Sensitivity of Phospholipase C (*Bacillus cereus*) Activity to Lipid Packing in Sonicated Lipid Mixtures

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Received July 7, 1992; Revised Manuscript Received May 18, 1993

ABSTRACT: We have recently demonstrated that phospholipase C (PLC) activity on membranes decreases in the presence of membrane-active peptides such as alamethicin, gramicidin S, and melittin [Rao, N. M. (1992) *Biochem. Biophys. Res. Commun. 182*, 682–688]. Since these peptides affect lipid packing in the membrane and induce nonbilayer phases depending on the lipid composition,we tested for the sensitivity of PLC activity to lipid packing. We monitored PLC activities on four lipid systems which demonstrate a transition from the bilayer to the nonbilayer phase as a function of one of the components. The four model systems are (1) dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE); (2) DOPE, DOPC, and cholesterol; (3) DOPE and lysophosphatidylcholine; and (4) DOPC and gramicidin D. On all four lipid systems, the PLC activity was high for lipid in the bilayer phase and decreased as the phase changed to the nonbilayer phase. The phase changes were also monitored in PLC assay conditions on the four model systems by ^{31}P NMR to confirm the observations made with PLC. These results suggest that the lipid in bilayer and nonbilayer phases was differentially susceptible to PLC; hence, PLC activity may be used to monitor isothermal phase transitions at physiological conditions.

The membrane/water interface is the site for several biological processes, and it has been known for some time that various physical and chemical parameters of the membrane, viz., intrinsic curvature, surface charge, lateral pressure, etc., influence these processes (Gruner, 1985, 1989; Gruner et. al., 1985; Seddon, 1990). Thus, elucidating the structure of the interface is crucial to the understanding of these processes. The intrinsic curvature of the interface is an important membrane parameter that is homeostatically controlled in organisms (Gruner, 1985, 1989; Hui & Sen, 1989). Interfacial curvature is dependent on the lipid composition of the membrane, since several biological lipids, viz., phosphatidylethanolamine (PE),[1] diacylglycerol, MGDG, etc., in isolation form structures of high interfacial curvature, i.e., H_{II} phases (Cullis et al., 1983; Gruner et al., 1985; Hui & Sen, 1989; Seddon, 1990). Agents that influence this parameter also have an ability to enhance processes such as fusion (Ellens et al., 1989; Seddon, 1990; Siegel et al., 1989; Tournois et al., 1990), trans/inter-bilayer movement of the lipids (Tournois et al., 1990), and exo- and endocytosis (Burger & Verkleij, 1990).

Phospholipases are widely distributed lipid-hydrolyzing enzymes (Waite, 1985). Their role in cell biological processes such as signal transduction is well established (Nishizuka, 1984). Recently, we have demonstrated that the activities of phospholipases C and D on membranes would decrease in the presence of membrane-active peptides whereas the activity of PLA_2 increases (Rao, 1992; Rao & Nagaraj, 1993). These peptides have an ability to induce H_{II} phases in the membrane depending on the lipid composition. The observed activity profiles with the phospholipases were suggested to be due to altered lipid packing in the membrane in the presence of the peptides. Several lines of evidence suggest that lipid packing in the liquid-crystalline phase (L_α), with zero curvature, and in the inverted hexagonal phase (H_{II}), with high curvature, is different [see Seddon, (1990)]. Interfacially active enzymes offer a sensitive tool to study the organization of the interface (Gennis, 1989). Phospholipases demonstrate interfacial activation, i.e., show high activity to lipid in a lipid/water interface compared to lipid monomers (Jain & Berg, 1989; Waite, 1985). To investigate whether the activity of phospholipase C is sensitive to the lipid packing differences that occur in bilayer and nonbilayer phases, we have monitored the activity of phospholipase C from *Bacillus cereus* on lipid mixtures, which demonstrate isothermal L_α to H_{II} transitions. In this paper, we report data that suggest that the activity of PLC is high on lipid in a bilayer phase and comparatively less on lipid in a nonbilayer phase.

MATERIALS AND METHODS

Materials

DOPC, DOPE, total PE from *Escherichia coli*, brain PE, and egg PC were purchased from Avanti Polar Ltd. Gramicidin D, Tris, ascorbic acid, lysophosphatidylcholine (stearoyl), and phospholipase C from *Bacillus cereus* and *Clostridium perfringens* were purchased from Sigma. All other reagents were of analytical grade. L-3-Phosphatidyl[2-^{14}C]ethanolamine, 1,2-dioleoyl (49 mCi/mmol), and L-3-phosphatidyl[*N*-*methyl*-^{3}H]choline, dipalmitoyl (55 Ci/mmol), were purchased from Amersham, England. The purity of the lipids was verified by TLC (Bergelson, 1980).

Methods

Small unilamellar vesicles were prepared by drying the lipid film under N_2 or in a Savant Speedvac and swollen in 50 mM Tris-HCl (pH 7.4) unless otherwise stated. The radioactive lipids at <0.01 mol % were added to the cold lipid before

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[1] Abbreviations: CHL, cholesterol; DLPE, dilinoleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; LUV, large unilamellar vesicle(s); MGDG, monoglucosyl diglyceride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SUV, small unilamellar vesicle(s).

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drying. The hydrated lipid was sonicated to clarity with a microtip probe fitted to a Branson sonifier (B30). Care was taken to avoid heating of the sample. Multilamellar vesicles were prepared by the same procedure without the sonication step and then centrifuged to remove small vesicles. With the lipid concentrations and volumes employed, the sonication time was less than 3 min, and the vesicles were prepared fresh each day and used in experiments within 2–3 h. Lipid concentration was calculated from the inorganic phosphate content in the sample. Phosphate was estimated after complete hydrolysis of the sample (McClare, 1971).

Enzyme Assay. Phospholipase C was assayed by estimation of the phosphate in the phosphate ester released from the substrate after phase separation and hydrolysis and/or by estimating the radioactive phosphate ester after phase separation (Klug & Kent, 1981; Rao, 1992). Both methods gave identical activity profiles. Enzyme (0.15 unit/assay; 800 units/mg) was added to lipid vesicles (2 mM) in the presence of 50 mM Tris-HCl (pH 7.4) and 10 mM $CaCl_2$ and incubated for 10 min at 37 °C in a shaking water bath. The reaction was terminated by addition of 20 mM EDTA and solvent and immediately put in ice. Extraction of the product was done as given earlier (Rao, 1992). Assays were performed to ensure linearity with respect to time and enzyme concentration. All the assays were done in duplicate, occasionally in triplicate, and the error associated with each data point was less than 5%. All the experiments were repeated to confirm the observations. Calculated correlation coefficients for peak intensity vs PLC activity were significant at $p < 0.05$, even when the sample sizes were small. To monitor the vesicle size changes during PLC activity, we have followed the 90° light scattering in a Hitachi spectrofluorometer (650–10S) with emission and excitation wavelengths set at 400 nm and the slit width set at 3 nm. The light scattering did not change by more than 10% of the initial value in all the lipid mixtures after incubating the sample for 20 min, suggesting appreciable aggregation or further vesiculation was absent. Radioactivity was counted in a Packard Tricarb 1500 liquid scintillation analyzer with Bray's cocktail. All the activities were expressed as percent hydrolysis, i.e., released phosphate/total phosphate or cpm released/total cpm incorporated. The total value of phosphate and/or radioactivity was obtained for each vesicle preparation to correct for variations in the extent of incorporation, if present.

NMR Spectroscopy. ^{31}P NMR measurements were carried out at 37 °C on a Bruker AM 300 pulsed high-resolution FT NMR machine operating at a resonance frequency of 121.5 MHz. FID's were collected using a 90° pulse width of 13.3 μs with a spectral width of 25 kHz. Approximately 8000–10 000 scans were accumulated with a repetition rate of 1 s and 8K data points. The composite pulse decoupling (CPD) technique using the WALTZ-16 sequence was employed for decoupling protons. Samples, in 10-mm tubes, were spun at 15 Hz. D_2O in a capillary served as an external lock. The FID's were multiplied with an exponential filter of 75-Hz line width and later Fourier-transformed with 8K memory size. In all NMR experiments, the lipid concentration used was 10 mM. The samples after hydration and vortexing were centrifuged at 50 000*g* for 15 min in Beckman table-top ultracentrifuge (TL 100), and the pellet was resuspended in the same buffer. The lipid concentration was estimated in the resuspended samples. All the spectra were referenced with respect to lysophosphatidylcholine, whose chemical shift was set at 0 ppm.

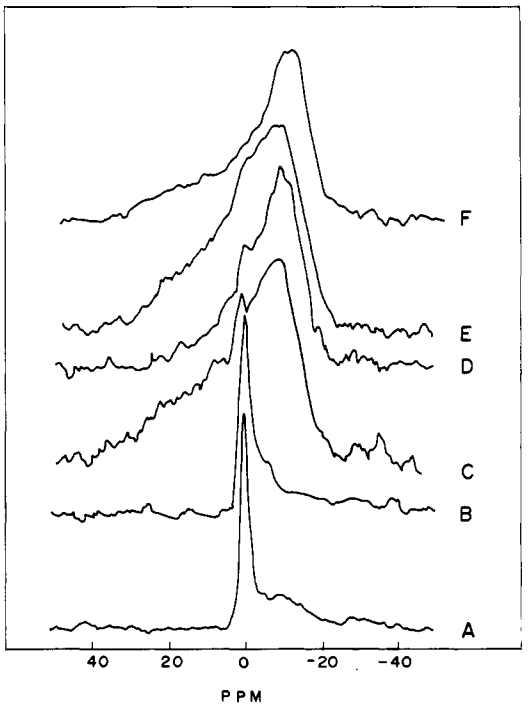

FIGURE 1: ^{31}P NMR spectra of MLV of DOPE and DOPC mixtures. MLV were prepared by codrying DOPE and DOPC in the required mole percent. (A) 0 mol %; (B) 20 mol %; (C) 30 mol %; (D) 40 mol %; (E) 60 mol % ; (F) 100 mol % of DOPC. After hydration, the MLV were spun down at 50000*g* for 15 min at room temperature and resuspended in hydration buffer. The lipid concentration in samples used for NMR was 10 mM.

RESULTS

Three lipid model systems that demonstrate isothermal phase transition as a function of one of the lipid components and a model system consisting of DOPC and gramicidin D were used to demonstrate the sensitivity of PLC activity to lipid present in either the bilayer or the nonbilayer phase. Lipid systems which show phase changes in the presence of divalent cations such as Ca^{2+} and with temperature were avoided since the interpretation would be complicated because these variables can affect the enzyme *per se*. Though MLV were used as substrate in one experiment, they were not the preferred form of the substrate due to their morphological effects; hence, sonicated lipid dispersions were used as the substrate for PLC. The experiments reported here were performed with PLC from *Bacillus cereus*.

Model 1: DOPC/DOPE Mixtures. The phase behavior of PC in PE was documented by various workers using ^{31}P NMR, differential scanning calorimetry, and electron microscopy (Batenburg et al., 1988; Cullis et al., 1978; Hui et al., 1981; Noordam et al., 1980). Since the published phase diagrams of binary mixtures of DOPC and DOPE were obtained in conditions different from PLC assay conditions, we have obtained ^{31}P NMR spectra of MLV containing varying amounts of DOPC and DOPE in PLC assay conditions (Figure 1). Pure DOPC MLV spectra have a typical bilayer profile with a high-field peak and low-field shoulder with σ (chemical shift anisotropy) $\simeq$60 ppm (Figure 1). Pure DOPE MLV spectra have a low-field peak and high-field shoulder with $\sigma \simeq 30$ ppm. As the DOPC mole percentage increases beyond 20% in the vesicles, the low-field peak almost disappears and the high-field peak appears, indicating the presence of the bilayer phase. Between 30 and 80 mol % of DOPC, coexistence of both phases was apparent.

The activity of PLC was monitored on sonicated dispersions containing varying ratios of DOPC and DOPE by estimating

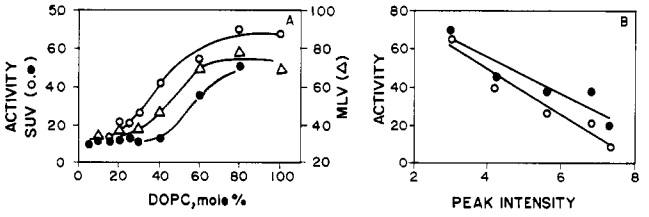

FIGURE 2: Activity of PLC on SUV and MLV of DOPE and DOPC mixtures. (A) Percent hydrolysis of lipid in SUV (○) and MLV (△) by PLC was plotted as a function of DOPC mole percent in the vesicles. Hydrolysis was calculated by estimating the released phosphate. Similarly, DOPE hydrolysis (●) was estimated by counting the released radioactive phosphoethanolamine. (B) The low-field intensity (in arbitrary units) of NMR spectra in Figure 1, which is indicative of the nonbilayer phase, shows an inverse relation with the PLC activity on MLV (●) and SUV (○) obtained from (A).

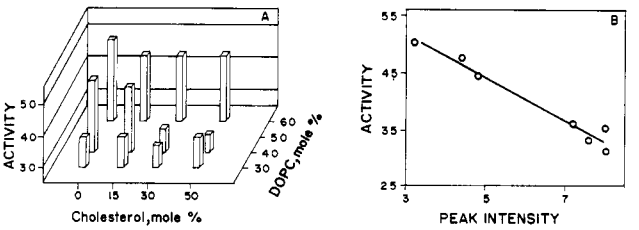

FIGURE 3: PLC activity on ternary mixtures of DOPC/DOPE/cholesterol. (A) effect of cholesterol in mixtures of DOPC and DOPE on PLC activity. Cholesterol mole percent was with respect to the total phospholipid in each sample. (B) The low-field peak intensity of the NMR spectra of MLV preparations [representing each bar in (A)] shows a strong inverse relation ($r = -0.98$) with the PLC activity on these preparations.

the released phosphate and radiometrically using radioactive DOPE at <0.01 mol % simultaneously. In Figure 2A, the activities of PLC were plotted against the mole percent of DOPC in the dispersions. The activity of PLC, as quantitated by phosphate estimation, increases as the DOPC mole percent increases from 20 to 80 mol %. The activity profile obtained using radioactive DOPE also demonstrates an increase in activity from 30 mol % DOPC. If hydrolysis was dependent on the substrate concentration alone, DOPE hydrolysis should decrease with decreasing concentrations of DOPE. However, DOPE hydrolysis increases with a decrease in the DOPE mole percent. This important observation suggests that the activity is primarily dependent on the accessibility of the substrate rather than the substrate concentration or substrate preference alone. To establish that this phenomenon is not exclusive to the strongly curved surfaces encounterd in SUV, we have studied the activity of PLC on MLV with lipid composition as in SUV. The profile obtained was similar to the profile obtained with SUV, suggesting the independence of activity on the number of lamella or surface curvature (Figure 2A). In general, the activity of PLC on unsonicated lipid preparations was always higher than on sonicated preparations. We had performed experiments with MLV and SUV at identical lipid to enzyme ratios to avoid complications in interpretation due to altered ratios. By adding less enzyme to MLV, the activity of PLC has become less, as would be expected; however, this did not influence either the activity profile or the point of transition. At concentrations of DOPC >20 mol %, the activity of PLC, both on SUV and on MLV, increases substantially. Since the transition observed in the activity profiles was not sharp, possibly the bilayer to H_{II} phase transition occurs at DOPC concentrations between 20 and 30 mol %. Though the NMR spectra show a sharp change from the nonbilayer to the bilayer phase at >20 mol % DOPC, further changes in the low-field peak were discernible. We have plotted the low-field intensity, which indicates the extent of nonbilayer phase, of a lipid mixture against the PLC activity on these mixtures (Figure 2B). We have obtained correlation coefficients of –0.88 with MLV and of –0.95 with SUV. These significant correlations indicate that both methods monitor apparently similar underlying packing arrangements during a phase change.

Model 2: DOPE/DOPC/Cholesterol System. On unsaturated PC and PE mixtures, cholesterol exerts a remarkable effect of destabilization and induction of H_{II} phases (Tilcock et al., 1982). Studies with cholesterol and other related sterols indicated that the conformation of the 3-hydroxy is irrelevant for sterol action; neither a coplanar ring system nor even the side chain is important (Tilcock, 1986). Cholesterol does not preferentially associate with either PC or PE in the mixture (Tilcock et al., 1982). The H_{II}-forming ability may partly reside in the distributed presence of cone-shaped cholesterol.

We have monitored PLC activity on ternary mixtures of DOPC, DOPE, and cholesterol (Figure 3A). In a DOPE/DOPC 7:3 system, the activity of PLC was low and independent of the cholesterol mole percent, and similarly in a DOPE/DOPC 4:6 system, the activity of PLC was high and independent of the cholesterol mole percent. On 6:4 DOPE/DOPC vesicles, the activity was high in 0–15 mol % cholesterol and decreased with increasing concentration of cholesterol to a value obtained with 30 mol % DOPC. Since the published NMR data were obtained at 40 °C and in different conditions, we have obtained the NMR spectra of these ternary mixtures in PLC assay conditions (data not presented). At 60 mol % DOPC, the presence of bilayer was unperturbed even at the highest cholesterol mole percent. Spectra at 30 mol % DOPC have an isotropic component, which disappears on addition of cholesterol, and the H_{II} phase was induced. However, in 40 mol % DOPC vesicles, the transition from bilayer to H_{II} phase was strongly dependent on the cholesterol mole percent in the vesicles. The origin of the isotropic component in 40 mol % DOPC may be due to structures where rapid motional averaging takes place; however, with NMR, it would be difficult to establish their structure unambiguously. We have obtained all the spectra within a fixed time after preparation of the sample, since this ternary lipid mixture was known to undergo time-dependent structural transitions (Tilcock et al., 1982). On comparison of the activity profiles of PLC with the NMR spectra of these lipid mixtures, a negative correlation between activity and the low-field peak is particularly noticeable (Figure 3B).

Model 3: Lysophosphatidylcholine and DOPE System. LysoPC has a bilayer-inducing effect on unsaturated phosphatidylethanolamine, a classic example of lipid shape complementation, wherein an H_I-preferring lipid and an H_{II}-preferring lipid together form an L_α phase, where this phase is not available to either lipid species in isolation (Kumar, 1991; Madden & Cullis, 1982). Figure 4 presents ^{31}P NMR spectra of lysoPC(stearoyl) and DOPE mixtures obtained in 100 mM NaCl and 10 mM Tris-HCl (pH 7.4). LysoPC at >15 mol % was able to form a bilayer phase with H_{II}-preferring DOPE. The presence of the isotropic component may be due to the reasons discussed above or also due to the presence of lysoPC in micellar form, whose formation depends on the membrane–buffer partitioning coefficient of lyso PC. Figure 5 presents the activity of PLC on sonicated lipid dispersions in 100 mM NaCl and 10 mM Tris-HCl (pH 7.4), with varying amounts of lyso PC in DOPE. The activity was monitored by phosphate estimation and release of radioactive product from radiolabeled DOPE. At a lysoPC:DOPE ratio of 1:9, the activity of PLC sharply increases and reaches a plateau value of lysoPC:DOPE ratio of 2:8. Replacing DOPE with either

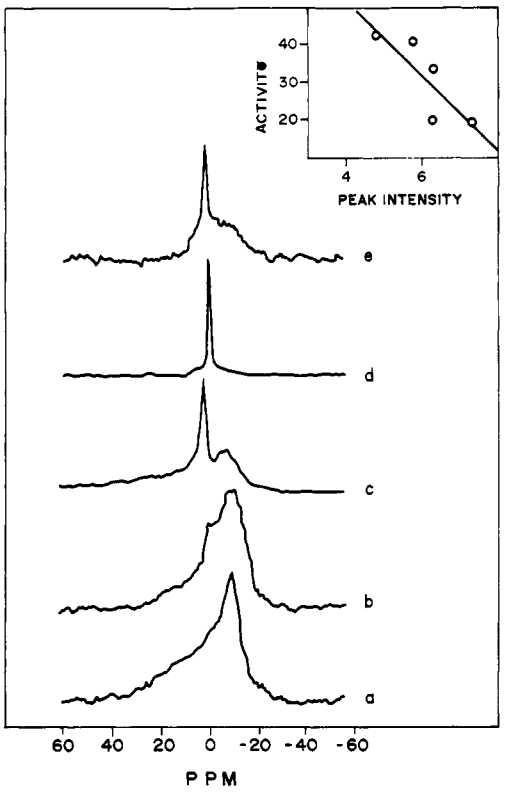

FIGURE 4: ^{31}P NMR spectra of lysoPC (stearoyl) and DOPE mixtures. Mole percent of lysoPC in the mixtures was 30 (a), 25 (b), 20 (c), 15 (d), and 0 (e). The inset shows the relation between the low-field peak intensity and PLC activity (r = –0.82).

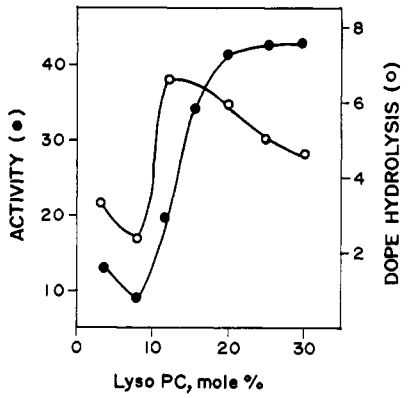

FIGURE 5: PLC activity on DOPE and lysoPC (stearoyl) mixtures. Phospholipid (●) and DOPE (○) hydrolysis by PLC in lipid mixtures containing varying amounts of lysoPC and DOPE.

brain PE or *E. coli* PE also gave similar activity profiles with an activity increase at a lysoPC:DOPE ratio of 1:9 (data not presented). The DOPE hydrolysis profile obtained simultaneously shows a sharp enhancement at a lysoPC:DOPE ratio of 1:9; however, the extent of DOPE hydrolysis was approximately 20% of the total hydrolysis as estimated by phosphate release, indicating that the accessibility of DOPE was lower compared to lysoPC in the concentration range of lysoPC used. A correlation coefficient of –0.82 was obtained between PLC activity and the low-field intensity (shown as the inset in Figure 4).

Model 4: Gramicidin D and DOPC System. PC is a strong bilayer-preferring lipid. However, gramicidin D, a peptide antibiotic, was shown to induce H_{II} phase at a 1:20 peptide to lipid ratio even in DOPC and cause fusion between vesicles (Killian et al., 1985; Tournoise et al., 1990). NMR spectra of MLV prepared by codrying DOPC and gramicidin D, presented in Figure 6, show that at ratios of gramicidin D to

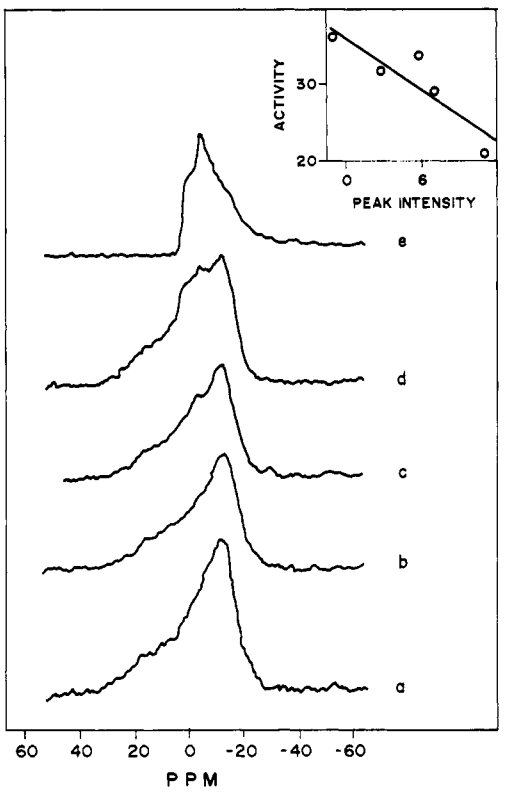

FIGURE 6: ^{31}P NMR spectra of gramicidin D and DOPC mixtures. NMR spectra were obtained on codried samples of ionophore and lipid. The ratio of gramicidin D to DOPC in the vesicles was 1:100 (a), 1:50 (b), 1:20 (c), 1:10 (d), and 1:5 (e).

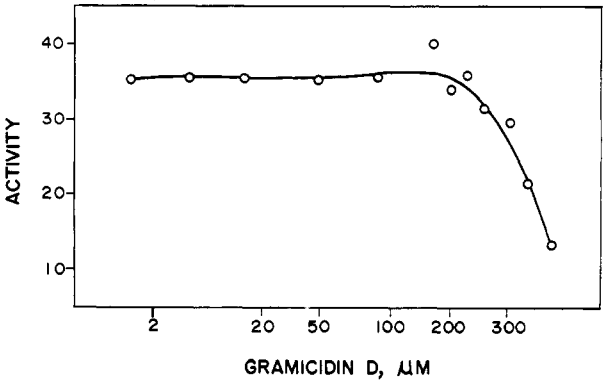

FIGURE 7: Activity of PLC on DOPC/gramicidin D mixtures. PLC activity decreases sharply at 200 μM gramicidin D concentration, which corresponds to a peptide to lipid ratio of 1:10.

DOPC <1:10, a strong H_{II} phase component appears. At a 1:5 ratio, gramicidin D forms a predominantly H_{II} phase with DOPC. The activity profile of PLC on DOPC vesicles containing varying amounts of gramicidin D sharply decreases as the peptide to lipid ratio decreases to 1:15, whereas the activity was unchanged in the peptide to lipid ratio range of 1:1000 to 1:15 (Figure 7). The existence of a nonbilayer phase, as a low-field peak in the NMR spectra, was inversely correlated with the PLC activity (r = –0.87).

DISCUSSION

Biological membranes contain lipids of several classes and adapt bilayer structure (Gennis, 1989). In a lipid mixture containing bilayer-preferring and nonbilayer-preferring lipids, with a tendency to form intrinsic curvature of zero value and a very large value, respectively, the packing of lipids creates stress and increases the bending energy of the bilayer (Hui & Sen, 1989; Seddon, 1991). The stress in a membrane

increases as the membrane approaches the $L_\alpha \rightarrow H_{II}$ transition (Sen et al., 1991). One of the ways of approaching the $L_\alpha \rightarrow H_{II}$ transition isothermally is to increase the proportion of H_{II}-prone lipid in the membrane (Hui & Sen, 1989). In this paper, we present data that suggest that the activity of PLC is sensitive to lipid-packing differences between a bilayer and an H_{II} phase and thus PLC activity can directly monitor the phase transitions. We have selected four lipid model systems, which demonstrate isothermal mesomorphism as a function of one of the components, to test the sensitivity of PLC activity to lipid present in bilayer and nonbilayer phases.

The enhancement in the activity of PLC in model I, with an increase in DOPC mole percent, suggests that the accessibility of the lipid head groups increases with an increase in the L_α phase in the membrane. An increase in an enzyme catalytic rate depends on several factors such as temperature, pH, "effective" substrate concentration, etc.; however, in the present situation, the variable of importance is the ratio of mole percent of DOPC and DOPE. The increase in the hydrolysis may be due to the preferential hydrolysis of DOPC, whose mole percent increases with an increase in the activity. However, the hydrolysis of DOPE increases when actually its concentration in the vesicles was decreasing. Further studies on the substrate preference of PLC demonstrated that both phosphatidylcholine and phosphatidylethanolamine were equally hydrolyzed in the presence of neutral diluants such as Triton X-100 (Roberts et al., 1978) and in human erythrocyte ghosts, small unilamellar vesicles of human erythrocyte ghosts (Roelofsen et al., 1971), and HeLa cell nuclei (Otnaess et al., 1976). These suggest that the enhanced rate is not due to the preferential hydrolysis of DOPC rather than DOPE by PLC. Another possibility is an increase in the "effective" concentration of the lipid as the vesicle composition changes. NMR (Cullis & de Kruijff, 1978; our data) and electron microscopic (Hui et al., 1981) data on MLV with varying amounts of PC and PE suggest that spectra typical of L_α begin to appear at DOPC >15 mol %, which corresponds well with the PLC activity profile.

On MLV containing DLPE and POPC mixtures, porcine PLA_2 was maximally active when the POPC concentration was 85 mol %, and DLPE was hydrolyzed to a greater extent compared to POPC. The results were interpreted, with the support of structural data and also based on calculations of bending energy, as due to the increase in the bilayer lipid packing stress and defects at the onset of the bilayer to nonbilayer transitions (Sen et al., 1991). Similarly, PLA_1 activity on PC and PE vesicles decreases as the PC mole percent in the vesicle increases (Robinson & Waite, 1983). The data reported in this paper on PC and PE vesicles suggest that the activity of PLC increases when there is a transition from a nonbilayer to a bilayer transition, an opposite trend compared to the observations made with PLA_2. With an increase in the interfacial curvature at the L_α to H_{II} transition, if the accessibility of the phosphate–ester bond decreases and the accessibility of the *sn*-2 bond increases, the observations with PLA_2 and PLC are easy to explain. Our observations with membrane-active peptides such as melittin, gramicidin S, and alamethicin and a number of signal peptides indicate that the activity of PLC and PLD decreases whereas the activity of PLA_2 increases on peptide-doped membranes, supporting these conclusions, since these peptides were known to induce nonbilayer phases in the membrane in a lipid composition-dependent manner (Rao, 1992; Rao & Nagaraj, 1993). The situation is comparable to the enhanced susceptibility of denatured proteins to proteases compared to the native proteins (Creighton, 1984). The activity of PLC on MLV suggests that the observations made are independent of the morphological differences between SUV and MLV; since the mole percent of DOPC at which the activities increase is similar (24 and 30 mol % in SUV and MLV, respectively), the substrate accessibility must be similar in these two types of vesicles.

At 40 mol % DOPC, the activity of PLC in the absence of cholesterol in the ternary mixture DOPC/DOPE/cholesterol was high, indicating the L_α phase of the substrate, and becomes progressively nonbilayer with an increase in the cholesterol content in the mixture. With the increase in cholesterol, the activity decreases from a bilayer value observed with 60 mol % DOPC and decreases to the value observed with 30 mol % DOPC. PLA_2 activity on vesicles containing DLPE and POPC vesicles in the presence of varying amounts of cholesterol suggests that the requirement of DLPE to form a H_{II} phase decreases as the cholesterol mole percent increases in these vesicles (Sen et al., 1991). Observation of several nonbilayer structures such as interlamellar attachments, cubic structures, and H_{II} cylinders in PC/PE/cholesterol films by cryoelectron microscopy further support the presence of nonbilayer structures in this ternary lipid system (Frederik et al., 1991).

The suggestion that the basis for phase preferrence is the molecular shape of the lipid was excellently supported with the formation of bilayers exclusively with H_I- and H_{II}-prone lipids (Kumar, 1991; Madden & Cullis, 1982). On mixtures of DOPE and lysoPC, PLC activity sharply increases at >10 mol % lysoPC in DOPE, confirming the observation that the PLC activity increases with the L_α phase in the membrane. ^{31}P NMR profiles of these lipid preparations revealed that the high-field peak typical of bilayer phases begins to appear at 10 mol % lysoPC. Egg yolk PE, whose bilayer to H_{II} phase transition temperature was approximately 15 °C higher than that for DOPE, probably requires lower mole percent lysoPC to make a bilayer (Madden & Cullis, 1982). Further, hydrolysis of DOPE was only 20% of the total phosphate released, suggesting that the accessibility of lysoPC for PLC was always higher compared to DOPE.

Gramicidin D, a peptide antibiotic, induces H_{II} phases in model and biological membranes (Tournois & de Kruijff, 1991). The peptide induces the H_{II} phase in model systems of saturated and unsaturated PC, provided that the acyl chain length exceeds 16 carbon atoms (Killian & de Kruijff, 1986). Gramicidin D has an ability to enhance fusion between vesicles (Tournois et al., 1990) and transbilayer movement of lipids across DOPC model membranes (Tournois et al., 1987) and erythrocyte membranes (Classen et al., 1987). The PLC activity on a sonicated lipid preparation codried with ethanolic solutions of gramicidin D decreases sharply when the peptide to lipid ratio is less than 1:20, suggesting a decreased accessibility of DOPC as H_{II} phases are introduced in the DOPC bilayer. It was proposed that gramicidin increases the interfacial curvature by contributing more to the hydrophobic volume than to the head-group region by taking its position between the acyl chains (Tournois & de Kruijff, 1991). Significant negative correlations between the low-field intensity of lipid mixtures and the activity of PLC on these support the conclusion that the activity of PLC decreases on inductions of nonbilayer phases in the membrane.

Membrane structural alterations at the L_α to H_{II} transition are suggested to play an important role in processes such as fusion, trans/inter-bilayer movement of lipids, etc. (Burger & Verkleij, 1990; Cullis et al., 1983). Membrane fusion is suggested to be mediated by structures of high curvature, and several membrane-active agents, such as PE, diacylglycerol, gramicidin D, melittin, etc., that induce fusion are capable of

inducing H_{II} phases in the membrane (Burger & Verkliej, 1990; Cunningham et al., 1989). Lipid transfer rates are enhanced in the presence of H_{II}-prone lipids such as monogalactosyl diglycerides (Rilfors et al., 1987; Bittman et al., 1990). These observations suggest that for these processes to occur the membrane structure loses its bilayer structure to form local nonbilayer structures. The data presented above suggest that in a bilayer the hydrolyzed phosphate–ester bond by PLC is freely accessible to the soluble PLC and with the increase in H_{II}-preferring lipids the accessibility tends to decrease as the interfacial curvature increases to form H_{II} phases, which probably occludes the lipid head group; consequently, the activity of PLC would be low on lipid in a nonbilayer phase compared to lipid in a bilayer phase. Thus, the "effective" concentration of the substrate, which reflects in the catalytic rate of an enzyme, becomes an important tool to study the structure of the substrate at the substrate/water interface. Since the membrane/water interface is extremely important for phospholipases, a rigorous definition is warranted to compare the catalytic efficiencies of the enzymes and the effects of substrate modifications (Jain & Berg, 1989; Otnaess et al., 1977; Roberts et al., 1978). We have used PLC from *Clostridium perfrengens* in two of our lipid mixtures, and the results were identical with the PLC from *Bacillus cereus*. With growing evidence on their importance in cell biological processes, H_{II} phases have been studied by several techniques, which helped in our understanding of these phases (Seddon, 1990). The emphasis in these studies is on temperature-induced L_α to H_{II} transitions. Isothermal phase transitions, which are biologically relevant, were less studied. Given the simplicity and the brevity of the PLC assay, this method offers a sensitive method to monitor bilayer to nonbilayer phase transitions. Phospholipases offer better tools to study phase transitions isothermally given the physiological nature of the assay, the high sensitivity, and an option to increase the resolution. PLC being an important enzyme in hormonal signal transduction, its regulation is crucial. The low activities on H_{II} phase lipid reported in this paper suggest that PLC activity may be self-regulatory, if the product diacylglycerol, a H_{II}-prone lipid, induces H_{II} phases locally.

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