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Role of Monomeric Activators in Cobra Venom Phospholipase A_2 Action†

Andreas Plückthun‡ and Edward A. Dennis*

ABSTRACT: Phospholipase A_2 from cobra venom (*Naja naja naja*), which acts poorly on phosphatidylethanolamine (PE) in mixed micelles, is activated toward PE by the monomeric phospholipid dibutyrylphosphatidylcholine (dibutyryl-PC) which is an even poorer substrate. Phosphorus-31 nuclear magnetic resonance spectroscopy was employed to show that only PE is hydrolyzed in mixtures of PE and dibutyryl-PC of various concentrations. The activation shows saturation behavior, and the fully activated enzyme hydrolyzes PE at a rate similar to its optimal substrate PC containing long chain fatty acid groups. Because dibutyryl-PC is not incorporated into the micelles, these results are consistent with a mechanism of direct activation of the enzyme by dibutyryl-PC rather than a change in the properties of the interface being responsible for the activation of phospholipase A_2. Furthermore, if either PC or PE as substrate is dispersed in mixed micelles, increasing amounts of the detergent Triton X-100 decrease the hydrolysis rate. The same detergent effect occurs if PE hydrolysis is activated by sphingomyelin (SPH). However, if the enzyme is activated by the monomeric dibutyryl-PC, this detergent effect can be overcome at high enough dibutyryl-PC concentrations. The hydrolysis of the monomeric dibutyryl-PC can also be stimulated by SPH in mixed micelles. This reaction shows no effect of detergent. Several models are considered to explain these observations, and it is suggested that the enzyme has two types of functional sites: an activator site and a catalytic site.

There is considerable current interest in understanding the role of the lipid/water interface in the mechanism of action of lipolytic enzymes such as the lipases and phospholipases (Dennis et al., 1981; Verger, 1980). Important information has been obtained from kinetic studies on monomeric phospholipids such as dibutyryl-PC,[1] which are very poor substrates for phospholipase A_2 (de Haas et al., 1971; Wells, 1974). Interestingly, this phospholipid appears to be able to activate

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[1] Abbreviations: PC, phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphorylcholine); lyso-PC, lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphorylcholine); PE, phosphatidylethanolamine (1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine); lyso-PE, lysophosphatidylethanolamine (1-acyl-*sn*-glycero-3-phosphorylethanolamine); SPH, sphingomyelin; cmc, critical micelle concentration; Tris, tris(hydroxymethyl)aminomethane.

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the phospholipase A_2 from cobra venom toward PE hydrolysis (Roberts et al., 1979). This report focuses on the mechanism of this activation and its implications for interfacial catalysis by phospholipase A_2.

Phospholipase A_2 from cobra venom (EC 3.1.1.4) hydrolyzes PC at a 10–20-fold higher rate than PE in micelles containing Triton X-100[2] and a single[3] type of phospholipid (Roberts et al., 1978b). In binary phospholipid mixtures, PC activates the enzyme toward PE so that the latter becomes the preferred substrate (Adamich et al., 1979; Roberts et al., 1979). In these binary mixtures, PE is hydrolyzed at approximately the same rate as PC in single-phospholipid mixed micelles, and PC is hydrolyzed more slowly than in single-phospholipid mixed micelles. In preliminary experiments (Roberts et al., 1979), an increased rate of phospholipase A_2 catalyzed hydrolysis of PE was also observed in the presence of dibutyryl-PC. Using ^{31}P NMR, we have now been able to follow the time course of phospholipase A_2 catalyzed hydrolysis of mixtures of PE and dibutyryl-PC for each phospholipid simultaneously. We (Plückthun & Dennis, 1981) have also shown that the dibutyryl-PC is monomeric in the presence of a large excess of Triton X-100 even in mixtures containing PE. This allows us to now characterize the activation process and its implication for the mechanism of action of phospholipase A_2.

Experimental Procedures

Materials. Lyophilized cobra venom, *Naja naja naja* (Pakistan) lot. no. NNP8STLZ, was obtained from the Miami Serpentarium. The phospholipase A_2 was purified as described elsewhere (Deems & Dennis, 1975, 1981). Protein was determined by the Lowry procedure using the appropriate correction factor (Deems & Dennis, 1981). PC was obtained from egg yolks by the method of Singleton et al. (1965). PE, prepared by transesterification of egg PC, and bovine brain sphingomyelin (SPH) were obtained from Avanti Biochemicals. Dibutyryl-PC was obtained from Calbiochem and purified by silicic acid chromatography (Unisil, Clarkson Chemical Co.) or prepared by the method of Patel et al. (1979). Lyso-PE and lysobutyryl-PC for NMR standards were prepared from the above phospholipids by the action of phospholipase A_2 as described elsewhere (Plückthun & Dennis, 1981).

1-Acyl-2-propionyl-*sn*-glycero-3-phosphorylcholine was prepared from lyso-PC, obtained from egg PC as described previously (Plückthun & Dennis, 1981), and propionic anhydride following the method of Khorana and co-workers (Gupta et al., 1977). After purification on silicic acid chromatography with a $CHCl_3$–CH_3OH gradient, the product gave a single spot on thin-layer chromatography ($CHCl_3$–CH_3OH–H_2O, 65:25:4 v/v/v). The product was characterized by ^1H NMR (10 mM in $CDCl_3$, chemical shifts relative to tetramethylsilane): ω-CH_3 of long fatty acyl chain, 0.89 ppm; ω-CH_3 of propionyl chain, 1.10 ppm; $-CH_2-$ of long fatty acyl chain, 1.25 ppm; β-CH_2 of long fatty acyl chain, 1.60 ppm; α-CH_2 (*sn*-1), 2.28 ppm (t); α-CH_2 (*sn*-2), 2.33 ppm (q); $-N(CH_3)_3$, 3.36 ppm; $-CH_2N-$, 3.80 ppm; $-CH_2CH_2N-$, 4.30 ppm; $-CH_2OCO-$, 4.10 ppm; $-CHOCO-$, 5.21 ppm; $-CH_2OP-$, 3.95 ppm.

[2] Triton X-100 is a polydisperse preparation of *p*-(1,1,3,3-tetramethylbutyl)phenoxypoly(oxyethylene) glycols, containing an average of 9.5 oxyethylene units per molecule.

[3] When one species (i.e., head group) of phospholipid is present in a detergent matrix, the mixed micelles are referred to as single-phospholipid mixed micelles. When two phospholipid species differing in head group are present in the mixed micelle system, it is referred to as a binary mixture.

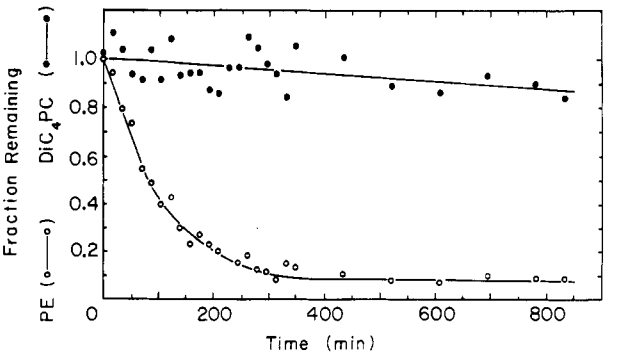

FIGURE 1: Time course for a typical hydrolysis of a binary mixture of 6.5 mM PE and 6.1 mM PC (DiC_4PC) in 96 mM Triton X-100 as followed by ^{31}P NMR spectroscopy. The fraction of remaining substrate is calculated for PE from the integrated intensities of the PE signal and the lyso-PE signal as described under Experimental Procedures. For dibutyryl-PC, no lyso product was observed, and therefore the intensity of the dibutyryl-PC signal relative to that at zero time is plotted.

Dodecylphosphorylcholine was the generous gift of Dr. H. S. Hendrickson, Saint Olaf College, Northfield, MN. Triton X-100 was obtained from Rohm and Haas. Mixed micelles were prepared by the addition of solutions of detergent to dry phospholipids; mixing was achieved by vortexing and then allowing the foam to subside before the sample was used.

^{31}P NMR. ^{31}P NMR spectra were obtained at 40.3 MHz and 40 ± 0.5 °C with a JEOL PFT-100 system equipped with a modified Nicolet 1080 computer and disk. The JNM-SD-HC hetero spin decoupler unit was used with a Schomandl ND-100M frequency generator. The phospholipid sample contained 30% D_2O for an internal lock. The assay contained phospholipid and Triton X-100 as indicated, 50 mM Tris-HCl, 10 mM $CaCl_2$, and 0.11 μg of phospholipase A_2. The measured pH was 8.0. The concentration of phospholipase A_2 employed was such that the time to acquire a spectrum was small compared to the entire enzymatic time course. The spin–lattice relaxation times are 2.5 s for PE and 12.4 s for dibutyryl-PC under the experimental conditions employed (Plückthun & Dennis, 1981). For optimization of the signal-to-noise ratios within a given time period, a delay time equal to the longer relaxation time was used. Integration via an electronic planimeter (Talos Systems) was used to quantitate hydrolysis. When sufficient lyso product was produced, the fractional intensity (integral of lyso product divided by the sum of the integrals of lyso product and unhydrolyzed phospholipid) was used as a measure of the extent of reaction. If no lyso product could be detected, the integral of the unhydrolyzed phospholipid was monitored. The latter values were compared with results obtained by computing the fractional intensity as unhydrolyzed phospholipid intensity divided by the sum of the integrals of all phospholipid peaks in order to test for machine instabilities. Both methods gave identical results.

pH Stat. Enzymatic hydrolysis was followed by the pH-stat technique (Dennis, 1973a; Deems & Dennis, 1981) where indicated. Standard assay conditions were 5 mM PC or 5 mM PE, dibutyryl-PC, SPH, or dodecylphosphorylcholine where indicated, 20 mM Triton X-100, 10 mM $CaCl_2$, pH 8.0, and 40 °C. Except where indicated, 0.11 μg of phospholipase A_2 was used. All experiments were conducted in at least duplicate, and the average values are reported.

Results

Activation by Dibutyryl-PC. In Figure 1, the time course for the enzymatic hydrolysis of an equimolar mixture of PE

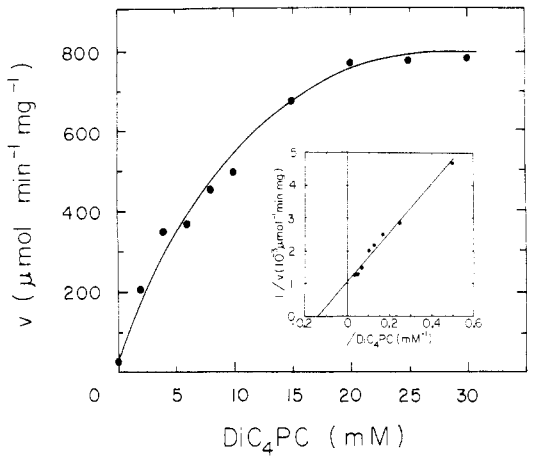

FIGURE 2: Specific activity of phospholipase A_2 catalyzed hydrolysis of PE (5 mM) in mixed micelles with Triton X-100 (20 mM) as a function of the concentration of added dibutyryl-PC (DiC_4PC) is shown. The pH-stat assay was employed. The insert is the double-reciprocal plot of the results.

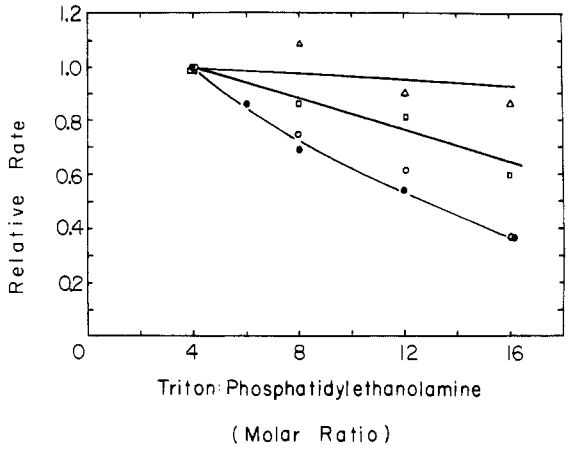

FIGURE 3: Rate of phospholipase A_2 catalyzed hydrolysis of PE as a function of the detergent–PE molar ratio. The pH-stat assay was employed with 5 mM PE as substrate and Triton X-100 at the mole ratio indicated. The Triton X-100 effect on the hydrolysis of PE (●), PE activated by 0.5 mM SPH (○), PE activated by 30 mM dibutyryl-PC (□), and PE activated by 60 mM dibutyryl-PC (△) is shown. The hydrolysis rate at a Triton to PE ratio of 4:1 was set at 1.0 in each case. For the nonactivated reaction, 1.3 μg of phospholipase A_2 was employed in each assay, whereas for the SPH and dibutyryl-PC activated reactions, 0.26 and 0.13 μg, respectively, of phospholipase A_2 were employed.

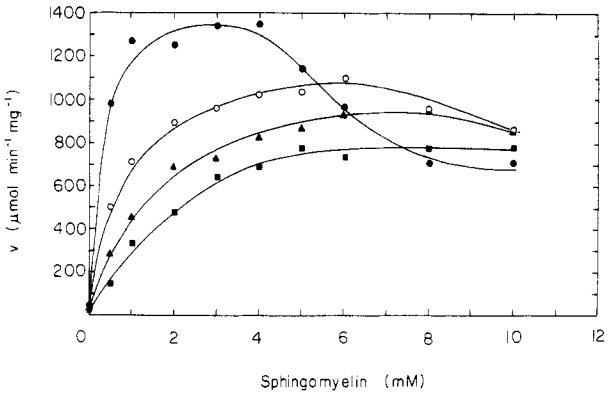

FIGURE 4: Specific activity of phospholipase A_2 catalyzed hydrolysis of PE (5 mM) as a function of SPH concentration in the presence of Triton X-100: (●) 20 mM; (○) 40 mM; (▲) 60 mM; (■) 80 mM. The pH-stat assay was employed.

and dibutyryl-PC is shown as determined by ^{31}P NMR. While the PE is rapidly hydrolyzed, the rate of hydrolysis of dibutyryl-PC is very low. From the line shown in Figure 1, it can be seen that less than 10% was hydrolyzed in 800 min. This can be accounted for mainly by nonenzymatic base hydrolysis using the value of base-catalyzed dibutyryl-PC hydrolysis of about 1.5 M^{-1} s^{-1} calculated from the data of Wells (1974). Thus, it appears that only a negligible amount of enzymatic hydrolysis of this compound occurs during activated PE hydrolysis. In a similar experiment, the enzymatic hydrolysis of an equimolar mixture of dibutyryl-PC and egg PC was followed by ^{31}P NMR, and as expected, only the egg PC was hydrolyzed (data not shown). On the other hand, 1-acyl-2-propionyl-PC (derived from egg PC, the *sn*-1 position contains predominantly palmitic acid) is hydrolyzed at about 35% of the rate of egg PC, whereas dibutyryl-PC is hydrolyzed at less than 1% of this rate. This confirms that the low rate against monomeric phospholipids is not due primarily to a specificity of the enzyme for the cleavage of a long chain fatty acid. The very high rate of enzymatic hydrolysis of this mixed-acyl phospholipid is presumably mainly due to its quantitative incorporation into the Triton micelles.

Figure 2 shows the rate of enzymatic hydrolysis of PE as a function of the concentration of dibutyryl-PC. Apparently, sufficiently large amounts of dibutyryl-PC can bring the enzyme to a fully activated state where the rate reaches a plateau region and the enzyme exhibits saturation behavior, with an apparent K_d of 7 mM. The maximum activity is about equal to the activity the enzyme has with egg PC alone as a substrate. Control experiments with identical mixtures assayed by ^{31}P NMR or in the absence of PE by pH stat show that no significant amount of dibutyryl-PC is hydrolyzed on this time scale even at the highest concentrations employed in Figure 2.

Effect of Triton X-100. The effect of the addition of Triton X-100 on the rate of hydrolysis of PE both in the absence and in the presence of several activators is shown in Figure 3. The enzymatic hydrolysis of PE and of PE in the presence of 10 mol % sphingomyelin shows a similar decrease as more Triton X-100 is added. Similar behavior had previously been found for dipalmitoyl-PC as a substrate in single-phospholipid mixed micelles (Dennis, 1973a). However, when the enzymatic hydrolysis of PE was activated by the monomeric activator dibutyryl-PC (30 mM), the dependence on Triton concentration was less, and it was nearly completely eliminated when 60 mM dibutyryl-PC was employed. Additionally, there was only a very small increase in rate when the substrate was increased from 2 mM PE to 10 mM PE, at a constant PE–Triton ratio of 8:1, both in the presence and in the absence of dibutyryl-PC, suggesting that the phospholipase is close to saturation with substrate in all experiments in Figure 3.

For determination of the dependence of the rate decrease on SPH concentration (both bulk and surface), a more extensive study was carried out. In Figure 4, activation profiles with SPH are shown for four different detergent concentrations. It appears that the amount of the rate decrease with increasing Triton becomes smaller with higher activator concentrations (going vertically between the lines). It must be noted, however, that at these low ratios of detergent to total phospholipid, the system is below the lamellar to micelle transition ratio (e.g., at 10 mM sphingomyelin, the ratios in Figure 4 are 1.33:1, 2.66:1, 4:1, and 5.33:1), so that the first two are expected to give anomalous results and lower rates (Dennis, 1973a,b; Robson & Dennis, 1979) because of incomplete solubilization of the phospholipid. Therefore, additional experiments were conducted with much higher detergent concentrations to overcome this problem.

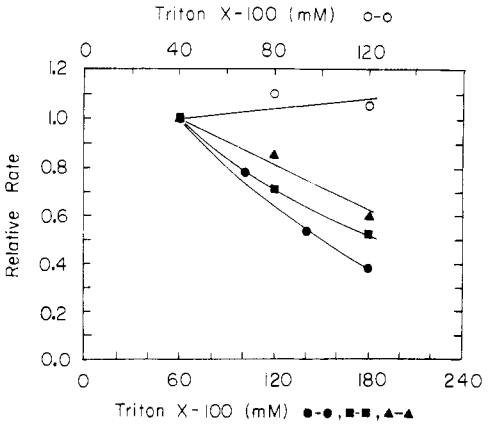

FIGURE 5: Effect of surface concentration of substrate PE and activator SPH on the hydrolysis rate of PE as a function of Triton X-100 concentration under four conditions: (i) (●) The bulk concentration of both PE (5 mM) and SPH (10 mM) are kept constant, and the amount of detergent is increased as indicated; hence, both activator and substrate are diluted in the surface. (ii) (■) The bulk concentration of PE is kept constant (10 mM), whereas the bulk concentration of SPH is increased with increasing Triton X-100 to keep the ratio of Triton–SPH constant at 12:1; hence, to a first approximation, only the substrate is diluted in the surface. (iii) (▲) The bulk concentration of SPH is kept constant (10 mM), whereas the bulk concentration of PE is increased with increasing Triton to keep the ratio of Triton–PE constant at 12:1; hence, to a first approximation only the activator is diluted in the surface. (iv) (○) The ratio of PE–SPH–Triton is kept constant at 1:1:8, but all bulk concentrations are increased; hence, the surface concentrations of both the substrate and the activator remain constant. Note that in all four experiments, the initial detergent–total lipid ratio was set at 4:1 and the concentration of detergent was varied over a 3-fold range. The pH-stat assay was employed.

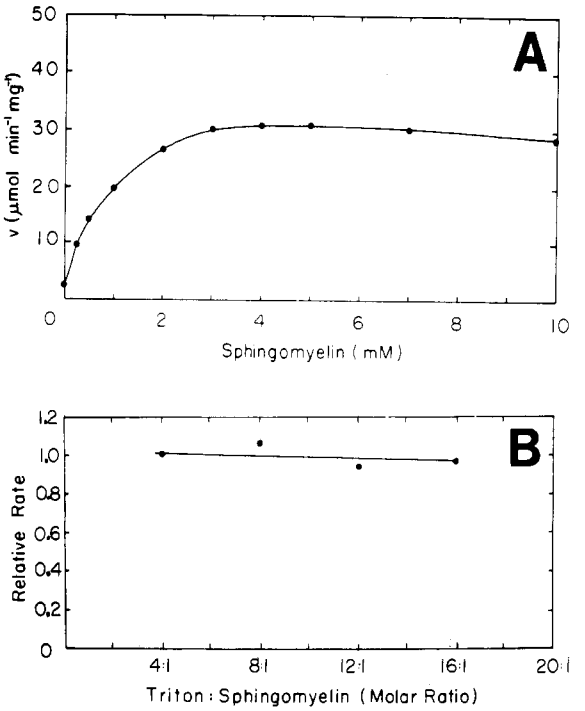

FIGURE 6: (A) Rate of phospholipase A_2 catalyzed hydrolysis of dibutyryl-PC (5 mM) in the presence of Triton X-100 (20 mM) as a function of SPH concentration. The pH-stat assay was employed. (B) Relative rate of phospholipase A_2 catalyzed hydrolysis of dibutyryl-PC (5 mM) in the presence of SPH (5 mM) as a function of the Triton X-100–SPH molar ratio. The pH-stat assay was employed.

If the bulk concentrations of both PE (substrate) and SPH (activator) are kept constant and the concentration of Triton is increased, a rate decrease identical with that in Figure 3 is observed as shown in Figure 5 (experiment i). It should be noted that the detergent effect appears similar, whether the dilution is carried out from 20 to 80 mM Triton (Figure 3) or from 60 to 180 mM (Figure 5). Although a change in micelle structure might occur over the range of Triton concentrations in each study, it would not be expected to be large (Robson & Dennis, 1978; Roberts et al., 1979), and therefore a dilution effect of the phospholipids in the surface is the simplest consistent explanation for the observed phenomena.

For examination of the kinetics when only one of the two phospholipids is surface diluted, the detergent and one phospholipid were added at a fixed ratio while keeping the bulk concentration of the other phospholipid constant as shown in Figure 5 (experiments ii and iii). Although these types of curves can be obtained in principle from the data in Figure 4 by going diagonally between the drawn lines, it must be noted that the dramatic dependence of the rate on bulk concentration of activator at low activator concentrations and the low detergent–phospholipid ratio at high activator concentrations prevent the data in Figure 4 from being used for this purpose. Therefore these investigations were carried out at both high activator concentrations (so that a plateau region of rate vs. bulk concentration is reached) *and* at high detergent concentrations. Since at even higher detergent concentrations, problems with the viscosity of the sample would arise, the range of useful concentrations of all components for kinetic experiments is limited.

The data in Figure 5 show that if only the substrate PE is surface diluted, but the ratio of Triton to SPH is kept constant, the rate still decreases with increasing Triton (experiment ii). The same effect is observed if only the activator is surface diluted, but the ratio of Triton to PE is kept constant (experiment iii). As expected, if the surface concentration of both PE and SPH is kept constant, no decrease in the rate is observed with increasing Triton (experiment iv).

Activation of Dibutyryl-PC Hydrolysis by Sphingomyelin. It was found that the very slow hydrolysis of dibutyryl-PC can be significantly activated by the presence of SPH/Triton mixed micelles as shown in Figure 6A. ^{31}P NMR shows that in these mixtures only dibutyryl-PC is hydrolyzed, yet the amount of dibutyryl-PC that is incorporated in the mixed micelles must be very small. The saturation behavior of the activation is a further indication that it is most probably due to a direct effect of SPH on the enzyme. In this system, with 5 mM dibutyryl-PC and 5 mM SPH, no change in rate was observed with increasing Triton concentrations (Figure 6B). This experiment suggests that there is no effect of detergent on the reaction when the substrate is monomeric and the activator is micellar. It is unlikely that the addition of SPH increases the very small amount of dibutyryl-PC in the micelle to a significant level and that it really is the micelle-bound dibutyryl-PC that is the substrate since the ^{31}P NMR chemical shift of this compound under these conditions was that of the monomer within experimental error (Plückthun & Dennis, 1981). However, if such an increase in solubilization did occur, the small amount of dibutyryl-PC should be proportional to the Triton concentration, and therefore, to a crude approximation, its surface concentration would stay constant. The surface concentration of SPH would, however, be diluted, and consequently a surface dilution effect analogous to Figure 5 (experiment iii) would be expected. This is not observed in Figure 6B. It can be seen from the data in Figures 3–6 that in order for the detergent to show surface dilution kinetics, *both* activator and substrate phospholipid must be micellar. If either the activator is monomeric (Figure 3) or the substrate is monomeric (Figure 6B), there is no surface dilution effect.

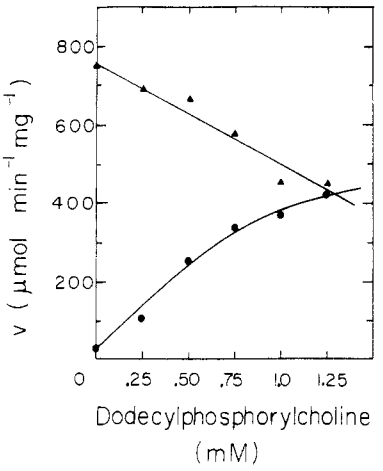

FIGURE 7: Rate of phospholipase A_2 hydrolysis as determined by pH stat is shown for mixed micelles composed of 20 mM Triton X-100 and either 5 mM PC (▲) or 5 mM PE (●) as a function of dodecylphosphorylcholine concentration.

Dodecylphosphorylcholine Effects. Various *n*-alkylphosphorylcholines have been used to protect the active site of phospholipase A_2 against chemical modification (van Dam-Mieras et al., 1975), as substrate analogues to measure binding to the enzyme (van Dam-Mieras et al., 1975; Verheij et al., 1980), as a detergent (Slotboom et al., 1976), or to determine the physical properties of micelle–enzyme complexes (Soares de Araujo et al., 1979). We tested the effect of dodecylphosphorylcholine on the rate of enzymatic hydrolysis of PC and PE. PC hydrolysis shows the expected decrease in rate with increasing amounts of dodecylphosphorylcholine (probably because of competitive inhibition). On the other hand, the hydrolysis of PE is activated by this compound (Figure 7). This observation shows that the minimum structural requirement for a molecule to act as an activator is the phosphorylcholine moiety and at least one hydrophobic chain, either a fatty acid or even an *n*-alkyl residue. Glycerophosphorylcholine alone has no effect on the rate. All attempts to activate the enzyme by mixtures of glycerophosphorylcholine and butyric acid, oleic acid, or oleoyl alcohol, with concentrations of glycerophosphorylcholine between 0.6 and 30 mM, butyric acid between 0.6 and 30 mM, oleic acid between 0.5 and 5 mM, or oleoyl alcohol between 0.6 and 2 mM, did not lead to a rate increase and in some cases led to a small rate decrease equivalent to that caused by the fatty acid or alcohol alone.

Discussion

Dibutyryl-PC as Monomeric Activator or Substrate. Dibutyryl-PC has now been shown by three independent methods to not be solubilized by Triton X-100 micelles (Plückthun & Dennis, 1981): (i) In a gel chromatography column that was equilibrated with dibutyryl-PC, and through which Triton X-100 micelles were passed, no binding of the phospholipid to the detergent micelles could be detected (Roberts et al., 1979), whereas with dihexanoyl-PC binding did occur. (ii) The ^1H NMR chemical shift difference between the α-methylene protons of the *sn*-1 and *sn*-2 fatty acid chains of dibutyryl-PC is 6–7 Hz and does not increase significantly upon the addition of Triton X-100, whereas for dihexanoyl-PC the chemical shift difference increases from 7–8 Hz to 17–18 Hz upon the addition of the same amount of Triton. This increase in chemical shift difference is presumably caused by a greater difference in environments of the *sn*-1 and *sn*-2 α-methylene groups in mixed micelles than in monomeric form (Roberts et al., 1978a; Burns & Roberts, 1980). (iii) ^{31}P NMR studies showed a negligible change in the chemical shift of dibutyryl-PC upon the addition of Triton X-100, even when 5 mM PE was also present, whereas the chemical shift of dihexanoyl-PC moves upfield by about 0.19 ppm in going from a monomeric state to the complete solubilization of all dihexanoyl-PC by Triton X-100. We therefore conclude that dibutyryl-PC is present as a monomer in solution under all experimental conditions employed in the work presented here.

Phospholipase A_2 catalyzed PE hydrolysis can be activated both by compounds that are quantitatively incorporated into Triton X-100 micelles (SPH and dodecylphosphorylcholine) and by monomeric compounds that are incorporated into the micelles to only a negligible extent (dibutyryl-PC). None of these activators is significantly hydrolyzed. Whereas the presence of the micelle-bound SPH did not alter the decrease in hydrolysis rate of PE with increasing concentrations of Triton X-100, the presence of monomeric dibutyryl-PC as either activator or substrate eliminates this detergent dependence. The rate of long chain substrate hydrolysis at full activation is about the same in all cases.

A consistent explanation for these effects is given by the model of "surface dilution" (Dennis, 1973b; Deems et al., 1975; Roberts et al., 1977). According to this model, for full activity, the enzyme must interact with an activator molecule prior to catalysis. This activator molecule can either be surface bound or monomeric. If the activator molecule is surface bound, then the activated enzyme is sequestered to the interface; the binding of the substrate molecule to the enzyme should then depend on the concentration of substrate in the two-dimensional interface, and a surface dilution effect could be responsible for the observed decrease in rate with increasing detergent concentration. If the activator molecule is monomeric, then the activated enzyme is not sequestered to the interface; thus the activity should not depend on the concentration of substrate in the interface, and surface dilution should not be observed. At 30 mM dibutyryl-PC, this compound acts as an activator, and the surface effect is diminished; at 60 mM dibutyryl-PC, the enzyme is completely activated and surface dilution eliminated. Even under these conditions, the dibutyryl-PC shows only negligible hydrolysis. In this case, the surface dependence is overcome, because every enzyme molecule binding to substrate in the mixed micelles is already activated. The lack of complete elimination of surface dilution at 30 mM dibutyryl-PC (which is about 4 times the apparent K_m) may be due to the presence of some PE in the activator site. With PC alone, this single type of phospholipid might serve as both an activator and a substrate for the enzyme, and full activity is expressed.

The concept of surface dilution is also consistent with the results on SPH/dibutyryl-PC mixtures. When the enzyme is activated by SPH, the reaction should only depend on the bulk concentration of lipid and enzyme since the activation on the surface occurs by simple collision, and since the substrate is in solution, then a diffusion within the surface is not necessary. Therefore this case is the mirror image of the reaction with PE and dibutyryl-PC.

The fact that surface dilution occurs only when both activator and substrate are surface bound is consistent with a model in which at least two phospholipid molecules are needed for catalysis. It also appears that both PC and PE can bind to the activation site since they both show surface dilution. The self-activation of the latter must, however, be poor or slow.

A particularly interesting case is obtained if only one of the two required phospholipids (either the activator or the substrate) is surface diluted (Figure 5, experiments ii and iii). In

both cases, the rate decrease is somewhat less than if both phospholipids are surface diluted (Figure 5, experiment i). Several possible contributory factors have to be considered: (a) If the bulk concentration of one phospholipid is increased, the enzyme gets closer to saturation, resulting in a slight rate increase (demonstrated in Figure 5, experiment iv). (b) If the enzyme binds both phospholipids in a random order, part of the time it will first bind the phospholipid whose bulk concentration is kept constant and therefore whose surface concentration is diluted. Thus, the phospholipid it binds in the second step would not be surface diluted, and no decrease in rate would be expected. The overall decrease in rate observed with added detergent would then reflect only that fraction of enzyme which first binds to the lipid that is not surface diluted (bulk concentration increased). Thus, the apparent decrease in rate with added detergent would be less than that in Figure 5 (experiment i). (c) Since the effective surface areas of both phospholipids and Triton are unknown, the effective surface concentrations of each component may be somewhat different in each experiment. This too would lead to differences in experiments i–iii.

The concept of surface dilution has been applied elsewhere (Wells, 1978) and has also been questioned (Sundler et al., 1978; Slotboom et al., 1976). While the kinetics of surface dilution may be indistinguishable from those of competitive inhibition (Dennis, 1973b), binding of the enzyme to Triton micelles devoid of phospholipid could not be detected (Roberts et al., 1977). Furthermore, if Triton were a competitive inhibitor of the enzyme, it is not clear how high concentrations of another phospholipid (dibutyryl-PC), which is itself not a substrate under these conditions, could reverse this competitive inhibition, without itself inhibiting the reaction.

If Triton competes for the "activator site", on the other hand, it would be difficult to explain why the enzyme, which is activated to about the same steady-state rate toward PE by SPH or dibutyryl-PC, is inhibited by Triton only in the case of SPH activation. Such a mechanism would only be consistent with the data reported here if SPH and dibutyryl-PC do not bind to the same activator site or if sphingomyelin acts only by modifying the surface rather than interacting with the enzyme. However, having each activator act in a different manner seems unlikely. It should also be noted that the effect of Triton on phospholipase C hydrolysis of PC (Eaton, 1975) or of still another phospholipase C that is specific for phosphatidylinositol (Sundler et al., 1978) falls practically on the same curve as the effect on phospholipase A_2 (Dennis, 1973a) with PC, PE, or PE in the presence of SPH. On the other hand, not all phospholipase A_2's from other sources showed the same behavior (A. Plückthun and E. A. Dennis, unpublished experiments), and we cannot exclude a very weak affinity of the enzyme for Triton.

Another explanation for the Triton effect would be that increasing amounts of Triton X-100 change the "quality of the interface" (Slotboom et al., 1976) and a two-step mechanism would not necessarily be required. The monomeric activator could then facilitate "penetration" of the interface by the enzyme (either by accelerating a slow surface binding step or by shifting a fast equilibrium), and no surface effect would be apparent. The interaction of the enzyme with the completely micelle-bound activator, on the other hand, would itself depend on the quality of the interface and therefore show a Triton effect. Since at the high dibutyryl-PC concentrations employed in some experiments, the percentage of micelle-bound dibutyryl-PC remains negligible but the total amount of dibutyryl-PC bound to the micelle might not, effects by micelle-bound dibutyryl-PC to change the quality of the interface might also fortuitously reverse the effect of Triton X-100. These possibilities are very difficult to rule out unequivocally at present.

Direct Enzyme Activation. While an alteration in micelle structure with changing penetrability for the enzyme or a direct lipid–lipid interaction appears at first to be a simple explanation for the activation of the hydrolysis of PE, evidence against these possibilities arises from the following experimental results: (i) At a molar ratio of PE–dibutyryl-PC of 1:1, the apparent hydrolysis rate is increased by about a factor of 10 compared to the hydrolysis of PE alone. The upper limit of dibutyryl-PC that could be bound to detergent micelles under these conditions is about 5% (Plückthun & Dennis, 1981) and probably only 2%. At the same concentration ratio (1:1), egg PC activates the enzymatic hydrolysis of 5 mM PE in mixed micelles by a factor of about 10. Egg PC has, however, been shown to be incorporated practically quantitatively into Triton X-100 micelles or PE/Triton X-100 micelles (Roberts et al., 1979). Postulating a lipid–enzyme interaction in which the lipid activator is required to be part of the interface to cause the activation phenomenon would require dibutyryl-PC to be a 20-fold better activator than egg PC. Although this does not rule out such a mechanism, it makes it appear very unlikely. (ii) Dodecylphosphorylcholine activates the enzymatic PE hydrolysis by a factor of about 4 at a concentration of only 5 mol % relative to PE and 1 mol % relative to Triton X-100, so that an effect of the activator on the substrate or the micelle structure is unlikely. Thus, all experimental results are consistent with a direct enzyme–activator interaction.

A model has been proposed for the mechanism of action of cobra venom phospholipase A_2 at lipid/water interfaces in which the enzyme undergoes a conformational change prior to catalysis, and this could involve a dimerization and/or an activator lipid binding step (Dennis et al., 1981). From the data presented here, PC would be a better substrate than PE for phospholipase A_2 according to this model not because it binds more tightly to the enzyme (the apparent dissociation constants of all phospholipids are about 1 mM) (Adamich et al., 1979) but rather because it is itself a good activator. The binding of more than one phospholipid molecule by the enzyme also appears very attractive in view of the fact that aggregated phospholipids are so much better substrates than monomeric ones.

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Lack of Transbilayer Coupling in Phase Transitions of Phosphatidylcholine Vesicles†

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ABSTRACT: Praseodymium and ytterbium chlorides were used as nuclear magnetic resonance shift reagents to resolve the inner and outer choline methyl resonances of single-walled dimyristoylphosphatidylcholine bilayer vesicles. The gel to liquid-crystalline phase transition of these vesicles was monitored by observing the proton and carbon-13 nuclear magnetic resonance line widths of the choline methyl group nuclei. In the absence of ions the transition occurred at 21.5 °C in both halves of the bilayer. With Pr^{3+} or Yb^{3+} added to the outside of the vesicles, the phase transition temperature of the outer half of the bilayer was raised several degrees, while the transition temperature of the inner half was unchanged. In vesicles containing 20 mol % cholesterol the phase transition of the outer monolayer was considerably broadened, while the inner half still melted sharply at 21.6 °C. By use of dipalmitoylphosphatidylcholine vesicles with UO_2^{2+} added to the outside, phase transitions at 41.5 and 44 °C were detected by electron spin resonance with the spin-label 2,2,6,6-tetramethylpiperidinyl-1-oxy. These results imply that the two halves of the bilayer in phospholipid vesicles are so weakly coupled that they can undergo the gel to liquid-crystalline phase transition independently.

A natural consequence of the bilayer model of biological membranes is the question of whether or not there is distributional symmetry of the lipid components across the midplane. It is widely believed that the glycolipids are located primarily in the outer monolayer (Hakomori, 1981), but the situation for both cholesterol and the phospholipids is still a matter of some study. Erythrocytes have been clearly shown to possess an asymmetric phospholipid distribution, but other membranes have not presented such an easily unraveled pattern (Op den Kamp, 1979). It appears that cholesterol exchanges between the bilayers with a time constant of several hours at 37 °C (Backer & Davidowicz, 1979; Bloj & Zilversmit, 1977); the measured exchange rates were found to depend strongly on the amount of acyl chain unsaturation, with increasing unsaturation favoring faster exchange.

Proteins and ions are not symmetrically disposed across the bilayer. Given this and the probable lipid asymmetries, it becomes relevant to attempt to ascertain the functional consequences of these structural features of the bilayer. Since the fluidity of the bilayer depends, in part, on the phospholipid head group and acyl chain composition, transbilayer phospholipid asymmetry may result in fluidity asymmetry as well. For asymmetric fluidity to exist, there must be weak coupling between the monolayers. This paper reports that phase transitions, and hence fluidity changes, are poorly transmitted across the bilayer for saturated synthetic phosphatidylcholines.

Materials and Methods

DMPC[1] was obtained from Sigma, while cholesterol, DPPC, and DMPE were purchased from Calbiochem. Deuterium oxide (99.7%) was supplied by Aldrich (Milwaukee, WI). Anhydrous praseodymium and ytterbium chlorides were obtained from PCR Inc. (Gainesville, FL) and handled under a dry argon atmosphere. Merck (St. Louis, MO) supplied $^{13}CH_3I$ enriched to 90% in ^{13}C. 2,2,6,6-Tetramethyl-

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine.

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