Activation of Phosphatidylinositol-Specific Phospholipase C toward Inositol 1,2-(Cyclic)-Phosphate†

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ABSTRACT: Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* catalyzes the hydrolysis of phosphatidylinositol (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. Water-soluble cIP was used as the substrate to study the cyclic phosphodiesterase activity and interfacial behavior of PI-PLC. Different detergent micelles and phospholipid vesicles were used to examine if "interfacial activation" of the enzyme could occur toward a soluble substrate. Almost all detergents examined activated the enzyme at least 2-fold, with PC species yielding the largest increases in PI-PLC specific activity. Kinetic parameters were measured in the absence and presence of several representative detergents (e.g., Triton X-100 and diheptanoylphosphatidylcholine (diC_7PC)). Gel filtration experiments showed that, under these conditions, the cIP did not partition to any measurable extent with these detergent micelles. The concentration at which half the maximum activation was observed occurred near the detergent CMC. Both K_m and V_{max} were altered by the presence of a surface: K_m decreased to different degrees depending on the detergent, while V_{max} increased substantially. The K_m for cIP was 90 mM without detergent and decreased to 29 mM with diC_7PC micelles added; V_{max} increased almost 7-fold in the presence of diC_7PC micelles. The enzyme efficiency (V_{max}/K_m) in the presence of diC_7PC increased more than 21-fold, but it was still 20-fold lower than initial phosphotransferase activity for monomeric dihexanoylphosphatidylinositol. The poor efficiency of the cyclic phosphodiesterase activity is largely due to substrate binding affinity. The dependence of rate on substrate concentration exhibits cooperative behavior, especially without detergent. This cooperativity is discussed in terms of protein aggregation and ligand binding sites on the enzyme.

Phosphatidylinositol-specific phospholipase C (PI-PLC)[1] catalyzes the cleavage of phosphatidylinositol (PI) to produce lipid-soluble diacylglycerol and water-soluble *myo*-inositol phosphates. Intracellular PI-PLCs are prevalent in mammalian cells (Takenawa & Nagai, 1981; Homma et al., 1988; Rhee et al., 1989) and are involved in phosphatidylinositol signal transduction pathways producing the 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 (Berridge, 1987; Nishizuka, 1986). Extracellular PI-PLCs are secreted by several microorganisms (Low, 1981; Ikezawa et al., 1981); these enzymes do not cleave the phosphorylated forms of PI. However, the bacterial phospholipases have the ability to cleave the glycosyl-PI moieties of membrane protein anchors (Low & Satiel, 1988; Ferguson et al., 1988; Ikezawa, 1991). PI-PLC enzymes 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).

Detailed information on the mechanism of the bacterial enzymes shows that this enzyme catalyzes an intramolecular phosphotransfer reaction to form inositol 1,2-(cyclic)-phosphate (cIP) (Volwerk et al., 1990). The cyclic inositol phosphodiester dissociates and is only slowly hydrolyzed to inositol 1-phosphate (I-1-P). A recent X-ray structure of PI-PLC from *Bacillus cereus* suggests that PI hydrolysis may be mediated by general acid and base catalysis using two conserved histidines (Heinz et al., 1995). The bacterial PI-PLC reactions occur independent of Ca^{2+} and with inversion of phosphorus configuration for cIP and retention of phosphorus configuration for I-1-P (Lin et al., 1990; Bruzik et al., 1992, 1994). This is rationalized as a result of a sequential mechanism. The second step in the mechanism, the cyclic phosphodiesterase activity, has not been well-characterized for any PI-PLC enzyme even though it involves a soluble substrate and a soluble protein. Furthermore, an explanation for the difference in the efficiency of these two steps, phosphotransferase and cyclic phosphodiesterase, is not apparent from the crystal structure of the enzyme. Mammalian PI-PLC enzymes also hydrolyze phosphatidylinositols to yield cyclic and noncyclic inositol phosphates at different ratios that depend on the isozyme, degree of phosphorylation of PI substrate, and other conditions such as pH and Ca^{2+} (Majerus et al., 1986, 1988; Kim et al., 1989). Since there is some sequence homology between the bacterial

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[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; PI, phosphatidylinositol; cIP, *myo*-inositol 1,2-(cyclic)-phosphate; I-1-P, *myo*-inositol 1-phosphate; PC, phosphatidylcholine; diC_nPC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PS, phosphatidylserine; PM, phosphatidylmethanol; DAG, diacylglycerol; TX-100, Triton X-100; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DOC, deoxycholate; NPIP, *myo*-inositol 1-(4-nitrophenyl phosphate); C_n-thio-PI, *rac*-(alkylthiophosphoryl)-1-*myo*-inositol; pyrene-PI, *rac*-4-((1-pyreno)butylphosphoryl)-1-*myo*-inositol; 2-NIP, 2-naphthyl *myo*-inositol 1-phosphate; CMC, critical micelle concentration.

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enzyme and the mammalian PI-PLC X-domain, the region essential for catalysis (Kuppe et al., 1989), there may be mechanistic similarities.

A common property of water-soluble phospholipases is "interfacial activation", the preference of aggregated over monomeric substrate. This kinetic effect has also been documented for the bacterial PI-PLC and was initially observed for short-chain PI (Lewis et al., 1993) and a PI analog (Hendrickson et al., 1992). Interfacial activation is thought to involve changes in enzyme conformation, presumably caused by the surface interacting with the enzyme at a site distinct from the active site. The surface binding switches the enzyme from a less active to a more active state, and hence represents allosteric regulation. In the case of the bacterial PI-PLC, there is a change in intrinsic fluorescence of the protein when nonsubstrate micelles are added to the enzyme (Volwerk et al., 1994). A mammalian isozyme, PI-PLC-δ, also exhibits interfacial activation (Rebecchi et al., 1993).

In the present work, water-soluble cIP was used as a substrate to study the phosphodiesterase activity of PI-PLC and to investigate the possibility of interfacial activation/surface allosteric activation toward a water-soluble, monomeric substrate. This is the first detailed study of a lipolytic enzyme acting on its authentic water-soluble substrate. Kinetic parameters for cIP hydrolysis were measured in the absence and presence of detergent (e.g., Triton X-100 and diC_7PC). The binding of water-soluble substrate exhibited cooperativity. Perhaps, more uniquely, the presence of detergents profoundly increased the enzyme efficiency, affecting both turnover number and, in the case of diC_7PC, substrate affinity. This represents allosteric activation of an enzyme toward a water-soluble substrate with the optimal allosteric "ligand" identified as a surface.

MATERIALS AND METHODS

Chemicals. L-α-Phosphatidylinositol and other phospholipids, including dibutyroyl-PC (diC_4PC), dihexanoyl-PC (diC_6PC), diheptanoyl-PC (diC_7PC), and dioctanoyl-PC (diC_8PC), were obtained from Avanti and used without further purification. Detergents and other reagents were purchased from Sigma and used without further purification.

Enzymatic Synthesis and Purification of Inositol 1,2-(Cyclic)-Phosphate. Crude phosphatidylinositol (50% PI) was purchased from Sigma. PI (1 g) was solubilized with 15 mL of 4% Triton X-100 in Tris buffer, pH 7.5, by bath sonication. PI-PLC enzyme (~20 μg) was added to this mixture and the reaction progress monitored by ^{31}P NMR spectroscopy. After the PI was completely hydrolyzed to cIP, further reaction (e.g., hydrolysis of cIP to I-1-P) was stopped by the addition of chloroform. A chloroform−methanol mixture was used to extract lipid (DAG and any unhydrolyzed PIs). The cIP, in the aqueous phase, was further purified using an AG1-X8 anion exchange column. The AG1-X8 resin (5 g of the chloride form, 100−200 mesh), purchased from Bio-Rad, was used for about 100 mg of crude cIP. The resin was converted to its formate form by rinsing with 1 N NaOH, washing with deionized water to a neutral pH, elution of 2 bed volumes of 1 M formic acid, followed by washing with deionized water to pH 7. The crude cIP was applied to the column and eluted with 40 mM of ammonium formate and 10 mM of formic acid with a linear flow rate of about 0.5 cm/min. About 110 mg of pure cIP was obtained from 1 g of crude PI.

Purification of PI-PLC Enzyme. A recombinant strain of *Bacillus subtilis* transfected with the *Bacillus 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). An additional ion exchange column, HiTrap SP, was used to further purify the enzyme. The column volume of this cation exchanger was 1 mL, and the elution rate was 1 mL/min. The enzyme was applied in acetate buffer, pH 4.5, and eluted with a NaCl gradient. The enzyme purity was checked by SDS gel electrophoresis; enzyme concentration was determined with the Bradford (1975) assay using bovine serum albumin as a standard. About 20 mg of pure PI-PLC enzyme could be obtained from an 8 L culture. The enzyme was stored at −20 °C in 50% glycerol (v/v), 20 mM Tris buffer, pH 8.5, containing 0.02% sodium azide. The concentrated stock solution was further diluted with 0.1% bovine serum albumin in 20 mM Tris buffer, pH 8.5, prior to enzyme assays.

^{31}P NMR Assays of PI-PLC. The buffer used in all PI-PLC activity assays was 50 mM HEPES; the sodium salt was adjusted to pH 7.5 with HCl or DCl. All stock solutions were prepared in D_2O and the pH meter reading was adjusted to 7.5 before addition to the assay solution. Mixed micelles of PI with Triton X-100, diC_7PC, or other detergents were prepared by addition of the detergent solution to dry PI and bath sonication of the mixture. All such solutions were optically clear. ^{31}P NMR spectra were acquired at 202.7 MHz on a Varian Unity 500 spectrometer using 5 mm sample tubes. The ^{31}P NMR parameters were optimized based on those previously used by Griffith and co-workers (Volwerk et al., 1990). These included a 4.5 μs pulse width (70°), recycle time of 2.6 s, 14 998 Hz sweep width, digital resolution at 0.3 Hz/point, and ambient probe temperature (22 °C). All chemical shifts were referenced to phosphoric acid (5%) as an external standard.

For all kinetic runs, a control spectrum (t = 0 min) was acquired prior to the addition of enzyme. The reaction was initiated by addition of 15 ng−20 μg of PI-PLC depending on whether the phosphotransferase or the cyclic phosphodiesterase activity was being monitored. After the addition of the appropriate amount of PI-PLC, an arrayed experiment which generally took 1−2 h was conducted. Initial rates were obtained from the progress curve for less than 10% hydrolysis. The rate (μmol/min) was calculated from the increasing (phosphotransferase) or decreasing (cyclic phosphodiesterase) integrated intensity of cIP with incubation time. Under all experimental conditions, the ratio of signal to noise was larger than 60. The systematic error in the integrated intensity of cIP in each spectrum was less than 5%. The larger error in the rates (<15%) reflects combined errors in enzyme and substrate concentrations as well as the standard deviation in the linear fits of cIP integrated intensity as a function of time.

Gel Filtration of cIP To Assess Its Partitioning in Micelles. A Sephadex G-25 (Sigma, 26 in. × 1.5 in.) column with a volume of 120 mL was used to assess cIP partitioning in Triton X-100 and diC_8PC detergent micelles. These micelles will elute in the void volume, while the water-soluble, monomeric cIP should elute at the column volume. The

column was equilibrated with 50 mM HEPES, pH 7.5, containing a constant level of detergent monomer (0.2 mM Triton X-100 or 0.2 mM diC_8PC). The presence of the monomeric detergent had no effect on any NMR parameters of cIP, indicating it did not form a complex with that molecule. The elution rate was 24 mL/h; 2 mL fractions were collected. The Triton X-100 content in each fraction was measured by its UV absorbance at 260 nm; cIP and diC_8-PC were quantified by ^{31}P NMR spectra of each fraction.

RESULTS

Inositol Cyclic Phosphate as a Substrate for PI-PLC. There is general agreement in the literature that PI-PLC catalyzed hydrolysis of cIP is less facile than enzymatic hydrolysis of PI (Dixon et al., 1987; Sekar et al., 1987; Volwerk et al., 1990). Since the enzyme specific activity is so low, there has been no quantitative kinetic study of this cyclic phosphodiesterase activity. cIP hydrolysis will release a H^+ which can be monitored by pH-stat. However, the low specific activities reported make this assay method difficult unless moderately large amounts of enzyme are used. In contrast, the long time scale for a ^{31}P NMR assay makes it a better technique for following the phosphodiesterase activity. Substrate and product resonances are well-separated, and since both compounds are water-soluble, linewidths do not change as the reaction occurs. The ^{31}P chemical shift of cIP is independent of pH, while the ^{31}P chemical shift of the product, I-1-P, is very sensitive to pH and can be used to monitor any pH changes in the assay solution. Under experimental conditions used in this work, the pH of the assay system did not change significantly within 10−30 min depending on substrate concentration. Thus, the initial rates based on ^{31}P intensities should be reasonably accurate. Rates of cIP hydrolysis were measured initially in the absence of detergent as a function of the substrate concentration. Some individual runs showed a variable lag phase that depended on substrate concentration. The lag phase was most obvious at low substrate concentrations; typical progress curves are shown in Figure 1. The lag phase decreased with the increasing cIP (compare the time course for 4 and 16 mM cIP samples, Figures 1A and -B, respectively) and was not detected at high cIP concentrations (see Figure 1C, 126 mM cIP). Some variability in the lag phase may be due to NMR error (difficulties in integrating small intensities for I-1-P product at early time points). However, the lag phase could also be the result of enzyme adhering to the glass after addition to the NMR tube and a slow off rate into the solution that is accelerated by higher concentrations of cIP or I-1-P. In any case, initial rates of cIP hydrolysis were calculated after the lag phase. The plot of the initial rate versus cIP concentration was sigmoidal (Figure 2). Similar sigmoidal behavior has been seen previously for PI-PLC cleavage of pyrene-PI (Hendrickson et al., 1992). These data for cIP hydrolysis were fit to the Hill equation, $v = V_{max}[S]^n/(K_m^n + [S]^n)$, with V_{max} = 20 μmol min^{-1} mg^{-1}, K_m = 90 mM, and the Hill coefficient $n = 1.8$. The high K_m indicates very poor affinity of PI-PLC enzyme for water-soluble substrate cIP.

Effect of Detergent or Vesicle Interface on PI-PLC Activity. A variety of detergents (typically at 8 mM to ensure a large fraction of micelles present) and vesicles were examined for their effect on PI-PLC hydrolysis of cIP. Specific activities are shown in Table 1. At least a 2-fold

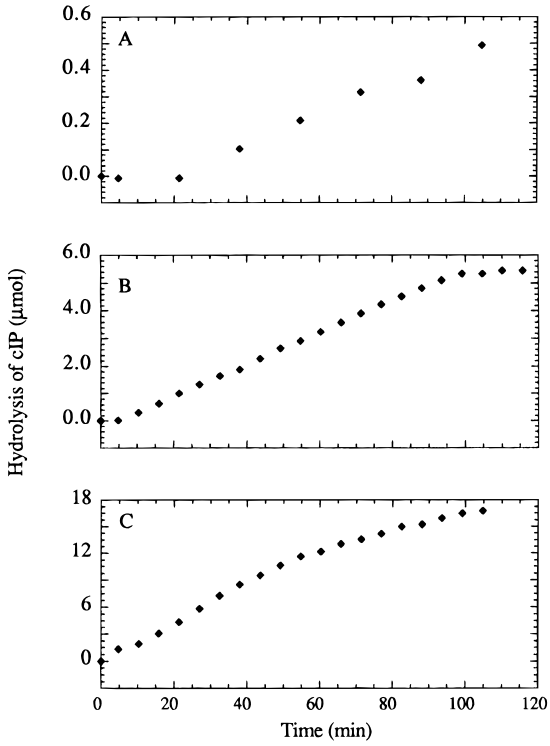

FIGURE 1: Reaction progress curves for the cyclic phosphodiesterase activity of PI-PLC (the hydrolysis of inositol cyclic 1,2-monophosphate (cIP) was calculated from the ^{31}P integrated intensity of cIP). Different lag phases are exhibited by samples with (A) 4 mM, (B) 16 mM, and (C) 126 mM cIP. Reaction conditions included 50 mM HEPES buffer, pH 7.5, and 20 μg of PI-PLC.

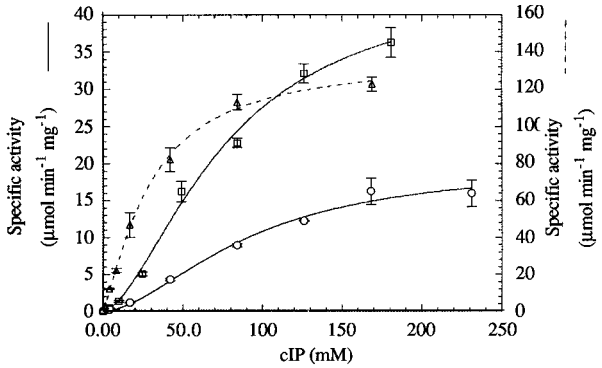

FIGURE 2: Cyclic phosphodiesterase activity of PI-PLC as a function of inositol cyclic 1,2-monophosphate (cIP) concentration in the absence of detergent (○) or presence of (□) 8 mM Triton X-100 or (△) 8 mM diC_7PC. The left axis label is for the points connected by a solid line; the right axis label is for points connected by a dashed line. Reaction conditions included 50 mM HEPES buffer, pH 7.5, 20 μg of PI-PLC enzyme in the absence of detergent or with Triton X-100, and 1 μg of enzyme in assays with diC_7PC present. The lines through data points represent fits with the Hill equation.

increase in the rate was observed for almost all the detergents examined. CHAPS and taurocholate, both bile salts, have the same hydrophobic part, and their activation of PI-PLC is almost same, although one is zwitterionic and the other is anionic. The effect of deoxycholate (DOC), another bile salt, is more complex. The PI-PLC reaction progressive curve was biphasic: there was an initial burst followed by a rate 20% lower than for that cIP concentration alone. DOC has a smaller negatively charged head group compared to taurocholate, and it could perhaps occupy the active site. All these bile salt detergents are well above their CMC at 8 mM

Table 1: Effect of Detergent and Phospholipid Aggregates on PI-PLC Phosphotransferase and Cyclic Phosphodiesterase Specific Activity[a]

detergent or phospholipids	CMC (mM)	cyclic phosphodiesterase (μmol min^{-1} mg^{-1})	phosphotransferase act. (μmol min^{-1} mg^{-1})
none		0.51 ± 0.04	
DOC	3	0.40 ± 0.02	783
taurocholate	3−6	1.25 ± 0.07	660
CHAPS	3−5	1.18 ± 0.06	845
TX-100	0.3	0.97 ± 0.06	528
β-OG	25	0.96 ± 0.05	
diC$_4$PC	∼280[b]	0.72 ± 0.04	
diC$_6$PC (4 mM)	14[b]	1.93 ± 0.08	
diC$_6$PC	14[b]	3.53 ± 0.30	367
diC$_7$PC	1.5[b]	15.5 ± 2.3	274
diC$_8$PC (2 mM)	0.27[b]	20.1 ± 0.3	
diC$_8$PS	<0.5[c]	0.80 ± 0.04	
diC$_8$PG	<0.5[c]	1.14 ± 0.1	
diC$_8$PA	0.45[d]	0.56 ± 0.08	
diC$_6$PE	12[c]	2.52 ± 0.20	
1-C$_{10}$-PC	6.4[b]	9.64 ± 1.0	
1-C$_{12}$-PC	0.67[b]	11.1 ± 1.7	
DMPM vesicles		0.05 ± 0.01	
DPPC vesicles		7.40 ± 0.7	

[a] Assay conditions include 8 mM detergent or phospholipid unless otherwise noted, 50 mM HEPES, pH 7.5, and 8 mM cIP or 4 mM PI; 20 μg or 58 ng of enzyme was used in the cyclic phosphodiesterase or phosphotransferase activity assay. Mixed micelles of PI with the detergents were prepared by bath sonication; the PM and PC vesicles were small unilamellar vesicles prepared by sonication. [b] Values from Bian and Roberts (1992). [c] Unpublished CMC values determined in this laboratory. [d] Value from Garigapati et al. (1995).

and activate PI-PLC enzyme toward cIP about 2−3-fold. The nonionic detergents TX-100 and β-octyl glucoside also produce a 2−3-fold increase in the PI-PLC specific activity under these conditions, suggesting little structural specificity for this general detergent activation of PI-PLC.

However, short-chain PC and lyso-PC micelles are much more potent activators of PI-PLC. The specific activity of enzyme increased 30-fold in the presence of diC$_7$PC at 8 mM (the CMC for this short-chain PC is 1.5 mM) and 40-fold in the presence of diC$_8$PC. DiC$_6$PC, with its CMC of 14 mM, activated the enzyme 4-fold at 4 mM and 7-fold at 8 mM. DiC$_4$PC, which is monomeric and has an extremely high CMC ∼280 mM (Bian & Roberts, 1992), does lead to some activation of PI-PLC (1.4-fold). These detergents may activate by binding the PI-PLC at the same site, since in the presence of TX-100 micelles (8 mM), diC$_4$PC (8 mM) has no further effect on the activation of the enzyme. To determine if the hydrophobic diacylglycerol moiety or lipid head group was important for the activation of enzyme, diC$_8$-PS, diC$_8$PG, diC$_8$PA micelles, diC$_6$PE monomers, dimyristoyl-PM and dipalmitoyl-PC small unilamellar vesicles, and lyso-PC micelles were examined. In contrast to the 30−40-fold increase in specific activity induced by short-chain PC micelles, diC$_8$PS produced a 1.5-fold activation. The PA and PG head groups also produced a small activation (1.1- and 2.2-fold, respectively). The diC$_6$PE, which was below its CMC under these conditions, activated PI-PLC 5-fold. This should be compared to the effect of 8 mM diC$_6$-PC, which gives rise to a 7-fold increase in PI-PLC specific activity. Thus, the PE head group, like the zwitterionic PC, activates the enzyme significantly. Dimyristoyl-PM vesicles inhibited the enzyme, while dipalmitoyl-PC vesicles activated PI-PLC to within a factor of 2 of the short-chain PC micelles. Lyso-PCs (1-C$_{10}$-PC and 1-C$_{12}$-PC) micelles, with CMC values considerably higher than those of the diacyl-PCs examined, activated the enzyme, but were less efficient than diC$_7$PC. Clearly, these data indicate that the polar head group is critical for activation or inhibition of the enzyme. More interestingly, a zwitterionic head group such as choline or ethanolamine appears necessary for optimum activation, while all other detergents activate PI-PLC 2−3-fold toward cIP. Two-chain PC micelles are slightly more effective than single-chain (lyso-PC), while both of these are more effective than PC in vesicles. The packing of phospholipid substrates in a micelle rather than a bilayer leads to a higher apparent rate of hydrolysis by phospholipases (Roberts & Dennis, 1989). This results come from the poor binding of enzyme to the zwitterionic PC substrate aggregate (Ramirez & Jain, 1991).

To investigate this further, the kinetic activation of PI-PLC toward cIP (8 mM) by micelles of diC$_8$PC, diC$_7$PC, diC$_6$PC, and TX-100 was examined (Figure 3). For all these detergents, the half-maximal activation occurred at a concentration around the CMC of the detergents (the CMC is 0.2 mM for diC$_8$PC, 1.5 mM for diC$_7$PC, 14 mM for diC$_6$-PC, and 0.23 mM for TX-100). A hyperbolic equation could be used to fit the data for diC$_8$PC and TX-100 detergent activation of PI-PLC, since there were no points below 0.1 mM. For these two detergents, the concentration for half of the maximum activation was near the CMC (see Figure

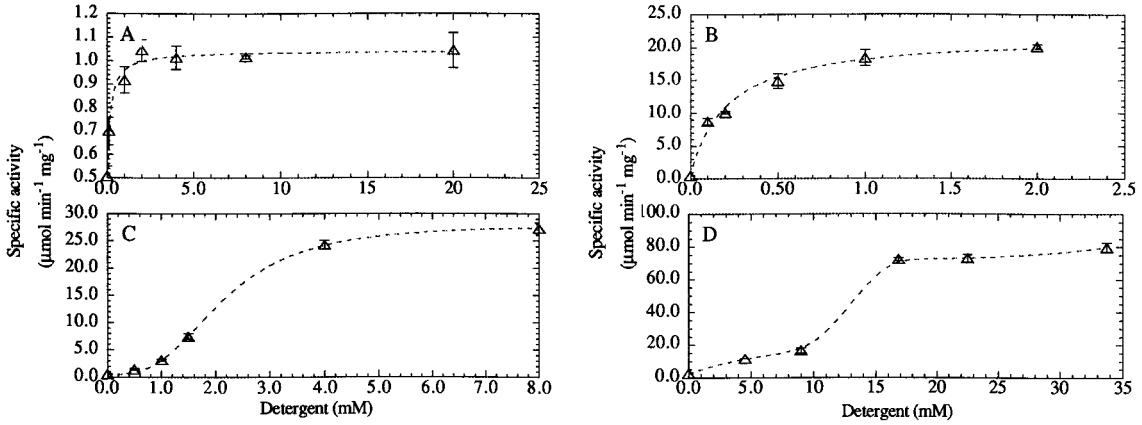

FIGURE 3: Change in cyclic phosphodiesterase activity as a function of the concentration of (A) Triton X-100, (B) diC$_8$PC, (C) diC$_7$PC, and (D) diC$_6$PC. The cIP concentration was 8 mM except in (D) where it was 15 mM. The enzyme concentration was 2.0 μg in (A), 1 μg in (B) and (C), and 2.2 μg in (D). Data in (A) and (B) were fit by a hyperbolic binding equation.

3A for TX-100 and Figure 3B for diC_8PC). When activators with higher CMC values were examined, the dependence of PI-PLC activity toward a fixed concentration of cIP became sigmoidal. With diC_7PC there was a small amount of activation below the CMC and a much larger increase around the CMC (Figure 3C). PI-PLC specific activity reached a plateau above the CMC. This same behavior was exhibited in PI-PLC activation by diC_6PC (Figure 3D). Below 10 mM, where diC_6PC is monomeric, activation of enzyme depended on the concentration of PC. It should be noted that the cIP concentration used in the diC_6PC activation experiment (15 mM) was about double that used for the TX-100, diC_8PC, and diC_7PC activation series. The maximal PI-PLC activity with activating diC_6PC was roughly double that observed with the diC_8PC system. The half-maximal PI-PLC activation occurred at 0.19 mM for Triton X-100, 0.21 mM for diC_8PC, 2 mM for diC_7PC, and 12 mM for diC_6PC; the correspondence to the CMC for each of those detergents should be noted.

The observation that micellar PC activates PI-PLC is consistent with an allosteric model for PC activation of PI-PLC with the surface binding at a site distinct from the active site. Monomeric PC appears to be less effective in binding to this allosteric site and promoting the appropriate change in the enzyme since it cannot activate PI-PLC as well. The presence of 8 mM short-chain PC causes a 30-fold increase in PI-PLC specific activity for diC_7PC, yet only a 7-fold increase for the comparable concentration of diC_6PC. These two lipids only differ by a methylene group on each chain. If PI-PLC activation were an allosteric event caused by the binding of a *single* PC molecule, then one would expect these two short-chain PCs to act similarly regardless of the fact that one is micellar and the other monomeric. That they do not (and that the shorter one activates as well as the slightly longer one once its concentration is above the CMC) argues against a single *isolated* PC ligand binding to an allosteric site as the optimal activator. This kinetic behavior is, however, consistent with the allosteric site interacting with a PC surface and the surface providing the optimal allosteric ligand. Three possible explanations of this are that (i) several PC molecules bind to the enzyme and enhance the conversion of PI-PLC from a low activity to a high activity form, (ii) one PC molecule in an interfacial conformation stabilized by the surface binds at the allosteric site, or (iii) the surface around the activator molecule(s) in the micelle slows down its off rate from the enzyme allosteric site, thus enhancing activation of the enzyme. Below the CMC of the allosteric PC ligand, the correct conformation of PC may be less probable or multiple molecules may not have as high an affinity for the allosteric site.

Whatever the actual mechanism of this allosteric interfacial activation, above the CMC, the short-chain PC micelle supplies a surface for the enzyme allosteric site to be easily saturated with an appropriate ligand. This is consistent with a fluorescence study that shows the intrinsic fluorescence intensity of PI-PLC is increased when the enzyme binds to a surface (Volwerk et al., 1994). With diC_8PC, an increase in fluorescence intensity was observed above 0.1 mM, and the half-maximal increase was around 0.2 mM (Volwerk et al., 1994). This type of interfacial activation can be viewed as classic allosterism, but it is the PC surface that causes the transition from a less active state ("T-state") to a more active form ("R-state"). The very cooperative increase in activation at the CMC of diC_7PC and diC_6PC (and other detergents) makes no sense unless one has the enzyme interacting with the PC surface. If it were purely a matter of K_D for the activator ligand depending on PC chain length, the dependence on PC concentration should be hyperbolic and one would not expect an increase at the CMC.

Table 2: Kinetic Parameters for PI-PLC Catalyzed Hydrolysis of cIP

	no detergent	TX-100 (8 mM)	diC_7PC (8 mM)
V_{max} (μmol min^{-1} mg^{-1})	20	45	136
K_m (mM)	90	76	29
V_{max}/K_m	0.22	0.59	4.69
Hill coeff, *n*	1.8	1.7	1.3
R	0.996	0.998	0.999

The dependence of PI-PLC activity on cIP concentration in the presence of diC_7PC and TX-100 micelles (8 mM) is shown in Figure 2 (for comparison to cIP hydrolysis in the absence of any interface). An 8 mM detergent concentration is well above that needed for each detergent to maximally activate PI-PLC, as determined from Figure 3. The plot of the initial rate versus cIP concentration was sigmoidal rather than hyperbolic with Triton X-100 present, with a Hill coefficient of 1.7 ± 0.2 compared to 1.8 ± 0.3 for cIP alone. TX-100 increased V_{max} 2-fold and decreased K_m marginally. However, with diC_7PC present, the activity versus substrate curve was much less sigmoidal (Hill coefficient $= 1.3 \pm 0.1$). DiC_7PC increased V_{max} about 7-fold and decreased K_m 3-fold. With the short-chain PC present, the data could also be fit by a hyperbolic equation with a slight increase in K_m ($\sim$30$-$34 mM). The significant decrease in the Hill coefficient with the more effective activating detergent (from 1.8 to 1.3) indicates that, in the presence of the PC, PI-PLC loses cooperativity in binding and cleaving cIP. The enzyme efficiency can be measured by the ratio of V_{max}/K_m. The presence of diC_7PC micelles increased the enzyme efficiency more than 21-fold. These kinetic results, summarized in Table 2, indicate that detergent can affect the catalytic rate constant and substrate binding for PI-PLC cleavage of its water-soluble substrate cIP.

This detergent activation of PI-PLC can certainly complicate kinetics if assay results with and without detergent are compared. It has been noted that PI stereochemistry (i.e., the D-inositol but not the L-inositol phospholipid as substrate) is critical; however, in a previous study (Volwerk et al., 1990) it was claimed that L-cIP may be an inhibitor of the phosphodiesterase activities of PI-PLC. Such a change in the stereochemical requirement for substrate binding is unusual. These researchers compared rates for PI-PLC hydrolysis of pure D-cIP and a synthetic racemic mixture of D- and L-cIP. The D-cIP was synthesized enzymatically from long-chain PI, and the detergent was not removed. It was noted that the enzyme specific activity was 2-fold higher for the D-cIP compared to the racemic system. The present work shows that detergent activation of PI-PLC toward cIP hydrolysis can be used to explain this kinetic difference. Thus, the stereochemical specificity for PI binding and hydrolysis is maintained in the enzymatic hydrolysis of cIP.

Interactions of cIP with Detergent Micelles or Monomers. An alternate explanation for the detergent activation of PI-PLC hydrolysis of cIP is that the soluble substrate, cIP, becomes associated with micelles and in this state is a better

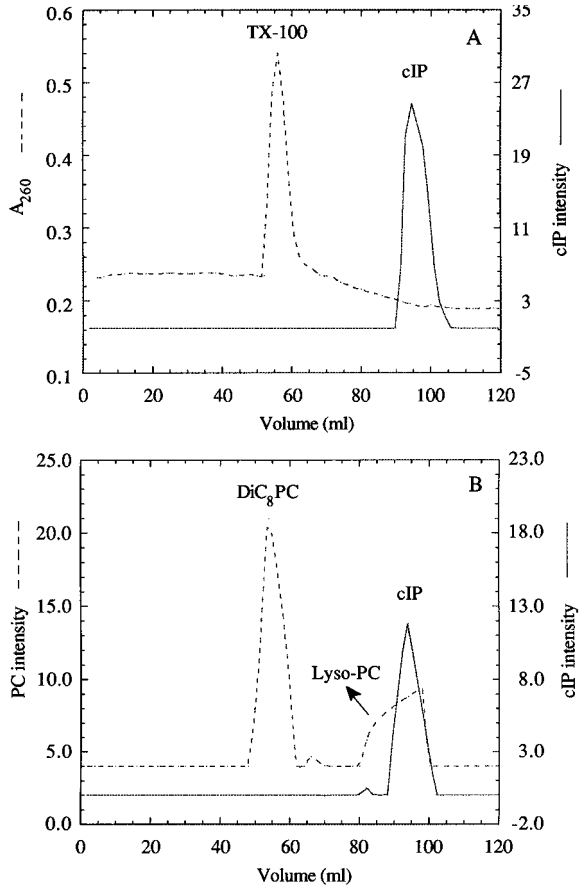

FIGURE 4: Elution profile of cIP mixed with detergent micelles and applied to a Sephadex G-25 column: (A) Triton X-100; (B) diC_8PC. The column was equilibrated and samples were eluted with 50 mM HEPES, pH 7.5, containing 0.2 mM detergent to maintain a constant monomer concentration.

substrate for PI-PLC. This was explored with gel filtration using a Sephadex G-25 column. As can be seen in Figure 4A, when a 1 mL sample of Triton micelles (8 mM) mixed with cIP (4 mM) was applied to the column, no detectable cIP eluted in the void volume with the detergent micelles. The micelles were well-separated from cIP by this column. Thus, the Triton X-100 activation cannot be explained by solubilization of substrate in micelles. The same experiment with diC_8PC as the detergent showed a similar result. Analysis of the different phosphate compounds in each fraction was determined from its ^{31}P NMR spectrum. Again, no cIP elutes with the PC micelles in the void volume. For the PC sample used, a small amount of lyso-PC (1-C_8PC) was present. Although this single-chain amphiphile has a higher CMC (63 mM) than the diacyl-PC (and a molecular weight (MW 383) larger than the cIP anion (MW 239)), it may form small aggregates below its CMC in the presence of another amphiphile such as monomeric diC_8PC. If it did so, its elution volume would decrease slightly compared to other small molecules. The cIP peak occurs at the same elution volume in both Triton X-100 and diC_7PC experiments (and in control columns with no detergent added). It does not overlap with the lyso-PC which elutes slightly ahead of it, a result that strongly suggests that cIP does not form small aggregates with detergent. Thus, the activation of PI-PLC toward soluble cIP cannot be the result of partitioning the cIP in a micelle. A reasonable interpretation is that the observed activation of PI-PLC is the result of an allosteric interaction of the detergent with the enzyme.

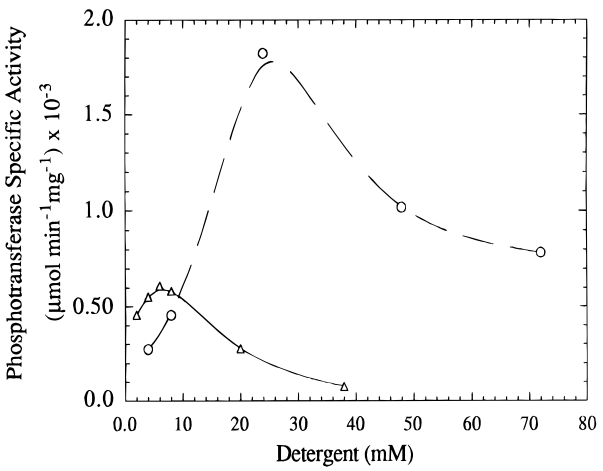

FIGURE 5: Effect of added detergent on the phosphotransferase activity of PI-PLC toward 4 mM phosphatidylinositol: (○) diC_7-PC, (△) TX-100. Assay conditions included 50 mM HEPES, pH 7.5, 4 mM phosphatidylinositol, variable detergent concentration, and 58 ng of enzyme.

PI in Detergent Mixed Micelles as Substrates for PI-PLC. Unilamellar vesicles of long-chain PI are poor substrates for PI-PLC. For an efficient assay of PI cleavage to cIP by PI-PLC, detergents are required to solubilize the long-chain phosphatidylinositol in an aggregate with the substrate accessible to the enzyme. For a detergent such as Triton X-100, a ratio of 2:1 detergent to phospholipid (in a liquid crystalline state) is needed to convert all lamellar structures to mixed micelles (Dennis, 1993; Carman & Dennis, 1995). The phosphotransferase rates for PI-PLC acting on PI in different detergent micelles at 2:1 detergent/PI are listed in Table 1. They vary somewhat with the identity of the detergent, but are all notably higher than PI-PLC specific activities toward cIP with the same detergents present. The differences in the phosphotransferase rates might reflect differences in the morphology of the mixed micelles, interaction of the detergent molecules with the enzyme, or differences in solubilization efficiency of the long-chain PI.

From this series, two detergent mixed micelle systems (TX-100 and diC_7PC) were chosen to examine how the surface concentration of PI affected PI-PLC specific activity. This type of experiment (referred to as a "surface dilution experiment") has been carried out for a number of lipolytic enzymes using Triton X-100/phospholipid mixed micelles (Dennis, 1973; Eaton et al., 1976; Roberts et al., 1977; Hendrickson et al., 1984; Carman & Dennis, 1995). For a fixed concentration of long-chain phospholipid, the detergent concentration is increased. There is invariably an initial increase in observed enzymatic activity that represents increasing the availability of substrate in micelles as opposed to multilamellar structures (which are poor substrates in general for many lipolytic enzymes). Once the phospholipid substrate is solubilized in mixed micelles, further increases in detergent decrease the surface concentration of the substrate, and if this parameter is kinetically important, the observed specific activity drops. Alternatively, the decrease could represent detergent binding to the enzyme as an inhibitor. Such an experiment is shown in Figure 5 for 4 mM PI solubilized by either Triton X-100 or diC_7PC. Both systems exhibited the characteristic initial increase and then decrease as detergent concentration was increased, although the maximum enzymatic activity and detergent concentration

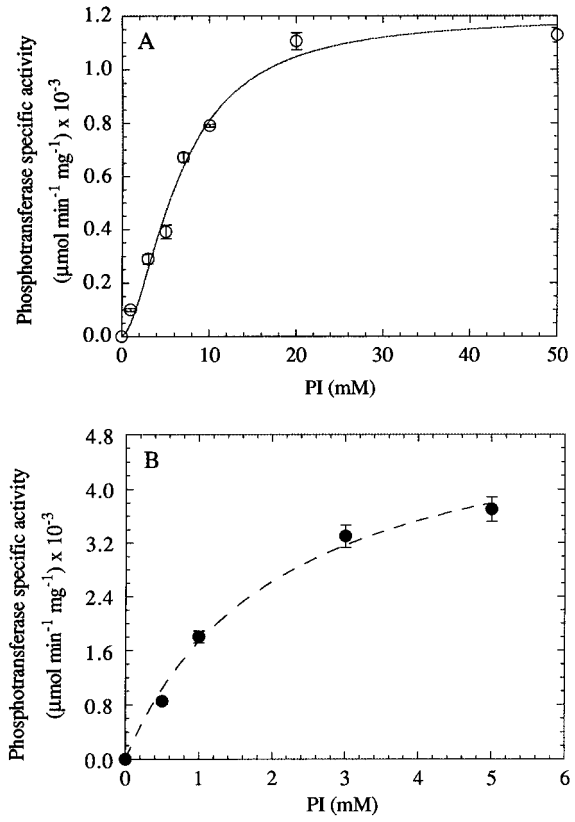

FIGURE 6: Phosphotransferase activity of PI-PLC as a function of PI concentration in diC_7PC mixed micelles with the ratio of PI/diC_7PC fixed at (A) 1:2 and (B) 1:8. Assay conditions included 50 mM HEPES, pH 7.5, and 58 ng of enzyme in (A) and 15 ng in (B). At 1:2 PI/diC_7PC, the Hill equation was used to fit the data with $K_m = 6.5 \pm 0.7$ mM, $V_{max} = 1200 \pm 70$ μmol min^{-1} mg^{-1}, and n, the Hill coefficient, as 1.7 ± 0.3; at 1:8 PI/diC_7PC, the fit with the Hill equation used $K_m = 1.2 \pm 0.02$ mM, $V_{max} = 4080 \pm 40$ μmol min^{-1} mg^{-1}, and $n = 1.6 \pm 0.3$.

at which activity begins to decrease depended on the identity of the detergent. For the Triton X-100 system, the maximum of this "surface dilution curve" is around 2:1 Triton X-100/PI. For the diC_7PC system, complete solubilization of PI is achieved at 2:1 diC_7PC/PI (as measured by the decrease in solution light scattering), but the maximum in PI-PLC activity does not occur until a much higher ratio of short-chain PC to PI (around 6 to 1). Comparing maximum activities for the two systems shows that long-chain PI in diC_7PC micelles is hydrolyzed 3.5-fold faster than in TX-100 micelles. Interestingly, this is comparable to what is observed with hydrolysis of the water-soluble substrate cIP: V_{max} with diC_7PC present is 3-fold larger than for TX-100.

The apparent K_m for PI was measured in the TX-100 and diC_7PC mixed micelles under the condition that the ratio of substrate to TX-100 or diC_7PC was fixed at 1:2, as well as for PI/diC_7PC fixed at 1:8. Plots of the reaction rate versus substrate concentration were sigmoidal (see Figure 6 for the curve for diC_7PC/PI). The apparent K_m for PI in TX-100 mixed micelles was estimated as 3 mM. PI-PLC exhibited an apparent K_m that varied with the ratio of PI to detergent in an interesting fashion in the diC_7PC/PI mixed micelle system (Figure 6). With the Hill equation to fit the data, the apparent K_m was 6.5 ± 0.7 mM and the V_{max} was 1200 ± 70 μmol min^{-1} mg^{-1} for the PI-PLC phosphotransferase reaction toward PI in diC_7PC/PI (2:1) mixed micelles. If a noncooperative model was used to fit the data, K_m was 9.4 $\pm$ 2.2 mM. When the diC_7PC/PI ratio was increased to 8:1, a value near the maximum of the "surface dilution" curve, the apparent K_m decreased to 1.2 ± 0.02 mM ($V_{max} = 4080 \pm 40$ μmol min^{-1} mg^{-1}). A noncooperative binding model yielded a K_m of 2.1 ± 0.4 mM. The Hill coefficient was similar for both these diC_7PC/PI ratios (1.7 ± 0.3 at 2:1 and 1.6 ± 0.03 at 8:1). The decrease in K_m as the surface concentration of the detergent is increased beyond that required for solubilization of the long-chain phospholipid is unusual. It indicates that the surface acts in a way that is kinetically distinct from just diluting the surface concentration of substrate. It is consistent with PC activating PI-PLC for PI cleavage to cIP as well.

A comparison of PI-PLC catalyzed cleavage of PI in Triton X-100 mixed micelles to cIP hydrolysis in the presence of this detergent also provides insights into the cause of the decreased activity at higher Triton X-100. There was no detectable inhibition at the high detergent concentrations in the cIP system (see Figure 3A; the PI-PLC specific activity is the same with 20 mM as well as 5 mM Triton X-100 present). However, inhibition was observed for the PI/detergent mixed micelle assays with the same detergent concentrations (again with TX-100 the PI-PLC activity with 20 mM detergent is roughly half of that at 5 mM). If Triton X-100 was noninhibitory in the cIP assay system, these molecules must be not be competitive inhibitors, in the PI system. The only way to explain the decreased activity toward PI in the Triton X-100/PI mixed micelle system is that the PI surface concentration is decreasing and this is an important parameter in PI cleavage. Competitive inhibition by Triton X-100 is not a problem for this phospholipase.

DISCUSSION

Many phospholipases are water-soluble enzymes, while their substrates are insoluble and tend to self-aggregate. The transfer of these enzymes from the aqueous phase to the lipid−water interface is necessary for optimal catalysis. Once at the lipid−water interface, the enzyme can recognize substrate and catalyze its cleavage. A common feature of these lipolytic enzymes is interfacial activation, an enhancement in k_{cat} caused by binding to an interface. This activation could be due to different mechanisms that include substrate-based (such as changes in the conformation or surface concentration of substrate) and enzyme-based (changes in the conformation or allosteric binding) effects. These effects are difficult to separate with an aggregated substrate. In the present work, the simplified kinetic analysis of the cyclic phosphodiesterase activity of PI-PLC toward its water-soluble substrate cIP was used to explore interfacial activation due to a direct interaction of the interface with the PI-PLC that cannot alter the physical state of substrate. Determination of the kinetic parameters in the absence and presence of detergents can be easily accomplished.

Kinetic constants for PI-PLC catalyzed hydrolysis of cIP in the absence and presence of detergent clearly show that the binding of enzyme to an interface enhances the catalytic step and for an optimal interface increases the binding affinity of the enzyme for the substrate. The results for PI-PLC represent a case of allosteric regulation where the optimal allosteric "ligand" is a PC molecule(s) presented in an interface. Interfacial activation toward a phospholipid substrate was first observed with phospholipase A_2 (Roholt

Table 3: Kinetic Constants for PI-PLC Activity toward Different PI Analogs

	D-NPIP[a]	2-NIP[b]	pyrene-PI[c]	C_{16}-thio-PI[c]	C_{12}-thio-PI[c]	diC_6PI,[d] monomer	diC_6PI,[d] micelle
V_{max} (μmol min^{-1} mg^{-1})	650	0.044[e]	68	6.25	6.17	100	560
K_m (mM)	5	6−8[f]	0.87	0.022	0.25	1	<1
V_{max}/K_m	130		78	284	25	100	>560
Hill coeff, *n*			1.9			>1[g]	

[a] *myo*-Inositol 1-(4-nitrophenyl phosphate); kinetic constants from Leigh et al. (1992). [b] 2-Naphythyl-*myo*-inositol 1-phosphate (Shashidhar et al., 1991). [c] From Hendrickson et al. (1992). [d] From Lewis et al. (1993). [e] Specific activity at a substrate concentration of 0.8 mM. [f] Estimate based on data in Shashidhar et al. (1991). [g] The kinetics toward monomeric diC_6PC are cooperative, although the data were insufficient to delineate an accurate assessment of *n*.

& Schlamowitz, 1961) and further investigated in detail by many authors. The binding of phospholipase A_2 to the interface enhances the affinity of the enzyme for active-site-directed ligands and is thought to affect the catalytic step to a lesser degree (Jain et al., 1993). A recent study of PI-PLC using a water-soluble substrate analog NPIP (*myo*-inositol 1-(4-nitrophenyl phosphate)) showed that binding PI-PLC to phospholipid vesicles changed the catalytic constant, k_{cat}, for dinitrophenol production without appreciably affecting the substrate binding (Volwerk et al., 1994). In the case of cIP, binding of PI-PLC to the detergent micelles presumably induces a conformational change that makes the PI-PLC active site more efficient in processing the aqueous substrate—a detergent induced conversion from a less active T-state to a more active R-state, in allostery parlance. An interface-induced conformational change has been confirmed using the intrinsic fluorescence of PI-PLC (Volwerk et al., 1994). Change in the intrinsic fluorescence of at least one tryptophan was correlated with high activity.

The kinetic parameters for cIP hydrolysis ($K_m = 90 \pm 17$ mM and $V_{max} = 20 \pm 3$ μmol min^{-1} mg^{-1}) can be compared to similar constants for monomeric PI cleavage to cIP (Table 3). The K_m for the PI-PLC cleavage of monomeric short-chain diC_6PI was estimated at 1 mM with a V_{max} of 90 μmol min^{-1} mg^{-1} (Lewis et al., 1993). The enzyme efficiency, V_{max}/K_m, for monomeric substrate diC_6PI hydrolysis to cIP is about 450-fold higher than for cIP hydrolysis to IP. Toward NPIP, which is also monomeric, PI-PLC exhibits a V_{max}/K_m ~600 times that for cIP without an interface present (Leigh et al., 1992). Clearly, comparing enzyme efficiencies for both these monomeric PI substrates in the phosphotransferase reaction to the hydrolysis of cIP in the absence of an interface indicates the largest discrepancy is in the K_m values.

Kinetic parameters for monomeric cIP hydrolysis when a diC_7PC interface is present to activate the enzyme ($K_m = 29 \pm 2$ mM, $V_{max} = 136 \pm 5$ μmol min^{-1} mg^{-1}) can be compared to similar constants for interfacial PI in the phosphotransferase reaction. There is a wide range of K_m and V_{max} values depending on the structure of the PI substrate and the aggregate assay system. For a pure PI micelle, the apparent V_{max} was 560 μmol min^{-1} mg^{-1} and the apparent K_m was estimated as <1 mM. The hydrophobic part of phosphatidylinositol is not recognized specifically by PI-PLC (Bruzik et al., 1992), and this has led to studies of a wide range of PI analogs (Table 3). For all of these substrates, the enzyme efficiency (V_{max}/K_m) ranges from 25 to 284, all higher than the efficiency of 4.7 calculated for cIP hydrolysis under optimal conditions. Interestingly, the V_{max} for cIP is considerably higher, suggesting that the catalytic cleavage of cIP is as effective and not the problem in the cyclic phosphodiesterase activity. It is the K_m term which is most different for the cyclic phosphodiesterase reaction. We can suggest that the K_m largely reflects substrate binding, since a cIP nonhydrolyzable analog exhibits a K_i roughly half of the K_m for cIP (Wu et al., 1997). The hydrophobic portions of PI analogs (the nitrophenyl group in NPIP, the pyrene or thioalkyl chains, or the diacylglycerol moiety in the short-chain PI molecules), while not interacting specifically with the enzyme, do have an effect on substrate binding.

It should be noted that the two enzymatic activities of bacterial PI-PLC, phosphotransferase and cyclic phosphodiesterase, have different responses to increasing detergent concentrations. This is not unreasonable and can be easily explained. When the substrate is soluble like cIP, the added detergent can bind to the enzyme at an allosteric site and activate it. Continuing to increase the detergent would have no effect on cIP hydrolysis if the cIP is soluble (i.e., there is no interfacial concentration of cIP that would decrease as more detergent is added). However, when the substrate is not soluble (e.g., any of the PI analogs) but must first be solubilized by the detergent, allosteric activation of the enzyme can still occur by the detergent, but folded into this effect is the fact that the surface concentration of the substrate changes as more detergent is added. If the surface concentration of substrate is important kinetically, then eventually the enzyme activity should drop.

While the kinetic studies cannot identify which region of the protein interacts with an interface, the recent crystal structure of PI-PLC from *B. cereus* (Heinz et al., 1995) may provide clues for further investigation. The short helix B and the loop comprising residues 237−243 show an unusual clustering of hydrophobic amino acids that are fully exposed to solvent in the crystal structure. Both helix B and loop 237−243 are weakly defined in the electron density map, which could indicate their potential role as a flexible lid during attack on the phospholipid substrate by the enzyme. Under the crystallization conditions used, the PI-PLC enzyme is clearly monomeric. If the cooperativity in cIP kinetics comes from enzyme aggregation, then it must be from substrate inducing aggregation to a more active form. However, attempts to cross-link the bacterial PI-PLC in the absence or presence of Triton X-100, diC_7PC, or cIP and diC_7PC yielded only monomers (C. Zhou and M. F. Roberts, unpublished results). The other reasonable explanation is that enzyme has an equilibrium distribution of conformations with different activities and multiple binding sites for substrate molecules. The introduction of multiple sites for substrate is necessary to account for cooperativity in a monomeric enzyme. As the concentration of the soluble substrate increases and occupies the "allosteric" site as well as the active site, the more active form of the enzyme is stabilized. It is possible that detergent, aggregated phos-

pholipid, or soluble substrate binds to helix B and loop 237−243 and stabilizes the enzyme in the more active form.

Bacterial PI-PLC is a very efficient enzyme for the phosphotransferase reaction but is very inefficient for the cyclic phosphodiesterase reaction. Mammalian PI-PLCs hydrolyze PI to yield cyclic and noncyclic inositol phosphate simultaneously. The catalytic mechanism is sequential for bacterial PI-PLC, but whether the mammalian PI-PLC enzymes follow a sequential or parallel mechanism is unclear (Lin et al., 1990; Bruzik et al., 1992; Lewis et al., 1993). In general, prolonged stimulation of cells or tissues tends to produce higher levels of cyclic phosphates, in part because these metabolites are metabolized slowly (Dixon et al., 1987; Sekar et al., 1987). The inefficient processing of cIP by PI-PLC may be one reason that cyclic inositol phosphohydrolase activities exist *in vivo* in a wide variety of cells and tissues (Majerus, 1992). All PI-PLC enzymes share a homologous region (∼80 amino acids) located in the X-domain (Kuppe et al., 1989) that is essential for catalytic activity. In the crystal structure of PI-PLC with *myo*-inositol bound, there are two conserved histidines that could be used for general acid and base catalysis (Heinz et al., 1995). If the mechanisms are similar for both types of PI-PLC enzymes, yet the mammalian enzymes produce I-1-P as well as cIP, one could suggest that the K_m for cIP must be much lower for the mammalian PI-PLC.

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