Allosteric Activation of Phosphatidylinositol-Specific Phospholipase C: Specific Phospholipid Binding Anchors the Enzyme to the Interface†

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ABSTRACT: Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* exhibits 'interfacial activation' toward the water-soluble substrate *myo*-inositol 1,2-(cyclic)phosphate [Zhou et al. (1997) *Biochemistry 36*, 347−355]. The activation of PI-PLC enzyme is optimal with PC or PE interfaces. NMR experiments (TRNOE and ^{31}P line width analyses) were carried out to investigate the interaction of PI-PLC with activator amphiphiles. These studies showed that the enzyme had high affinity for phosphatidylcholine (or PE) molecules with dissociation constants of 0.5 and 0.3 mM for diC_6PC and diC_7PC, respectively. TRNOE cross-peaks of bound PC were confirmed to represent intramolecular relaxation pathways using partially perdeuterated PC molecules consistent with a single molecule binding tightly. The large activation by a PC interface can be explained by a single PC molecule binding specifically to PI-PLC and anchoring the enzyme−lipid complex to the interface. Other interfaces, such as micellar diC_8PS, can activate PI-PLC about 2−3-fold; however, the monomers of these detergents showed little affinity for the enzyme as measured by TRNOE or ^{31}P NMR line widths. The 3.6-fold activation produced by polymerized vesicles of 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (compared to the 15-fold activation generated by nonpolymerized PC vesicles) was comparable to the nonspecific activation of other detergents. This confirmed that single-PC molecule binding was allosteric and anchored the enzyme in the interface. The conformation of interfacially activated enzyme is discussed in term of the stabilization of a critical surface loop and helix B observed with weak intensity in the X-ray crystal structure.

A common property of water-soluble phospholipases is 'interfacial activation', the preferential hydrolysis of aggregated phospholipid substrate. Interpretations of this kinetic phenomenon require two binding events for optimal enzyme activity. Enzyme is proposed to bind initially to the interface, an event that can induce a conformational change in the enzyme to a more active state. Subsequent binding of a substrate at the active site of the enzyme results in the efficient cleavage of substrate to product. Stable attachment of an enzyme at the interface is also critical for multiple catalytic cycles ('scooting mode' kinetics) performed by the enzyme before detaching from the interface (Berg et al., 1991; Jain & Gelb, 1991). The affinity of enzyme for the interface largely depends on the physicochemical nature as well as the organization and dynamics of the interface. For example, the interfacial organization of phospholipids (Kensil & Dennis, 1985; Jain et al., 1989; Burack et al., 1993) and the interfacial electrostatics (Jain & Berg, 1989) greatly influence the interfacial binding and catalysis of phospholipase A_2.

Phosphatidylinositol-specific phospholipase C (PI-PLC)[1] enzymes are a distinct class of water-soluble phospholipases. Cytosolic PI-PLC enzymes play key roles in phosphatidylinositol signal transduction pathways by producing two second-messenger molecules: diacylglycerol, which is responsible for the activation of protein kinase C, and inositol 1,4,5-trisphosphate, which is involved in intracellular calcium mobilization (Homma et al., 1988; Rhee et al., 1989; Rhee & Choi, 1992; Berridge, 1987; Nishizuka, 1986). The physiological functions of PI-PLCs secreted by several microorganisms (Low, 1981; Ikezawa et al., 1981) are not clear, but the ability to cleave the glycosyl-PI moieties of membrane protein anchors (Low & Saltiel, 1988; Ferguson et al., 1988; Ikezawa, 1991) suggests that bacterial PI-PLC may play an essential role as a virulence factor in *Staphylococcus aureus* (Marques et al., 1989) and *Listeria monocytogenes* (Camilli et al., 1991; Mengaud et al., 1991).

PI-PLC catalyzes the cleavage of PI in discrete steps: (i) an intramolecular phosphotransferase reaction to form inositol 1,2-(cyclic)phosphate (cIP) followed by (ii) a cyclic phosphodiesterase activity that converts cIP to inositol 1-phosphate. The phosphodiesterase reaction exhibits interfacial activation. There is a 5−6-fold increase in V_{max} when a given substrate is presented in a micellar versus monomeric state (Lewis et al., 1993; Rebecchi et al., 1993; Hendrickson et al., 1992). This kinetic behavior is similar to that observed for secretory phospholipase A_2 and nonspecific phospholipase C enzymes (Roberts & Zhou, 1996). The cyclic phosphodiesterase activity, which can be studied separately from the first step using water-soluble cIP as substrate, is also sensitive to interfaces (Zhou et al., 1997). This represents a novel allosteric activation of PI-PLC toward a substrate that does not partition in the interface. Almost

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[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C: cIP, *myo*-inositol 1,2-(cyclic)phosphate; PC, phosphatidylcholine; BLPC, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TRNOE, transferred nuclear Overhauser effect; diC_nPC, diacylphosphatidylcholine; TX-100, Triton X-100; CMC, critical micelle concentration; 1D, one-dimensional; 2D, two-dimensional.

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all detergents examined activated PI-PLC toward cIP at least 2-fold, with PC or PE species yielding the largest increases in PI-PLC V_{max}. In the present work, NMR and fluorescence techniques have been used to study activator molecule interactions with PI-PLC from *Bacillus thuringiensis*. The results show that enzyme has a strong affinity for monomeric PC (or PE) and not for PS or other phospholipids. The off-rate of PC from the enzyme−PC complex is relatively slow as detected by chemical exchange line broadening and TRNOE contour plots that exhibited extensive cross-peaks due to intramolecular spin-diffusion. Fluorescence studies of the enzyme showed that a conformational change altering the environment of one or more tryptophans occurred in the presence of PC interface. Extents of activation by mixed PC/TX-100 or polymerized PC vesicle interfaces are consistent with a single PC molecule bound to enzyme (at a site distinct from the catalytic site) forming a stable point of anchorage of the enzyme to the interface. The enzyme conformational change upon anchoring to an interface, which is key for generating activated enzyme, is possibly related to helix B and the loop 237−243 penetration into the hydrophobic core.

MATERIALS AND METHODS

Materials. Short-chain phosphatidylcholine (dihexanoyl-PC, diheptanoyl-PC), dihexanoyl-PE, and phosphatidylserine (dihexanoyl-PS, dioctanoyl-PS) were purchased from Avanti and used without further purification. 1,2-Bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (BLPC) was obtained from Dr. Wonhwa Cho of the University of Illinois at Chicago (Wu & Cho, 1993). D_2O (99.9% enriched), *N,N′*-dicyclohexylcarbodiimide, glycerophosphorylcholine ($CdCl_2$ complex), and detergents were obtained from Sigma. The deuterated compounds NaOD, DCl, Tris-d_{11}, hexanoic acid-d_{11} (D, 98%), and methyl iodide (D, 99.5%) were purchased from Cambridge Isotope Laboratories. 4-(Dimethylamino)pyridine and tributylamine were obtained from Aldrich. Dimethylformamide (DMF), chloroform, and methanol were HPLC grade from Aldrich. DMF was stored over 4 Å molecular sieves for at least 24 h before use.

Purification of PI-PLC Enzyme. A recombinant strain of *Bacillus subtilis* transfected with the *B. thuringiensis* PI-PLC gene for overproduction of PI-PLC enzyme was obtained from Dr. Martin G. Low (Columbia University). The enzyme was isolated from culture supernatants and purified as described previously (Low et al., 1988; Zhou et al., 1997).

Synthesis of Chain-Perdeuterated DiC_6PC (DiC_6PC-d_{22}). Chain-perdeuterated diC_6PC was synthesized from perdeuterated hexanoic acid (133.5 mg, 1.05 mmol) and glycerophosphorylcholine (116.8 mg, 0.27 mmol) using 4-(dimethylamino)pyridine (64.3 mg, 0.53 mmol) as the catalyst. Dicyclohexylcarbodiimide (238.5 mg, 1.16 mmol) was used to generate the fatty acid anhydride. The reaction mixture was stirred for 48 h at room temperature under a nitrogen atmosphere. The catalyst was removed from the reaction mixture by an AGMP-50 column using the solvent system $CHCl_3$:CH_3OH:H_2O (4:5:1). The product was purified by elution through a silicic acid column. A ^1H NMR spectrum confirmed that the acyl chains were perdeuterated.

Synthesis of N-Methyl-Perdeuterated DiC_6PC [$N(CD_3)_3$-diC_6PC]. Methyl iodide (174.5 mg, 1.20 mmol) was added gradually to 2 mL of dimethylformamide solution containing diC_6PE (82.4 mg, 0.20 mmol) and tributylamine (222.4 mg, 1.20 mmol). The reaction mixture was stirred overnight at room temperature. The reaction was monitored by ^{31}P NMR spectroscopy (the resonances for PC and PE are separated by 1 ppm). The product PC was purified by elution from a silicic acid column using a $CHCl_3$:CH_3OH:H_2O solvent system with a linear gradient of H_2O (adjusted to pH 8.0). Appropriate fractions were collected and further purified on another silicic acid column eluted with $CHCl_3$:CH_3OH:CH_3-$COOH$:H_2O (170:25:25:6). The ^{31}P NMR spectrum showed a single peak for PC (−0.3 ppm); in the ^1H NMR spectrum the resonance for the *N*-methyl group at 3.36 ppm was absent.

Enzymatic Synthesis and Purification of cIP. cIP was enzymatically synthesized and purified as described previously (Zhou et al., 1997; Wu et al., 1997). Crude phosphatidylinositol (50% PI) was purchased from Sigma. PI-PLC enzyme was added to PI/TX-100 mixed micelles, and reaction progress was monitored by ^{31}P NMR spectroscopy. After PI was completely converted to cIP, the cIP, in the aqueous phase, was further purified using an AG1-X8 anion exchange column.

Gel Filtration Chromatography of DiC_6PC Partitioning in TX-100 Micelles. Size exclusion chromatography was carried out at room temperature with a column (66 cm × 2.0 cm) containing Sephadex G-25 (Sigma) to separate detergent micelles and monomers or small aggregates. The column was equilibrated with a monomer detergent solution (0.2 mM TX-100 in 50 mM HEPES, pH 7.5). The sample, 1.0 mL, was loaded, and 2.0 mL fractions were collected (the flow rate was set at 24 mL/h). TX-100 was monitored by its UV absorption at 260 nm, and phosphatidylcholine was detected by ^{31}P NMR spectra.

Preparation of Samples for NMR Measurements. Purified PI-PLC enzyme was dialyzed overnight against 50 mM NaCl, pH 7.0, and then frozen and lyophilized. The sample was rehydrated in D_2O containing 20 mM Tris-d_{11} buffer, pH 7.5. A typical sample used for TRNOE measurements contained 0.2 mM enzyme; for ^{31}P line shape analysis, NMR samples contained 0.03−0.1 mM enzyme and 0.2 mM EDTA.

NMR Spectroscopy. One- or two-dimensional NMR experiments were carried out on a Varian Unity 500 NMR spectrometer. ^1H NMR spectra were acquired using an indirect probe; ^{31}P NMR spectra were obtained with a broadband probe. Parameters for ^{31}P NMR spectra are similar to those for assaying PI-PLC (Zhou et al., 1997) but with a variable number of transients (depending on the ratio of PC to enzyme).

TRNOE, developed primarily by Clore and Gronenborn (1982, 1983), provides an indirect approach to the determination of bound ligand conformation. TRNOE is a powerful method to obtain the bound conformation of small, flexible ligand molecules that can bind reversibly to macromolecules. This exchange on the relaxation time scale allows the transfer of population information from one molecular environment to another. Generally a large excess of ligand molecule is used in a TRNOE experiment. Transferred NOE spectra were acquired using the two-dimensional NOESY pulse sequence in the phase-sensitive mode by collecting hypercomplex data. Residual water suppression was achieved by selectively saturating the water resonance during the relaxation delay (1.5 s) and the mixing time (0.1 ms) of the NOESY sequence. Spectra were obtained at 30 °C without spinning the sample. There were 2048 points in *F*2 and 256 points in *F*1 dimensions. Zero filling was employed, and

final spectra contained 2K × 2K real points. A Gaussian weighting function was used in both dimensions.

An analysis of the line shape of the ^{31}P resonance of phosphatidylcholine in the presence of PI-PLC enzyme was also carried out. The chemical exchange of monomeric PC can be described by the simple model

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL$$

If this exchange proceeds rapidly on the NMR time scale, the dissociation constant, $K_d = k_1/k_{-1}$, can be estimated by monitoring the chemical shift or line width change as function of ligand and enzyme. When the bound ligand fraction, f_{EL}, is small compared to total ligand (f_L), the observed NMR parameter P_{obs} (chemical shift, transverse relaxation time, or line width) can be expressed in terms of apparent values for bound state (P^*_{EL}) according to:

$$P_{obs} = f_L P_L + f_{EL} P^*_{EL}$$

$$f_L + f_{EL} = 1$$

By introducing another two parameters, $\Delta = P_{obs} - P_L$ and $\Delta_o = P^*_{EL} - P_L$, then $f_{EL} = \Delta/\Delta_o$. From the equilibrium reaction and [L] ≫ [EL], where Lt ≈ [L],

$$\frac{P_{obs} - P_L}{P^*_{EL} - P_L} = \frac{E_t}{K_d + L_t}$$

$$P_{obs} = \frac{E_t}{K_d + L_t}(P^*_{EL} - P_L) + P_L \qquad (1)$$

If the condition [L] >> [EL] is not satisfied, [L] can be calculated using an estimated K_d. A plot of $L_t(P_{obs} - P_L)$ versus [L] can be used to correct the error due to the difference of [L] and L_t. This process can be iterated several times until K_d does not change:

$$L_t(P_{obs} - P_L) = \frac{E_t[L](P^*_{EL} - P_L)}{(K_d + [L])} \qquad (2)$$

The chemical shift difference between free and bound environments is very small for the PC/PI-PLC system (less than 3 Hz), while the change in line shape is relatively large. Phosphatidylcholine was titrated into the enzyme solution in the NMR tube, and the ^{31}P line width was measured at 30 °C. Since the volume change was less than 10%, the enzyme concentration can be considered to be constant. At each PC concentration three measurements were made at signal-to-noise greater than 10 in order to minimize errors from shim differences. Experimental data were fit to eq 1, and parameters K_d and P^*_{EL} were obtained. If P^*_{EL} is the apparent line width of the bound ligand, the following relationship can be derived (Perlman et al., 1994; Davis et al., 1994):

$$\pi P^*_{EL} = \frac{1}{T_{2EL}}\left[\frac{\left(1 + \frac{\tau_{EL}}{T_{2EL}}\right) + T_{2EL}\tau_{EL}\Delta\omega^2}{\left(1 + \frac{\tau_{EL}}{T_{2EL}}\right)^2 + (\tau_{EL}\Delta\omega)^2}\right] \qquad (3)$$

where T_{2EL} is transverse relaxation rate of bound ligand, $\tau_{EL} = 1/k_{-1}$ is the lifetime or off-rate of the bound ligand, and $\Delta\omega = 2\pi(\delta_{EL} - \delta_L)$ is the chemical shift difference of ligand resonance between unbound and bound states. One can calculate τ_{EL} using eq 3 if parameters T_{2EL} and $\Delta\omega$ are known.

If the enzyme has multiple binding sites for the ligand, the chemical exchange reaction can be described by the model:

$$E + nL \rightleftharpoons EL_n$$

This model is more complicated than for a single ligand-binding site. We assume that the interaction between bound ligands is small and can be ignored, i.e., the *n* ligands bind independently. Equation 1 can be rewritten to yield the following equation:

$$\frac{P_{obs} - P_L}{P^*_{EL} - P_L} = \frac{nE_t L_t^{n-1}}{K_d + L_t^n} \qquad (4)$$

From the best fit to eq 4, one can assess how many molecules bind to enzyme.

^{31}P NMR Assay of PI-PLC Activity. The cyclic phosphodiesterase activity of PI-PLC was monitored by ^{31}P NMR spectrospcopy. The buffer used in all PI-PLC assays was 50 mM HEPES, pH 7.5. All stock solutions were prepared in D_2O, and the pH meter reading was adjusted to 7.5 before addition to the assay solution. The ^{31}P NMR parameters were optimized based on those previously used by Griffith and co-workers (Volwerk et al., 1990) as modified by Zhou et al. (1997). The reaction, initiated by addition of the appropriate amount of PI-PLC, was monitored for 1−2 h at 30 °C. The rate of cIP hydrolysis (μmol min^{-1} mg^{-1}) was calculated from integrated intensity of the I-1-P (increasing) or cIP (decreasing).

Fluorescence Measurements. The concentration of PI-PLC for fluorescence measurements was kept at about 1 μM in Tris-acetate buffer, pH 7.4. Steady-state fluorescence measurements were performed with a Shimadzu RF 5000 V spectrofluorimeter (with a xenon light source) at 23 °C. The excitation wavelength was 290 nm, with both excitation and emission slit widths set at 3 nm. The emission was scanned from 295 to 500 nm.

RESULTS

TRNOE Studies of Phospholipids Interacting with PI-PLC. Previous studies (Zhou et al., 1997) showed that PCs are the best interfacial activators of PI-PLC-catalyzed hydrolysis of cIP. The activation by monomeric PC was much smaller compared to that induced by micellar PCs, although it was significant. DiC_6PC has a high CMC (14 mM) and is a good choice to see how a monomeric PC ligand binds to PI-PLC. Therefore, diC_6PC was used for the TRNOE experiments with PI-PLC. The 1D ^{1}H NMR spectrum of diC_6PC (4 mM) showed resonances with very narrow line shapes (Figure 1A); the 2D NOESY (mixing time of 1.0 s) experiment showed a few positive NOEs (Figure 2A). When 0.2 mM PI-PLC was added, the line width of the PC resonances was clearly increased as shown in Figure 1B. This indicated that monomeric PC molecules bind to PI-PLC and that the binding is in intermediate to fast exchange on the NMR time scale. TRNOE spectra were acquired at a 40:1 ratio of diC_6-PC:PI-PLC with a 0.1 s mixing time. There were many cross-peaks all with the same phase as the diagonal peaks

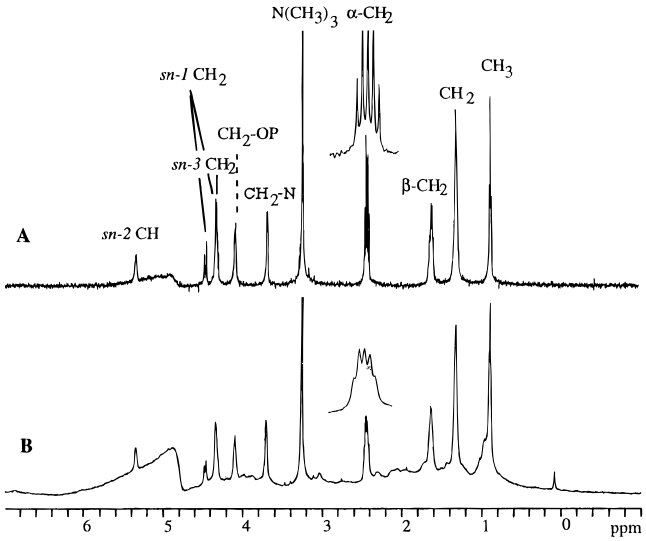

FIGURE 1: 500 MHz ^{1}H NMR spectra of diC_6PC (4 mM) in the absence (A) and presence (B) of PI-PLC enzyme (0.2 mM) in 20 mM Tris-d_{11}-DCl buffer, pH 7.5.

in Figure 2B. This meant that the NOEs had changed from positive to negative, indicating that bound PC information was transferred to free PC molecules. It was not possible to derive the conformation for a single bound PC molecule by analysis of NOESY spectra of PC with PI-PLC because almost all PC resonances were correlated. For example, the terminal methyl protons had cross-peaks with every single proton in the PC molecule. The failure of the TRNOE data to provide distance constraints which were consistent with a single, bound conformation of the molecule has several potential explanations (Perlman et al., 1994). (i) Indirect relaxation pathways mediated through either the ligand protons or protons on the enzyme exist. Generally the correlation time (τ_c) of molecules in nonviscous solvent is proportional to MW $\times$ 10^{-12} (Neuhaus & Williamson, 1989). For our system τ_c is around 3×10^{-8} s, and dipolar relaxation should dominate within 100 ms mixing time. (ii) Ligand exchange is insufficiently rapid compared with relevant cross-relaxation rates of the bound species, and therefore indirect dipolar coupling pathways are overestimated (London et al., 1992). (iii) There may be multiple modes of binding or multiple binding sites. This last possibility could be a likely explanation for the diC_6PC/PI-PLC system. If enzyme is bound to a membrane surface, it may bind to a patch of lipids and not a single molecule. In this case, several PC molecules could be bound to the enzyme and the observed NOEs could be intermolecular as well as intramolecular.

In order to determine if the observed NOEs represent intramolecular or intermolecular interactions, partially perdeuterated diC_6PC molecules were synthesized. Chain-perdeuterated/proteo-headgroup (diC_6PC-d_{22}) and *N*-methyl-perdeuterated/chain-proteo ($N(CD_3)_3$-diC_6PC) lipids were mixed in a 1:1 ratio. A TRNOE experiment was performed at a mixing time of 0.1 s with 0.2 mM PI-PLC and a total of 4 mM PC species. The contour plot for this experiment is shown in Figure 2C. Most of the intensity of the cross-peaks between the *N*-methyl group and acyl chain protons has disappeared. If the cross-peak between the two CH_2 groups in the choline headgroup is used as a standard, the cross-peak intensities of *N*-methyl with chain protons α-CH_2, β-CH_2, $(CH_2)_3$, and CH_3 are 41%, 20%, 53%, and 32%, respectively, for normal diC_6PC. For deuterated mixed species there are small detectable cross-peaks of the *N*-methyl

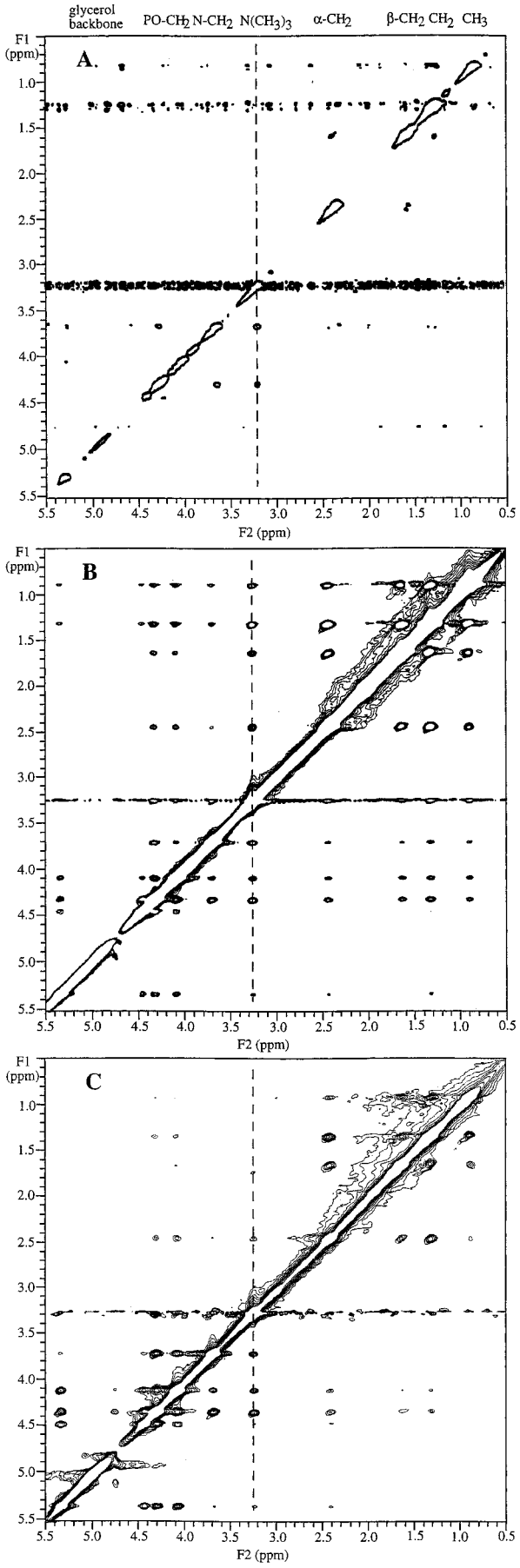

FIGURE 2: NOESY contour plots for diC_6PC: (A) 4 mM diC_6PC, (B) 4 mM diC_6PC with 0.2 mM PI-PLC, and (C) total 4 mM chain-perdeuterated and *N*-methyl-perdeuterated diC_6PC (1:1) mixture with 0.2 mM PI-PLC. The mixing time was 1.0 s in panel A and 0.1 s in panels B and C. Cross-peaks are positive in panel A and negative in panels B and C.

protons with α-CH_2 and β-CH_2 protons but not with $(CH_2)_3$ or CH_3 groups. The intensities of these two cross-peaks are less than 5% of the standard. It is possible that α- and β-carbons in chain perdeuterated diC_6PC were partially protonated in solution or that these cross-peaks were due to NMR artifacts. The fact that phosphatidylethanolamine (PE) can activate PI-PLC to the same extent as PC (Zhou et al., 1997) indicated that PE can also bind to PI-PLC. A mixture of chain-perdeuterated diC_6PC and diC_6PE was also mixed with PI-PLC and examined by TRNOE. The spectra (not shown) were almost identical to the deuterated PC mixture. This further confirmed that most of the NOEs observed were intramolecular in origin.

Most other detergents above their CMCs can activate PI-PLC 2-fold. Phosphatidylserine (PS) is an example of one of these nonspecific detergents; hence it was chosen for TRNOE studies with PI-PLC. DiC_6PS, monomeric in solution at 4 mM, exhibited a sharp line shape and positive NOEs in NOESY spectra with mixing time 1.0 s; this behavior was similar to that of 4 mM diC_6PC. There was no observable change in line width in the 1D ^{1}H NMR spectrum, and positive NOEs were observed in the TRNOE experiment in the presence of 0.2 mM PI-PLC. This indicated that monomeric PS did not bind to the enzyme or that individual PS molecules have a weak affinity for the enzyme that is beyond NMR detection. In contrast, micelles formed by PS molecules may have relatively higher affinity for PI-PLC and could partially stabilize the active form of the enzyme. DiC_4PC, with a CMC > 200 mM, was also examined in the absence and presence of 0.2 mM PI-PLC. The result was the same as PS, consistent with the observation that this molecule as a monomer does not appreciably activate PI-PLC toward cIP.

^{31}P Line Widths Monitor PC Binding to PI-PLC. The ^{1}H TRNOE results indicated that one or more PC molecules bind to PI-PLC and most of the observed NOEs are intramolecular in origin. Other NMR parameters for PC such as ^{31}P chemical shift or line width changes in the presence of enzyme can be used to assess how many molecules bind to PI-PLC. Since the interaction of the phospholipid with PI-PLC appears to depend on the headgroup, the phosphorus nucleus should be sensitive to binding. The ^{31}P chemical shift of the diC_6PC−enzyme complex was estimated as 2 Hz downfield of monomeric diC_6PC. The micellization of diC_6PC can generate a 3−4 Hz downfield shift, so changes in the ^{31}P chemical shift were not useful in monitoring the number of PC molecules binding to PI-PLC. Changes in the line width of the ^{31}P resonance, if dramatically larger, would be more useful. In terms of its contribution to line width, the interaction of PC molecules with one another can be omitted even if there are multiple binding sites on the enzyme for PC. The ^{31}P line width does not increase significantly for diC_6PC when it aggregates to form nearly spherical micelles. The ^{31}P line width for diC_7PC (20 mM), which forms rod-shaped micelles, does increase by 1.5 Hz compared to monomeric diC_7PC. If binding is accompanied by large line width changes, this would be a useful parameter for determination of the number of PC molecules binding to the enzyme. With this in mind, the line widths of diC_6-PC or diC_7PC were followed in the presence of PI-PLC and eq 4 was used to assess how many PCs were bound to PI-PLC.

PI-PLC concentration was limited to <0.1 mM by the observation that some of the protein precipitated in the presence of high concentrations of PC molecules (the PC concentrations ranged from 0.1 to 20 mM). Titration of PI-PLC with diC_6PC was carried out at two different enzyme concentrations, 0.10 and 0.06 mM. The best fit using eq 4 showed that the average number of bound PC molecules per enzyme was 0.96 and 0.99 ± 0.15, respectively. Titration of PI-PLC with diC_7PC was also accomplished at two enzyme concentrations (0.075 and 0.046 mM), and the average numbers of bound PC molecules obtained from eq 4 were 1.2 and 1.1 ± 0.1, respectively. These results are consistent with a single-PC molecule binding mode.

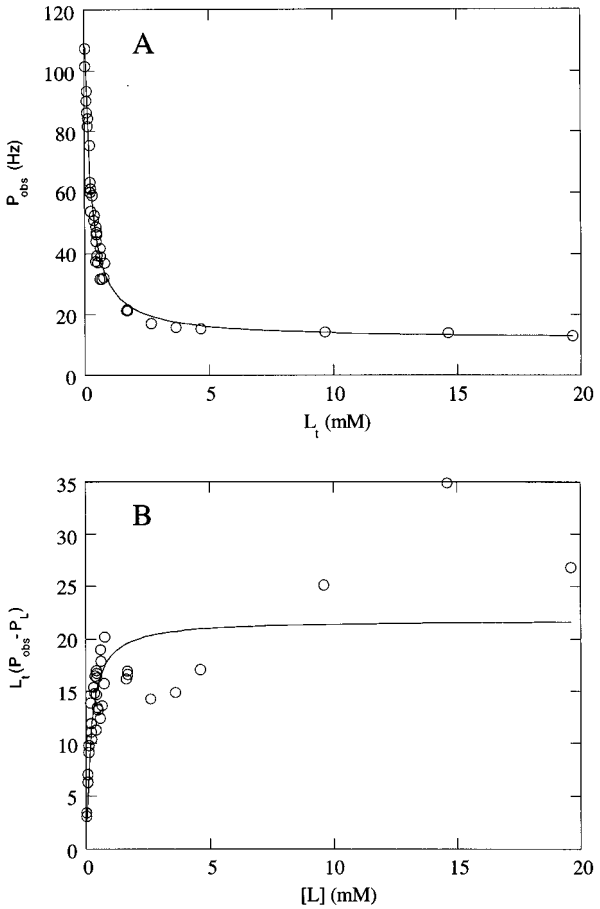

FIGURE 3: ^{31}P line width of diC_7PC in the presence of PI-PLC. (A) Observed line width, P_{obs}, versus total ligand concentration, L_t; the solid line represents the best fit of eq 1 with K_d = 0.20 ± 0.01 mM, P^*_{EL} = 481 ± 21 Hz. (B) Plot of $L_t(P_{obs} - P_L)$ versus free ligand concentration [L] with the solid line the best fit to eq 2 (K_d = 0.19 ± 0.04 mM, P^*_{EL} = 476 ± 28 Hz).

Titration curves were well fit by eq 1. However, at low PC concentrations, the condition [L] >> [EL] was not satisfied. The error can be as high as 20%. The other data-processing method using eq 2 was tried, and [L] was calculated using K_d. The two different data treatment methods are shown in Figure 3. The average dissociation constant (K_d) and apparent line width of the PC−enzyme complex (P^*_{EL}) are listed in Table 1. Dissociation constants for PI-PLC interacting with diC_6PC and diC_7PC are below 1 mM. For comparison, the concentrations at which half-maximum enzyme activation occurs were 12 mM for diC_6-PC and 2 mM for diC_7PC. This indicated that the binding of a single PC molecule can not switch PI-PLC from an inactive form to the optimally active form. Interestingly, there was 1 order magnitude difference in apparent bound line width for the two PC−enzyme complexes.

Table 1: Dissociation Constants and Apparent ^{31}P Line Widths of the DiC_nPC−Enzyme Complex

phosphatidylcholine	dissociation constant K_d (mM)	apparent line width $P\pi_{EL}$[a] (Hz)
DiC_6PC	0.5 ± 0.2	39 ± 4
DiC_7PC	0.3 ± 0.2	482 ± 41

[a] Estimated as described in Materials and Methods.

Enzyme Fluorescence Properties in the Presence of PCs. The TRNOE and ^{31}P NMR line width analyses of bound PC indicated that PI-PLC has high affinity for monomeric PC molecules with a dissociation constant under 1 mM. If this single-PC molecule binding is an allosteric effector of enzyme, the concentration for half-maximum activation by diC_6PC and diC_7PC should be similar. However, this is not the case (Zhou et al., 1997). Previous fluorescence studies of bacterial PI-PLC (Volwerk et al., 1994) showed that the protein intrinsic fluorescence emission intensity increased in the presence of PC vesicles and micelles. For diC_8PC micelles, the fluorescence intensity increase was observed above 0.1 mM indicating a K_d which is close to the CMC of the lipid. With diC_8PC, it was unclear whether single-PC molecule binding or interfacial PC binding induced the increase in fluorescence intensity. Both diC_6PC and diC_7-PC have higher CMCs and provide good model systems for examining PC monomer- versus micelle-induced fluorescence changes. Figure 4 shows the PI-PLC fluorescence intensity as a function of added diC_6PC. At high PC concentrations, the broad emission peak was split and exhibited fine structure. The intensity at shorter wavelength (327 nm) was higher than that at longer wavelength (340 nm). Plots of the fluorescence intensity at 327 nm versus PC concentration are shown in Figure 5. The fluorescence intensity at 340 nm exhibited the same trends. Clearly the fluorescence change induced by PC is biphasic. There was a fairly small increase in fluorescence intensity at concentrations below the CMC (14 mM) and a large increase around the CMC for the PC. These fluorescence changes parallel the effect of PC on PI-PLC activity (Zhou et al., 1997). This suggests that only interfacial PC binding can stabilize PI-PLC in its active form. Interestingly the shape of the fluorescence binding curve at high PC concentration does not quite match that of the enzyme activation curves (Zhou et al., 1997). Activation reaches a plateau when PI-PLC is saturated by PC micelles. However, the fluorescence increase is more gradual and does not reach a plateau for either diC_6PC or diC_7PC. This indicates that the environment of tryptophans changes in 'polarity'/'hydrophobicity' in the presence of PC micelles over and above that necessary to activate the enzyme. Furthermore, one or more of the tryptophans in the enzyme sense this activation. Presumably, the affinity of PI-PLC for a PC monomer keeps the enzyme associated with the interface. This phenomenon may be reminiscent of the scooting behavior of phospholipase A_2 in kinetic analyses (Jain & Gelb, 1991; Berg et al., 1991), except in this case the PC is not a substrate but an allosteric effector that anchors the enzyme to the interface.

Activation by DiC_6PC/TX-100 Aggregates or a Polymerized PC Interface. The interaction of PI-PLC with PC or other detergents should occur at the same site on the enzyme. Different efficiences in activation would depend on the affinity of the amphiphile for the enzyme. With this in mind, the cyclic phosphodiesterase activity of PI-PLC was examined under different conditions (Table 2): 1.8-or 4.0-fold activation toward cIP was observed with TX-100 micelles (4 mM) or diC_6PC monomers (4 mM) present with PI-PLC. A mixture of TX-100 and diC_6PC at the same concentrations produced a much larger activation, a 12-fold activation of PI-PLC toward cIP. If diC_6PC does not form small aggregates with TX-100, the maximum activation should be ~7-fold. The distribution of amphiphiles in this sample was explored with gel filtration using a Sephadex G-25 column (Figure 6). The column was equilibrated with 0.2 mM TX-100, a concentration close to the CMC of the detergent. When 1 mL of micellar TX-100 (4 mM) mixed with diC_6PC (4 mM) was applied to the column, there were two elution peaks for TX-100. The first eluted in the void volume with no detectable diC_6PC; the other was broad and overlapped with the diC_6PC elution peak whose maximum was slightly before the column volume (the arrow indicates where cIP elutes, marking the column volume). This behavior was different from that observed for the TX-100 and cIP system where TX-100 eluted in the void volume and was well separated from the cIP peak (Zhou et al., 1997). DiC_6PC eluted earlier than cIP in the presence of TX-100; this clearly showed that diC_6PC forms small aggregates with TX-100 micelles. A 12-fold kinetic activation was observed, presumably because diC_6PC anchored the enzyme to this mixed interface. Comparing activation by this mixed interface to the pure diC_6PC (20 mM) interface suggests that TX-100 functions like a neutral surface dilution reagent in these small mixed aggregates. Activation by this mixed interface is lower than by a pure diC_6PC (20 mM) micellar interface.

One way to distinguish what an individual PC molecule contributes to the kinetic activation from the role of the interface is to use a chemically cross-linked interface. If the binding of a single PC molecule is important for anchoring enzyme in the interface, a polymerized PC interface should not exhibit the same activation as micellar or vesicle PC surface. The presence of such an interface may be expected to generate the same activation as other nonspecific detergent interfaces but much less than un-cross-linked PC vesicle activation (Zhou et al., 1997). BLPC forms stable small unilamellar vesicles when cross-linked (Wu & Cho, 1993). These can be examined for their effect on activating PI-PLC toward cIP. As shown in Table 2, the activation by the BLPC interface was 3.6-fold, a value comparable to other nonspecific detergent interfaces. Cross-linking the PC molecules largely decreased the activation efficiency. This result strongly suggests that tight binding of a single PC molecule is critical for stabilizing PI-PLC at the interface where it must adopt an active conformation.

DISCUSSION

Bacterial PI-PLC, like other water-soluble phospholipases, works at the lipid/water interface during PI cleavage. The cIP released into the bulk solution is further hydrolyzed to I-1-P. This second catalytic step involves both soluble enzyme and soluble substrate (cIP has no tendency to partition into interfaces). However, the enzyme activity toward cIP can be dramatically altered by the presence of an interface (Zhou et al., 1997). Most detergent interfaces examined activated the enzyme 2−3-fold, but an interface composed of PC increased enzyme efficiency 20-fold, decreasing K_m and increasing V_{max}. The specificity of this activation must involve a single molecule of PC (or PE) binding to PI-PLC. The dissociation constant, K_d, of a single PC molecule binding to PI-PLC is less than 1 mM for both

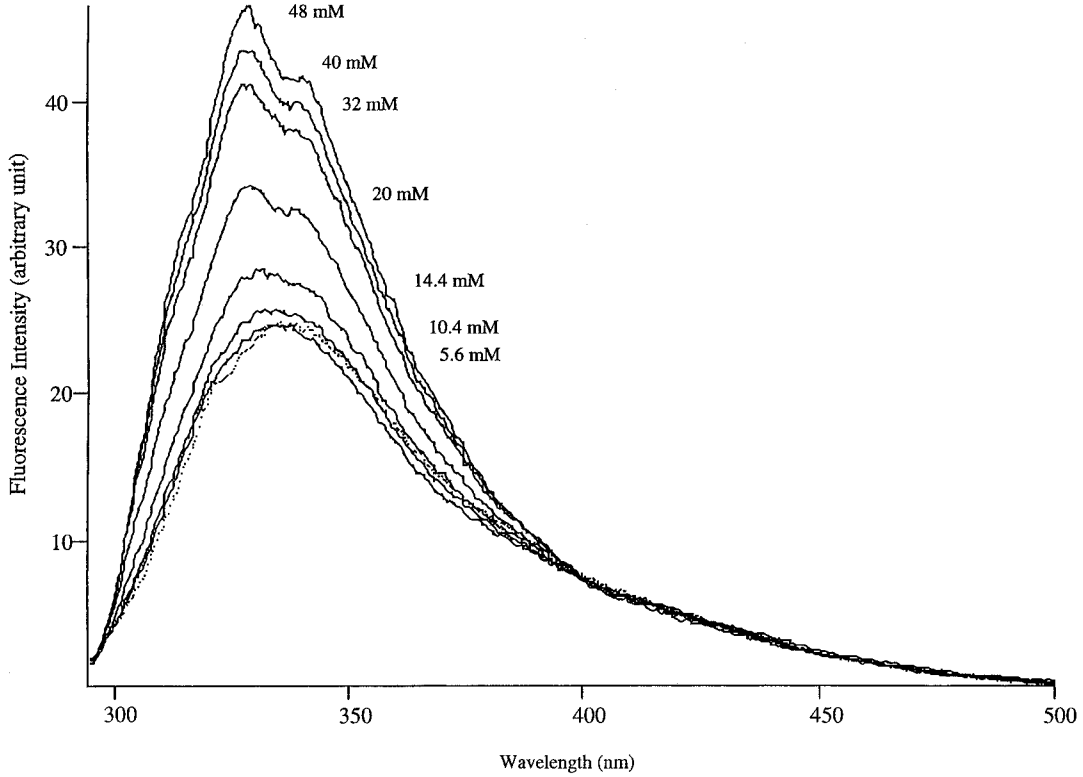

FIGURE 4: Fluorescence emission spectra of PI-PLC (1 μM) (dotted line) as a function of increasing concentrations of diC_6PC (labeled on the spectra). The excitation wavelength was 290 nm.

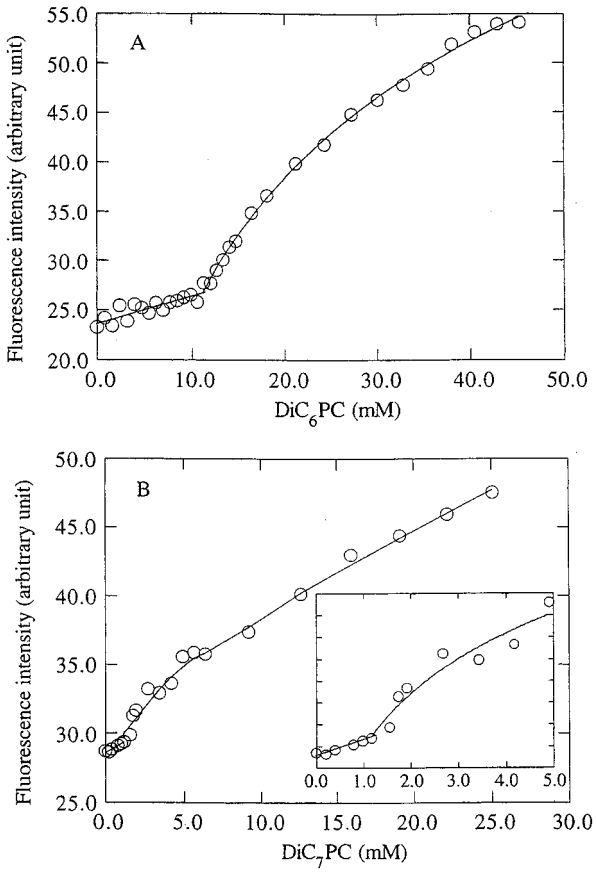

FIGURE 5: PI-PLC intrinsic fluorescence intensity as a function of the concentration of (A) diC_6PC and (B) diC_7PC. The inset shows the low-PC concentration region enlarged.

diC_6PC and diC_7PC. This binding constant, which is not very dependent on chain length, fails to correlate with the kinetic activation and also does not induce a large change in the fluorescence intensity of PI-PLC. Other phospholipids and detergents do not bind tightly to PI-PLC as detected by NMR techniques, although they do induce an increase in the protein fluorescence (Volwerk et al., 1994). Thus, the activation of PI-PLC appears to arise from specific interaction of the enzyme with the PC headgroup. Also consistent with this is the observation that sphingomyelin, which has a choline headgroup, activated PI-PLC to the same extent as PC in Table 2.

Table 2: Phosphodiesterase Activity of PI-PLC with cIP as Substrate in the Presence of Different Amphiphiles

additives[a]	specific activity[b] (μmol min^{-1} mg^{-1})	relative activity
–	0.99	1.0
TX-100	1.74	1.8
diC_6PC	3.96	4.0
TX-100 + diC_6PC	11.6	12
diC_6PC (20 mM)	17.0	17
diC_7PC	19.8	20
diC_{14}PC	12.1	12
sphingomyelin	11.9	12.0
BLPC[c]	3.56	3.6

[a] Solutes present at 4 mM unless otherwise indicated. [b] Assay conditions included 50 mM HEPES, pH 7.5, 15 mM cIP, and 7 μg of enzyme. [c] BLPC, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine, cross-linked in small unilamellar vesicles.

Binding of a single PC molecule can not switch the enzyme from an inactive to a fully activated form. An interface is needed to drive this conformational change of PI-PLC, and this interaction shows much less specificity. The mechanism proposed for this interfacial activation is illustrated in Figure 7. Specific binding of PI-PLC to the PC headgroup (and possibly the glycerol backbone and part of the acyl chains) drives the enzyme to an interface. In such a model, the activation efficiency depends on the affinity of the enzyme for a molecule localized at the interface. The higher the affinity of the enzyme for a PC molecule at the

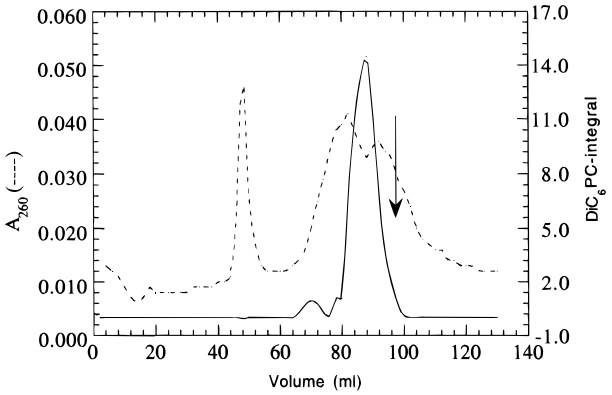

FIGURE 6: Elution profile of a TX-100 (4 mM) and diC_6PC (4 mM) mixture applied to a Sephadex G-25 column. The column was equilibrated and eluted with 50 mM HEPES, pH 7.5, containing 0.2 mM TX-100 to maintain a constant monomer concentration. The arrow indicates where cIP elutes.

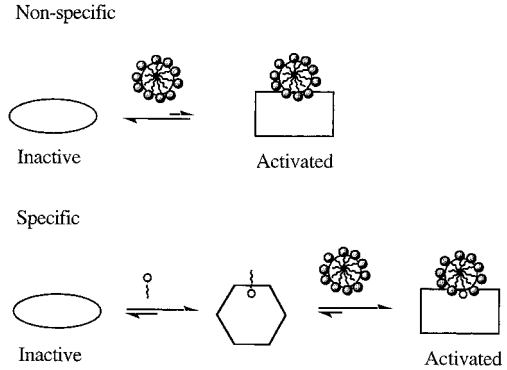

FIGURE 7: Proposed scheme for the mechanism by which PC activates PI-PLC toward cIP.

interface, the longer the enzyme will be kept in an active form. That is why a mixed diC_6PC/TX-100 interface yields a larger activation than TX-100 itself. With the cross-linked PC interface, binding to an isolated PC molecule is hindered, since all molecules are cross-linked and vertical motions of the lipids are damped. The interaction of PI-PLC with this interface is weaker; hence the activation is only 3.6-fold, less than the 20-fold maximum seen with the PC micelles or vesicles.

The bound line width estimated for the diC_7PC binding to PI-PLC is quite large. The chemical shift difference of free and bound PC molecules is less than 3 Hz; hence the broadening due to chemical exchange should not be larger than 3 Hz if the exchange is fast. Clearly this is not the case; hence chemical exchange among PC species must be in an intermediate regime. Generally the relaxation of ^{31}P nuclei in a macromolecular complex is within 20−30 ms, and the line width is around 11−16 Hz without chemical exchange. The exchange rate can be expressed as the reciprocal of the residence time, and in some cases this value can be extracted from the bound line width. However, that is difficult to do for the PI-PLC/diC_7PC system since the lipid is exchanging between many different sites. The CMC of diC_7PC is relatively low, and there are monomers and micelles in solution as shown in Figure 8. Chemical exchange can occur between tightly bound PC ($PC_{tightly\ bound}$) with monomeric PC (PC_{mon}) or with PC in the same micelles ($PC_{interface\ site}$) or different micelles (PC_{mic}). The exchange with PC in the same micelles could be fast and may largely decrease the residence time of the complex. DiC_6PC has a much higher CMC and only monomeric PC exists in the

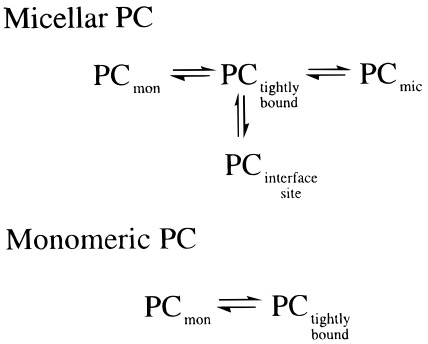

FIGURE 8: Illustration of all the exchange equilibria involving PC.

experimental region; fewer discrete species are in exchange with the PI-PLC/diC_6PC system. This may be why bound diC_7PC had a much broader linewidth in the presence of PI-PLC. Presumably, exchange of enzyme-bound PC with interface or micellar PC is responsible for the 10-fold increase in line width.

A previous study of PI-PLC activity toward water-soluble cIP showed that all anionic (taurocholate and phosphatidylglycerol), nonionic (TX-100 and β-OG), and zwitterionic (CHAPS) detergent interfaces examined gave a 2-fold activation of enzyme activity with little effect on K_m (Zhou et al., 1997), while zwitterionic PC or PE interfaces produced a much larger activation. This result indicated that activation of cyclic phosphodiesterase activity of PI-PLC by general interfaces did not depend on the electrostatics of the monomer in the interface. The organization and dynamics of the PC interface did, however, have some influence on the overall catalytic process since PC micelles were somewhat better than PC vesicles in the activation of the enzyme. There are at least two factors which could lessen the efficiency of vesicle activation: (i) PI-PLC may have difficulty binding a single PC molecule packed in a vesicle, and (ii) any enzyme interfacial penetration which is governed by the packing density of the interface will be slower in vesicles than in micelles. Helix B and a nearby loop possess hydrophobic amino acid clusters. These were suggested to be involved in the interfacial binding by X-ray crystallography. The penetration of the helix B and the loop into the hydrophobic core of a membrane or micelle is favored thermodynamically. The environment of both tryptophans in helix B and the loop would change under such conditions, and the intrinsic protein fluorescence would increase as the fluorophores are transferred from a hydrophilic to a hydrophobic environment.

The use of a lipid activator to increase the affinity of water-soluble enzymes for membranes may be a common theme in signal transduction pathways. DAG is an activator of protein kinase C. Binding measurements showed that the key role of phorbol esters or DAG is to increase the affinity of protein kinase C for membranes by several orders of magnitude (Mosior et al., 1996). The data revealed that the direct binding of protein kinase C to activators provides a major contribution to the free energy change involved in the association of protein kinase C with membranes. The PC (or PE) binding to PI-PLC could play the same role as DAG or phorbol esters binding to protein kinase C. The binding of a PC molecule increases the affinity of PI-PLC for the interface. Once anchored at the interface, the active conformation of the enzyme is stabilized.

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