higher than that for phosphatidylcholine. In binary mixtures of the two phospholipids, the pK_a for the reaction is 5.8 for *both* phospholipids. Thus, phosphatidylcholine activation of the enzyme lowers the pK_a of an ionizable group on the enzyme important for catalysis.

Whatever the detailed mechanism for preferential phosphatidylethanolamine hydrolysis in phospholipid mixtures, phospholipid activation appears to be a phenomenon common to other snake venoms (*Naja naja* species and *Crotalus atrox*). Furthermore, phospholipase A_2 from a variety of sources displays preferential hydrolysis of phosphatidylethanolamine in the presence of other lipids (Sundler et al., 1977; Takeshi et al., 1978; Barzilay et al., 1978). These findings suggest mechanistic similarities for the different phospholipases.

Acknowledgments

We thank Dennis Templeton for help in the purification of the *Naja naja kaouthia* enzyme.

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Differential Effects on Phospholipid Phase Transitions Produced by Structurally Related Long-Chain Alcohols†

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ABSTRACT: The thermotropic behavior of aqueous dispersions of dipalmitoylphosphatidylcholine and, in a few cases, dimyristoylphosphatidylcholine and distearoylphosphatidylcholine, was measured spectroscopically by using the spin probe, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo[1]). From the resulting sigmoidal phase transition profiles, the main gel to liquid-crystalline transition temperature (T_m) was obtained, an estimate was made of the mean transition half-width ($\bar{W}_{1/2}$) and, where it was observed, the small pretransition (T_l) was also determined. The effects on these parameters of incorporating the long-chain alcohols, $C_{14:0}$, *cis*- and *trans*-$C_{14:1}$, $C_{16:0}$, and *cis*- and *trans*-$C_{16:1}$, were studied as a function of the concentration of alcohol. In DPL, the saturated alcohols produced a concentration-dependent elevation, the trans unsaturated alcohols, a smaller elevation, while the cis unsaturated alcohols produced a substantial depression of T_m. All six alcohols broadened the main transition. The latter effect was large in the case of the saturated alcohols but significantly smaller in the case of three out of four of the unsaturated alcohols. The unsaturated hexadecenols were also incorporated into DML and DSL. As with DPL, the trans isomer raised, while the cis isomer lowered, the main transition temperature. In each case, there was an increase in the mean transition half-width ($\bar{W}_{1/2}$). Spin-labeled phospholipid (PC(7,6)) was used to determine the order parameter of DPL vesicles in the presence and absence of 33 mol % *cis*- and *trans*-hexadecenol. Above T_m, both alcohols ordered the lipid membrane slightly, whereas, below T_m, the cis isomer disordered, while the trans isomer expelled the spin label from the lipid bilayer. In contrast to their effect on T_m, all three of the C_{16} alcohols shifted the pretransition (T_l) to higher temperatures such that $|\Delta T_l|$ was usually greater than $|\Delta T_m|$. The manner and extent to which the phase transition parameters were modified were found to depend not only on the length and shape of the added alcohol but also on the chain length of the lipid into which it was incorporated. The results are discussed in terms of a thermodynamic model describing the differential partitioning of the alcohols into the gel and liquid-crystalline phases of the respective lipids.

The thermotropic phase transitions of aqueous phospholipid dispersions have been the subject of numerous studies in the last few years (for a recent review, see Lee (1977)). Impetus for such studies derives from the conjecture that a large variety of biological phenomena, having a biomembrane as their locus, are mediated by lateral phase separations in the lipid portion of the membrane. For example, lateral diffusion (Cullis, 1976), transport (Thilo et al., 1977), and membrane fusion (Poste & Allison, 1973) have been shown to be markedly enhanced in the temperature region of the gel to liquid-crystalline transition of the membrane lipids. Also, breaks in Arrhenius plots of glucagon-stimulated adenylate cyclase (Houslay et al., 1976), calcium-dependent ATPase in sarcoplasmic reticulum (Hidalgo et al., 1976), and phospholipase activity of β-bungarotoxin (Strong & Kelly, 1977) attest to the importance of this phenomenon in modulating enzyme activity. Furthermore, certain bacterial membranes such as *Acholeplasma laidlawii* (Verkleij et al., 1972), *Halobacterium cutirubrum* (Esser & Lanyi, 1973), and *Escherichia coli* (Träuble & Overath, 1973; Jackson & Sturtevant, 1977), as well as erythrocyte ghosts at low temperature (Verma & Wallach, 1976), have, themselves, been shown to undergo thermotropic phase transitions. The role of perturber molecules in shifting and broadening the phase transition has attracted considerable attention recently both because of the information yielded concerning the transition process and because of the possible involvement of phase transitions in drug action. Thus, Jain & Wu (1977) and Lee (1977) have carried out an extensive study of the effects on the DPL phase transition of a wide variety of organic and inorganic compounds. In particular, the effects of incorporating aliphatic alcohols into both pure and mixed lipid dispersions have been examined by several biophysical techniques, including light scattering (Hill, 1974), fluorescence (Lee, 1976), differential scanning calorimetry (Hui & Barton, 1973; Eliasz et al., 1976; Jain & Wu, 1977; Jain et al., 1978), and dilatometry (MacDonald, 1978). Taken together, the above studies show that short-chain alcohols depress, whereas long-chain alcohols elevate, the main gel to liquid-crystalline transition temperature (T_m) of the phospholipids into which they are intercalated. In

† From the Harvard Medical School and Massachusetts General Hospital, Departments of Pharmacology and Anesthesia, Boston, Massachusetts 02114. *Received February 16, 1979*. This research was supported by a grant from the National Institute of General Medical Sciences (GM 22671).

* K. W. Miller is a Research Career Development Awardee (GM 00199).

[1] Abbreviations used: DPL, dipalmitoylphosphatidylcholine; DML, dimyristoylphosphatidylcholine; DSL, distearoylphosphatidylcholine; PC(7,6), 1-acyl-2-[8-(4,4-dimethyloxazolidine-*N*-oxyl)]palmitoylphosphatidylcholine; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl; $C_{14:0}$, tetradecanol; $C_{14:1}$, *cis*- or *trans*-9,10-tetradecenol; $C_{16:0}$, hexadecanol; $C_{16:1}$, *cis*- or *trans*-9,10-hexadecenol; T_m, temperature of gel to liquid-crystalline phase transition; $\bar{W}_{1/2}$, mean transition half-width; T_l, temperature of pretransition; *n*, hydrocarbon chain length; *S*, order parameter; ESR, electron spin resonance.

0006-2960/79/0418-3314$01.00/0