# Phosphatidylcholine Activation of Bacterial Phosphatidylinositol-Specific Phospholipase C toward PI Vesicles†

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ABSTRACT: The effect of different phospholipids on the kinetic behavior of phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* toward PI vesicles has been investigated. Cosonicated PC/PI vesicles displayed enhanced hydrolysis of PI when less than 0.20 mole fraction PC was incorporated into the vesicle; higher mole fractions of PC led to a decrease from the maximum activity mimicking surface dilution of substrate. Since the PC could affect PI-PLC binding to vesicles, the effect of separate PC vesicles on enzymatic hydrolysis of PI vesicles was examined. Separate phosphatidylcholine vesicles were found to activate PI-PLC-catalyzed cleavage of PI vesicles up to 7-fold. The activation was completely abolished when the PC vesicle was composed of cross-linked molecules. In the absence of enzyme, fluorescence resonance energy transfer studies did not detect any fusion between PI and PC vesicles if the total lipid concentration was below 2 mM. Higher total lipid concentrations (>20 mM) increased PC transfer between PC and PI vesicles, producing a PI vesicle population with small amounts of PC in the outer monolayer. This suggested that the activation of PI-PLC toward PI vesicles reflects the time scale of transfer of PC from PC vesicles to PI vesicles. Cosonicated PC/PI vesicles provide a measure of enzyme activity versus mole fraction of PC that can be used to estimate the extent of vesicle exchange or fusion between separate vesicle pools. The effects of other phospholipid vesicles on PI-PLC hydrolysis of PI were also examined; zwitterionic lipids were activators while anionic phospholipids inhibited activity. The results indicated that PC molecules in the PI interface allosterically bind to PI-PLC and help anchor enzyme in a more active conformation to the PI interface.

Phospholipase C enzymes specific for phosphatidylinositols (PI-PLC)[1] catalyze the hydrolysis of phosphoester bonds of phosphatidylinositols to produce two second messenger molecules. One is diacylglycerol, which is membrane-localized and a potent activator of protein kinase C. The other is a soluble inositol phosphate, most notably inositol 1,4,5-trisphosphate formed from $PIP_2$ cleavage, which plays an important regulatory role in cellular activation (*1*−*3*). PI-PLC enzymes exist both in intracellular and in extracellular forms in a wide variety of tissues and organisms. Intracellular PI-PLC isozymes are prevalent in mammalian cells and are involved in second messenger metabolism (*4*, *5*), while extracellular PI-PLCs have been isolated from the culture media of several microorganisms (*6*, *7*).

Extracellular PI-PLC enzymes are water-soluble and specific for nonphosphorylated PI. They catalyze the cleavage of the glycerophosphate linkage of PI in a $Ca^{2+}$-independent manner in two steps: (i) an intramolecular phosphotransfer reaction to form inositol cyclic 1,2-monophosphate (cIP); (ii) hydrolysis of the cyclic phosphodiester to produce inositol 1-phosphate (*8*, *9*). These enzymes are modulated by interfaces in several ways. PI-PLC from *Bacillus thuringiensis* exhibits interfacial activation when its substrate, PI, is present in an interface (e.g., micelle) as opposed to existing as a monomer in solution (*10*). When water-soluble cIP was used to study the cyclic phosphodiesterase activity, a different type of interfacial allosteric activation of PI-PLC was observed. Almost all detergents examined activated the enzyme at least 2-fold. However, phosphatidylcholine (PC) species, either in micelles or in vesicles, led to a large increase in PI-PLC specific activity toward cIP (*11*). Aggregated PC was also found to increase the phosphotransferase activity of the enzyme toward long-chain PI in PC/PI mixed micelles (*11*).

PI aggregated in bilayer vesicles is the natural substrate for PI-PLC. Kinetics with vesicles are complicated by slow exchange kinetics and discrete binding and catalytic events that can be difficult to separate. For PI-PLC, other phospholipids or products can alter both vesicle association as well as specific binding of PI substrate to the enzyme. Moreover, the presence of other phospholipids may affect the activity of PI-PLC toward PI vesicles if the enzyme has

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[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; cIP, *myo*-inositol 1,2-(cyclic)-phosphate; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; DMPC, dimyristoyl-*sn*-glycero-3-phosphatidylcholine; BLPC, 1,2-bis[12-(lipoyloxyl)dodecanoyl]-*sn*-glycero-3-phosphatidylcholine; PS, phosphatidylserine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; DOPG, 1,2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; SPM, sphingomyelin; TX-100, Triton X-100; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)-PE; NBD-PE, *N*-(7-nitro-2,1,3-benzyoxadiazol-4-yl)-PE; CF, carboxyfluorescein; SUV, small unilamellar vesicle; SDVB, styrene-divinylbenzene beads.

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some affinity for the other phospholipids. In this work, the characteristics of PI-PLC action toward PI in vesicles and the role of other nonsubstrate phospholipids packed in separate vesicles in activating the enzyme have been examined. This allows us to put constraints on how the PC allosterically activates the enzyme.

## MATERIALS AND METHODS

*Materials*. L-α-Phosphatidylinositol and other phospholipids, including POPC, DMPC, POPE, POPS, POPA, DOPG, Rho-PE, and NBD-PE, were obtained from Avanti in chloroform solutions. Carboxyfluorescein (CF), sphingomyelin and $D_2O$ (99.9%) were purchased from Sigma. Small unilamellar vesicles formed of cross-linked 1,2-bis-[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-PC (BLPC) were obtained from Dr. Wonhwa Cho, University of Illinois at Chicago (*12*). Styrene-divinylbenzene (SDVB) beads (5.2 ± 0.3 μm diameter) were purchased from Seradyn, Inc. (Indianapolis, IN).

*Purification of PI-PLC.* 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 (*11*, *13*). Enzyme purity was checked by SDS gel electrophoresis; enzyme concentration was determined with the Bradford assay (*14*) using bovine serum albumin as a standard.

*Vesicle Preparation.* Lipid stock solutions in chloroform were obtained from Avanti and used without further purification. All samples were initially placed in glass scintillation vials, and the chloroform was removed by a stream of $N_2$. The lipid film was lyophilized overnight and then rehydrated with buffer. Small unilamellar vesicles (SUVs) were generated by sonication (Branson Sonifier Cell Disrupter) of these aqueous solutions with a 30% duty cycle until maximum optical clarity was obtained. The solutions were then centrifuged at 13 000 rpm for 20 min to remove large particles. These lipid vesicles were stable (i.e., no detectable aggregation or fusion) for at least 1−2 days at room temperature. Large unilamellar vesicles of DMPC were formed by multiple passages of the aqueous lipid solution through polycarbonate membranes (100 nm pore diameter).

*$^{31}$P NMR Assays of PI-PLC.* The buffer used in all PI-PLC assays was 50 mM HEPES (pH 7.5) in $D_2O$. $^{31}$P NMR spectra were acquired at 202.7 MHz on a Varian Unity 500 spectrometer, using 5 mm sample tubes. The total volume of the enzyme assay was 350 μL. The $^{31}$P NMR parameters were optimized based on those used previously (*11*). These included a 4.5 μs pulse width (70°), recycle time of 2.6 s, 14 998 Hz sweep width, and 30 °C. All chemical shifts were referenced to phosphoric acid (5%) as an external standard. For all kinetic runs, a control spectrum ($t = 0$ min), which contained only phospholipid vesicles, was acquired prior to the addition of enzyme. The reaction was initiated by addition of 100-400 ng of PI-PLC depending on the reaction rate. After the addition of the appropriate amount of PI-PLC, an arrayed experiment that typically took 1−2 h was conducted. Initial rates were obtained from the progress curve for less than 10% PI hydrolysis. The rate was calculated from the increase in integrated intensity of product cIP with incubation time ($\mu$mol·min$^{-1}$). As a control, PI-PLC activity toward 10 mM PI vesicles was determined at each spectrometer session.

*Fluorescence Assays for Vesicle Fusion and Leakage.* Vesicle fusion in the absence and presence of PI-PLC was monitored by resonance energy transfer assays using SUVs containing 0.25−4.0 mol % fluorescent labeled lipid, NBD-PE, Rho-PE, or a 1:1 combination of both. The appropriate amounts of dyes were cosolubilized with nonlabeled phospholipids in chloroform prior to vesicle formation. Final nonlabeled lipid concentrations were 2−110 mM. Fluorescence emission was monitored before and after mixing the vesicles containing different lipid dyes. Steady-state fluorescence measurements were carried out with a Shimadzu RF 5000V spectrofluorimeter (with a Xenon light source) using 1-cm path length cells. Sample fluorescence emission was monitored from 470 to 650 nm using an excitation wavelength of 450 nm; intensities for both Rho-PE and NBD-PE emissions were measured.

Vesicle leakage under different conditions was assessed using entrapped, self-quenched CF dye (excitation at 490 nm); 100 mM CF in 50 mM HEPES, pH 7.5, was used to hydrate the lipid film for formation of SUVs. Nonencapsulated CF was removed by elution through a Sephadex G-25 column. If the CF is released into the external medium by disruption of SUV structure (general leakiness or fusion), the intensity of the CF emission peak (520 nm) would be expected to increase since the concentration of extravesicular CF would now be sufficiently diluted.

*Fluorescence Measurements of PC Binding to PI-PLC.* PC binding to PI-PLC increases the intrinsic fluorescence of the protein (*15*, *16*). Analysis of the protein fluorescence intensity as a function of added PC provides an estimate of $K_d$. The concentration of PI-PLC for fluorescence measurements was 4 μM in 10 mM HEPES, pH 7.5 at 25 °C. Steady-state fluorescence measurements were carried out with the same spectrofluorimeter as above. The excitation wavelength was at 290 nm, with both excitation and emission slit widths set at 3 nm; emission was scanned from 295 to 500 nm.

*Binding of PI-PLC to Phospholipid-Coated SDVB Beads.* SDVB beads were washed with 20% NaOH to remove the silica emulsion, and then coated with phospholipid as described by Cho and co-workers (*17*). After coating with phospholipid, the beads were washed twice with water followed by washing with 10 mM HEPES buffer, pH 7.5, to remove the phospholipid in the aqueous solution not bound to the beads. For binding measurements, 40 mg of POPC (or SPM)-coated beads was resuspended in 2 mL of the binding assay buffer (10 mM HEPES, 1 mg/mL BSA, pH 7.5); 100 μL aliquots of this dispersion were transferred to 0.5 mL microcentrifuge tubes and incubated with 0−20 μM enzyme for 10 min. Following incubation, the beads were separated by centrifugation. The concentration of free enzyme ($E_f$) in the supernatant was determined by measuring the enzyme activity toward PI/di$C_7$PC (1:6) mixed micelles using $^{31}$P NMR spectroscopy (*11*). Total enzyme concentration, $E_t$, was determined from the activity after incubation of enzyme with binding assay buffer but with no beads added. In the enzyme concentration range used in the assays, PI hydrolysis rates varied linearly with PI-PLC. From the dependence of bound PI-PLC concentration, $E_b$ (determined

as $E_t - E_f$) with $E_f$, $K_d$ was determined by fitting the curve to the following equation: $E_b = L_t/(n + K_d/E_f)$. In this equation, $L_t$ represents the total concentration of phospholipids in the solution, and $n$ is the number of phospholipid molecules which are bound to or covered by one PI-PLC molecule. This equation assumes that each phospholipid molecule binds independently with a dissociation constant of $K_d$ to $n$ equivalent sites on a PI-PLC molecule.

*Gel Filtration of PI-PLC Partitioned with Phospholipid Vesicles.* Different amounts of phospholipid unilamellar vesicles were incubated with 50 $\mu$g of PI-PLC for 10 min, then applied to a Sephadex G-150 column, and eluted with buffer composed of 10 mM HEPES, pH 7.5. The vesicles with bound PI-PLC eluted in the void volume, and were well separated from the free enzyme. The activities of bound enzyme fractions were examined (using PI/di$C_7$PC mixed micelles and $^{31}$P NMR) to determine the amount of PI-PLC associated with the phospholipid vesicles, $E_b$. The substrate mixed micelles were far in excess of the phospholipid concentration added when an aliquot from the column fraction was assayed; under these conditions, the vesicles would be rapidly micellized but would be so low in concentration as to not contribute to the total substrate pool. An apparent $K_d$ value was determined from a plot of $E_b$ versus $L_t$ (the total concentration of phospholipid) using the following equation: $E_b = L_tE_t/(K_d + L_t)$, where $E_t$ is total enzyme concentration. This equation assumes that each phospholipid molecule binds independently with a dissociation constant of $K_d$ to $n$ equivalent sites on a PI-PLC molecule, and that $nE_b \ll L_t$ (i.e., the phospholipid ligand concentration is in the millimolar range while the enzyme sites are micromolar at most).

## RESULTS

*Kinetic Characteristics of PI-PLC Action toward PI Vesicles.* Like most other water soluble lipolytic enzymes, PI-PLC can act on substrates at organized lipid/water interfaces. However, the activity of PI-PLC toward PI vesicles was not as high as that for micellar PI. The reaction progress curves for the hydrolysis of PI vesicles were linear for 20−30 min with no lag phase or burst detected. After that time period, the enzyme activity leveled off. The initial part of each progress curve was used to estimate PI-PLC specific activity. The linearity suggested that the time scale for PI-PLC binding to the substrate PI vesicles was relatively short. It also showed, in contrast to $PLA_2$ acting on PC SUVs, that the products had little effect on the activity of PI-PLC (at least to 15−20% hydrolysis). Bacterial PI-PLC shows cooperativity when it processes water-soluble substrate cIP (*11*). Similar cooperativity in the substrate dependence of activity has been seen for PI-PLC-catalyzed hydrolysis of pyrene-labeled PI (*18*) and for PI in Triton X-100 mixed micelles at fixed Triton X-100/PI (*11*). With this as a background, we chose to fit the dependence of PI-PLC activity on bulk PI vesicle concentration with both a Michaelis−Menten equation and the Hill equation. In the Michaelis−Menten treatment, the apparent $K_m$ was 3.8 ± 1.0 mM with a $V_{max}$ of 156 ± 19 $\mu$mol min$^{-1}$ mg$^{-1}$. With the Hill equation, the apparent $K_m$ was 2.6 ± 0.5 mM with a $V_{max}$ of 126 ± 14 $\mu$mol min$^{-1}$ mg$^{-1}$ and $n$ = 1.7 ± 0.5 (the fit with the Hill equation is shown in Figure 1). The decreased error in $K_m$ using the Hill equation suggests that vesicular PI cleavage is better fit with a cooperative model than with a simple hyperbolic binding profile. The apparent $K_m$ obtained with these equations is complex because it has contributions from both interfacial affinity and active site affinity. However, it is still is a useful parameter to compare different substrates and the effect of adding a second component.

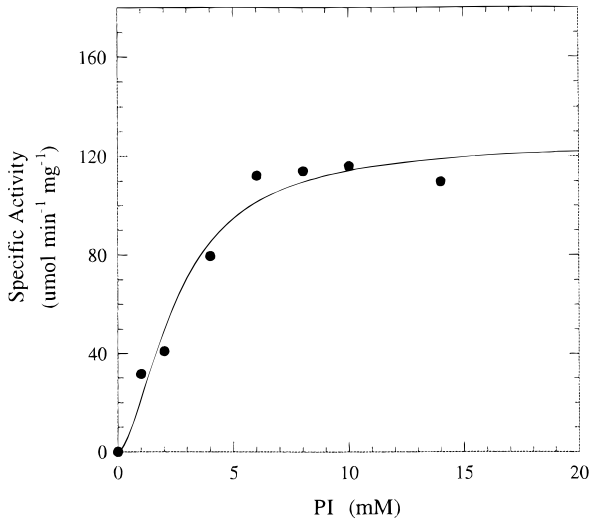


FIGURE 1: Dependence of *B. thuringiensis* PI-PLC specific activity on the bulk concentration of PI vesicles; the enzyme concentration was 0.29 $\mu$g/mL. The solid line is the best-fit curve using the Hill equation $v = V_{max}[S]^n/(K_m^n + [S]^n)$, with $K_m$ = 2.6 ± 0.5 mM and $n$ = 1.7 ± 0.5.

*Binary PI/PC Vesicles as Substrates for PI-PLC.* Since PI-PLC exhibited higher activity toward PI solubilized in di$C_7$PC micelles than in TX-100 micelles (*11*), the effect of PC cosonicated with PI vesicles was examined. At fixed PI, as the mole fraction PC increases, one would expect the PI-PLC activity to decrease if the surface concentration of substrate is important ('surface dilution'). In fact, studies with the enzyme from *B. cereus* documented surface dilution for $X_{PC} > 0.2$ (*16*). However, at sufficiently low mole fractions PC ($X_{PC}$), some degree of activation might be observed. The concentration of PI was kept at 10 mM (as seen in Figure 1, the activity at this substrate concentration appears to be $V_{max}$), and cosonicated vesicles with different mole fractions of DMPC were prepared for assays of PI-PLC activity. As shown in Figure 2, between 0 and 0.2 mole fraction DMPC ($X_{DMPC}$), PI-PLC activity toward the PI increased. The maximum activation was 7-fold at $X_{DMPC}$ of about 0.2. Above 0.2 mole fraction DMPC, PI-PLC activity decreased from its maximum, behavior reminiscent of surface dilution. Above 0.6 mole fraction DMPC, the specific activity of PI-PLC was reduced to nearly the same as toward PI vesicles in the absence of PC. The decrease in PI-PLC activity as the PI surface concentration is decreased above the maximum activity observed at 0.2 mole fraction DMPC (0.8 mole fraction PI) is not quite linear with PI surface concentration. At 0.8 mole fraction PI, the relative activity is ~7; at 0.4 mole fraction PI (0.6 mole fraction DMPC), the relative activity has decreased to that of the enzyme toward pure PI vesicles, rather than decreased 2-fold as expected if surface concentration were the key parameter in PI-PLC activity. Several factors could contribute to this effect. In the SUVs, the distribution of PI on both monolayers could vary with PC concentration. If less PI is

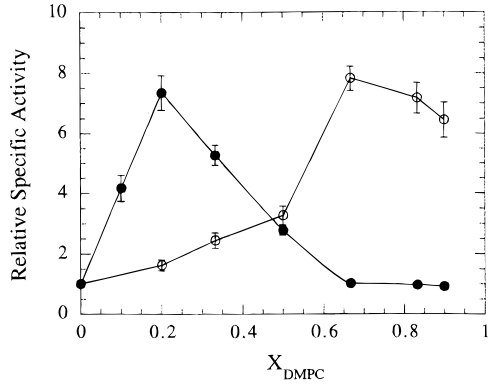


FIGURE 2: Relative activity of *B. thuringiensis* PI-PLC toward (●) covesicles of DMPC and PI and (○) mixtures of separately sonicated DMPC and PI small unilamellar vesicles as a function of the mole fraction of DMPC. In each case, the concentration of PI was kept constant at 10 mM while the [PC] varied from 0 to 90 mM. The specific activity is normalized to that for the enzyme acting on pure PI vesicles, 116 ± 12 $\mu$mol·min$^{-1}$·mg$^{-1}$.

available on the outer leaflet at higher PC, then the observed activity would decrease more than expected, especially since the bulk concentration of surface PI is not that far above the apparent $K_m$. Measuring the ratio of $PI_{out}/PI_{in}$ is difficult in these mixed phospholipid vesicles. For example, the addition of lanthanide shift reagents (which can accurately monitor sidedness for PC vesicles) tends to induce fusion/massive precipitation in the mostly PI vesicles. PI packed in a PC matrix could also have different diffusion characteristics than in a PI matrix, and this could affect the rate of enzymatic hydrolysis of PI. Increasing surface PC could also bias the distribution of PI-PLC (vesicle-bound versus free in solution), again affecting the observed rate. The key to these experiments with covesicles is to realize that the bulk concentration of substrate PI has not changed, only the surface concentration. PC has little affinity for the active site. As long as PI-PLC can dissociate from one vesicle surface and bind to another, then there would be a limiting activity for the enzyme as it leaves one vesicle and binds to another. Since the bulk PI is the same, there is the same probability that a PI molecule will encounter the enzyme via collisions. This might also suggest that the surface concentration of PC affects enzyme scooting/hopping behavior. All of these complicating factors mean that a quantitative interpretation of the observed 'surface dilution' behavior is problematic.

These results were different from those reported in a previous study by Volwerk et al. (*16*), who used PI-PLC from *Bacillus cereus* expressed in *E. coli.* In their work, DOPC showed the typical surface dilution effect when DOPC was cosonicated with PI (comparable to what is seen in the present work). No activation was detected (although $X_{PC} < 0.2$ was not examined). The different results may be due to the different expression and purification methods of the enzyme. Expressing PI-PLC in *E. coli* may allow some activator membrane lipids, such as PE, to bind the enzyme. That zwitterionic phospholipid has also been shown to be an activator of PI-PLC-catalyzed hydrolysis of cIP (*11*). POPE, at 0.2 mole fraction of the total lipid, cosonicated with PI also activated PI-PLC 3.2-fold toward PI (Table 1).

*Effect of Separate PC Vesicles on PI-PLC Activity toward PI Vesicles.* Since PC in the same vesicle was an activator of PI-PLC, we decided to examine the effect of a separate pool of PC vesicles on PI-PLC hydrolysis of PI vesicles. This might address (i) relative affinities of the enzyme for PC and PI as well as (ii) a mechanism for the PC activation by exploring whether enzyme can access substrate when it binds to nonsubstrate vesicles. Hydrolysis of PI vesicles (10 mM) by PI-PLC from *B. thuringiensis* was examined in the presence of varying concentrations of other nonsubstrate phospholipid vesicles (Table 1). The addition of PC vesicles, such as DMPC and POPC, to the solution of PI vesicles clearly increased PI-PLC specific activity, although the maximum activation and the bulk concentration of PC vesicles required for optimum activation depended on the nature of the PC chains. The PI-PLC specific activity toward PI was increased 7-fold when the ratio of DMPC vesicles to PI vesicles was about 2:1; as shown in Figure 2, this activation was comparable to what was observed with cosonicated binary vesicles with PC/PI equal to 0.25. Also notable with the mixed separate vesicles is the lack of strong 'surface dilution' that is observed in covesicles as DMPC/PI is increased above 0.25.

Table 1: Phosphotransferase Activity of PI-PLC toward PI Vesicles (10 mM) in the Absence and Presence of Other Phospholipid Vesicles

| added SUVs (mM) | specific activity[a] ($\mu$mol·min$^{-1}$·mg$^{-1}$) | | relative activity[b] | |
|---|---|---|---|---|
| | separate vesicle | cosonicated vesicle | separate | cosonicated |
| – | 116 ± 12 | | 1.0 | |
| DMPC 2.5 | 190 ± 21 | 853 ± 67 | 1.6 | 7.4 |
| 5 | 284 ± 30 | 612 ± 39 | 2.4 | 5.3 |
| 10 | 379 ± 35 | 322 ± 19 | 3.3 | 2.8 |
| 20 | 907 ± 47 | 119 ± 7 | 7.8 | 1.0 |
| 50 | 832 ± 59 | 113 ± 10 | 7.2 | 0.97 |
| 90 | 748 ± 68 | 107 ± 9 | 6.4 | 0.93 |
| POPC 2.5 | 295 ± 21 | | 2.6 | |
| 5 | 263 ± 26 | | 2.3 | |
| 10 | 327 ± 21 | | 2.8 | |
| POPE 2.5 | | 370 ± 40 | | 3.2 |
| POPS[c] 2.5 | 105 ± 18 | | 0.91 | |
| 5 | 95 ± 19 | | 0.82 | |
| 10 | 52 ± 22 | 21 ± 10 | 0.45 | 0.20 |
| BLPC 10 | 121 ± 13 | | 1.0 | |
| 2.5 | | 596 ± 68 | | 5.1 |
| SPM 10 | 71 ± 11 | | 0.61 | |
| 2.5 | | 300 ± 22 | | 2.6 |
| DOPG 10 | –[d] | | –[d] | |
| POPA 10 | <20 | –[d] | 0.17 | –[d] |

[a] Assay conditions include 50 mM HEPES, pH 7.5, 10 mM PI, and 0.2 $\mu$g of enzyme unless otherwise noted. [b] The relative activity is the ratio of observed activity to that for PI-PLC acting on 10 mM PI SUVs (116 ± 12 $\mu$mol·min$^{-1}$·mg$^{-1}$). [c] 1 $\mu$g of enzyme was used. [d] There was no detectable cleavage in these vesicle mixtures within 2 h.

The activation of PI-PLC toward PI vesicles exhibited specificity as to the phospholipid headgroup. If POPS vesicles were added into the system instead of PC or PE, the initial rate of PI hydrolysis decreased slightly. Increasing the bulk concentration of POPS vesicles decreased PI-PLC specific activity toward PI vesicles more significantly. A likely explanation is that the PI-PLC was bound to PS vesicles as well as PI vesicles. The activity of PI-PLC toward PI vesicles was nearly abolished by the presence of equimolar DOPG or POPA vesicles; covesicles of these lipids with PI also abolished PI-PLC hydrolysis of PI. Under these conditions, it is possible that the enzyme binds to DOPG or POPA vesicles tightly and is no longer available to bind to

PI in separate vesicles, although one needs relative binding affinities to assess this. SPM exhibited distinctly different behavior. This lipid has the same choline headgroup as PC and leads to activation of PI-PLC when SPM and PI exist in the same vesicle. However, there was no activation when separate SPM vesicles were added to the solution of PI vesicles; inhibition of enzyme activity was observed.

*Integrity of PC and PI Vesicles.* To understand the mechanism of PC vesicle activation of PI-PLC toward separate PI vesicles, the integrity of both vesicle populations was examined. If PI vesicles and PC vesicles fuse or if exchange of outer layer lipids occurs between the two vesicle populations, the activation by PC vesicles may due to the incorporation of PC molecules into the PI vesicle surface which in turn activates the PI-PLC bound to the PI substrate vesicles. Fluorescence resonance energy transfer was used to detect such vesicle fusion. Two fluorescence probes, N-NBD-PE (donor) and N-Rho-PE (acceptor), were incorporated into separate PC or PI vesicles. If these two fluorescent labeled lipids mixed within the same vesicle because of vesicle fusion, an increase in energy transfer efficiency would be detected. Alternatively, if the two probes were initially mixed in the same vesicle, and then incubated with an excess of unlabeled lipid vesicles, a decrease in Rho-PE fluorescence would indicate transfer of phospholipids from one pool to another, either by fusion or by exchange of outer monolayer components. Two conditions for vesicle mixing were examined: (i) relatively low total vesicle concentrations; and (ii) high vesicle concentrations equivalent to those used in the assays.

Under low SUV concentration conditions, 2 mM DMPC containing 1.0 mol % NBD-PE and 2 mM DMPC containing 1.0 mol % Rho-PE, the emission intensity of both Rho-PE and NBD-PE in the mixed separate vesicles showed little change for 2 h; small changes occurred over the time scale of days (Figure 3A). The addition of PI-PLC to the PC vesicle mixture had no effect on the emission intensities. This result showed that at this concentration and on this time scale DMPC vesicles do not fuse in the presence of PI-PLC (similar results were observed for POPC vesicles). The mixing of millimolar POPC vesicles containing NBD-PE and PI vesicles containing Rho-PE was also examined. The emission intensities were stable over a 2 h period, indicating that the two kinds of vesicles did not fuse (in the absence of enzyme) under these conditions. Fusion studies were also done by diluting 100 $\mu$M PI or PC vesicles containing both 1 mol % NBD-PE and 1 mol % Rho-PE with unlabeled 1 mM POPC vesicles. The fluorescence spectra of vesicles containing both NBD-PE and Rho-PE showed a large Rho-PE emission peak at 590 nm and a small NBD-PE peak at 515 nm, indicating efficient resonance energy transfer. Dilution of the labeled vesicle sample with the same volume of 1 mM POPC vesicles did not alter the fluorescence intensities over a 1 h period. Addition of calcium ion to induce fusion caused a slow decrease in the fluorescence intensity (Figure 3B). The same experiments with PI vesicles containing NBD-PE and Rho-PE (no calcium) showed that the two vesicle populations were stable for over 2 h when mixed at these concentrations in the absence of enzyme.

However, at higher total lipid concentrations, collisional lipid exchange/fusion does occur. PI SUVs containing 4 mol % NBD-PE and 4 mol % Rho-PE (from a stock solution of

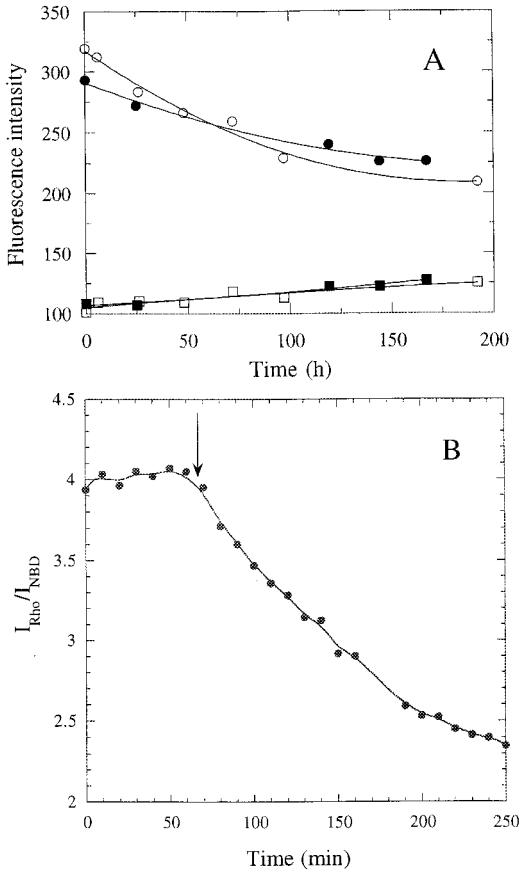


FIGURE 3: Vesicle fusion as measured by NBD-PE and Rho-PE resonance energy transfer. (A) Incubation of 1 mM POPC vesicles containing 0.02 mM NBD-PE with 1 mM POPC vesicles containing 0.02 mM Rho-PE: fluorescence intensity at 522 nm (○, NBD-PE) and 593 nm (□, Rho-PE) in the absence (open symbols) and presence (filled symbols) of 9.5 $\mu$g of PI-PLC. (B) Vesicles containing 100 $\mu$M PI/NBD-PE/Rho-PE (100:1:1) solution were diluted 1:1 with unlabeled PC vesicles (1 mM total PC); the arrow indicates the addition of 2 mM $Ca^{2+}$ to induce vesicle fusion.

16.7 mM) were mixed with DMPC SUVs (from a stock of 200 mM) to a final concentration of 10 mM PI and 90 mM PC. Aliquots were removed from the sample as a function of time and diluted to 0.22 mM PI and 2 mM DMPC, where no further mixing/exchange of lipids occurred (as shown above). The efficiency of energy transfer from NBD-PE to Rho-PE is significantly reduced over the time scale of 20 min at the highest concentration of DMPC vesicles (Figure 4); with a 2:1 ratio of PC/PI pools (and a smaller total phospholipid pool), the time scale for fusion is longer, but loss of energy transfer is still observed. If no PC vesicles were added, the ratio of Rho-PE/NBD-PE fluorescence was unaltered (Figure 4). Therefore, when mixing the separate vesicle pools, significant exchange of phospholipids (presumably from the outer monolayers) does occur. This strongly suggests that the PC SUV activation of PI SUVs involves altering the PI vesicle surface so that a significant concentration of PC is available to activate the enzyme. Since small amounts of PC, less than 0.2 mole fraction, lead to measurable enhancement of PI hydrolysis by PI-PLC, it does not take much PC transfer to yield increased PI-PLC in a mixed population of separate PI and PC vesicles.

Is the inserted PC the result of vesicle fusion or exchange? Vesicle stability was also examined by looking for leakage of entrapped CF dye. When a sample of vesicles containing

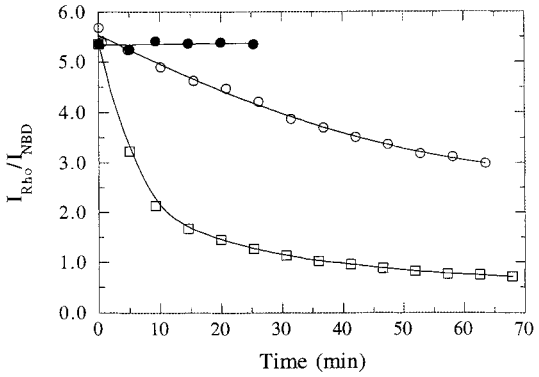


FIGURE 4: Ratio of Rho-PE to NBD-PE fluorescence intensities in vesicles containing 10 mM PI/4 mol % NBD-PE/4 mol % Rho-PE as a function of incubation time at 30 °C alone (●) and with (○) 20 mM DMPC or (□) 100 mM DMPC.

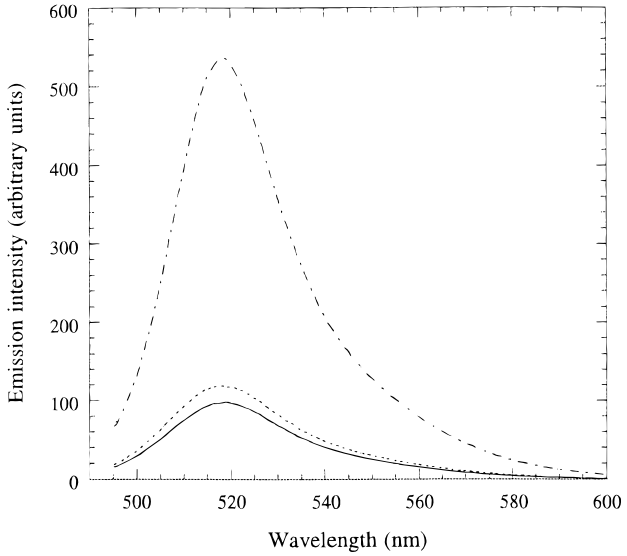


FIGURE 5: Fluorescence spectra of DMPC vesicles containing 100 mM CF: (—) prior to the addition of enzyme; (···) after adding PI-PLC (1.9 $\mu$g); and (—·—·) after adding TX-100 (8 mM).

high concentrations of CF (100 mM) was excited at 490 nm, the CF emission intensity at 520 nm was very low because of fluorophore self-quenching. If any CF is diluted into the external medium by disruption of SUV structure (general leakiness or fusion), the intensity of CF emission would be expected to increase. For DMPC, POPC, and PI vesicles containing 100 mM CF, the emission intensity at 520 nm was constant for at least 2 h; 1.9 $\mu$g of PI-PLC (120 nM) was added to a solution of DMPC vesicles; after an initial small increase in the emission intensity, the CF fluorescence was stable for 1 day. For comparison, the addition of a detergent, such as TX-100, to the vesicles with entrapped CF produced a much larger increase in CF fluorescence (Figure 5). Thus, DMPC vesicles do not leak significantly and are very stable in the presence of the PI-PLC, even though under these conditions significant exchange of PC can occur. The small change in the CF intensity after initial addition of PI-PLC may be due to the interaction of the vesicles and enzyme.

*PC LUV Activation of PI-PLC toward PI SUVs.* Another way of probing how critical phospholipid exchange/vesicle fusion is to activating PI-PLC toward PI vesicles is to examine the effect of PC LUVs, where vesicle curvature is no longer severe, on PI-PLC activity toward separate PI vesicles. The DMPC LUV preparation had an average diameter of ~1000 Å. $^{31}$P line width measurements of the PC LUVs are consistent with a population of large vesicles: the PC resonance is shifted upfield 10 ppm and has a line width of ~3000 Hz. In contrast, the population of PI SUVs has a much sharper line width of 78 Hz. When the two vesicle populations are mixed (in the absence of enzyme), significant fusion of the PC with the PI vesicles would be detected by an increased line width of the PI resonances and/or loss of intensity. As shown in Table 2, the activity of PI-PLC toward PI vesicles increases significantly upon incubation with the DMPC LUVs. The magnitudes of the activation are comparable to what was observed with added PC SUVs. More critical to the interpretation of these results is that the $^{31}$P line width of the PI vesicles also increased upon mixing the two populations in the absence of enzyme, however, not enough to indicate a new population of PI/PC large vesicles. Without the added PC LUVs, the PI vesicle line width is unaltered over the time scale of hours. Thus, at these high vesicle concentrations there appears to be some transfer/exchange of PC from the LUV population to the PI SUV pool. On average, the PI SUVs must still be moderately small, since the line width does not approach that of the PC (or PI) LUVs. This strongly suggests that the magnitude of PC activation of PI-PLC toward PI vesicles reflects the transfer of PC into the PI surface. A separate resonance for the PC is not observed; however, less than 5 mol % PC in the PI outer monolayer would be sufficient to activate the enzyme 3-fold.

Table 2: Effect of Incubating DMPC Large Unilamellar Vesicles on $^{31}$P Line Widths of Small Unilamellar Vesicles of PI (10 mM) and Phosphotransferase Activity of PI-PLC toward the PI Vesicles

| [DMPC] (mM) | relative activity | PI $^{31}$P line width (Hz) |
|---|---|---|
| 0 | 1.0 | 78 |
| 2.5 | 1.3 | 86 |
| 20 | 4.8 | 138 |
| 100 | 10 | 200 |

*Polymerizable Phosphatidylcholines as Activators for PI-PLC Activity.* If PC vesicles can activate PI-PLC toward separate PI vesicles by transferring PC to the PI interface, then no activation should occur when a PC vesicle population that is incapable of fusion is used. BLPC (1,2-bis[12-(lipoyloxyl)dodecanoyl]-*sn*-glycero-3-phosphocholine) vesicles can be used to test this hypothesis. The structure of this cross-linked PC is shown in Figure 6. The headgroup is the same as in PC, while the fatty acid chains possess a five-membered ring containing a disulfide bond at the terminus. Once packed in SUVs, this PC can be cross-linked through ring opening polymerization, initiated by using a catalytic amount of dithiothreitol (*19*). Cross-linking does not change the bulk physical properties of BLPC vesicles (*12*). However, this polymerization effectively prevents collisional exchange of individual PCs or fusion with other vesicle pools. Therefore, tethering the fatty acyl chains through the formation of disulfide cross-linking should not allow PC activation of PI-PLC toward PI vesicles. For an assay system that contained 10 mM PI vesicles and separate 10 mM polymerized BLPC vesicles, the initial rate for PI-PLC was 121 $\mu$mol·min$^{-1}$ mg$^{-1}$ (Figure 7). This value was essentially the same as that for 10 mM PI vesicles alone (116

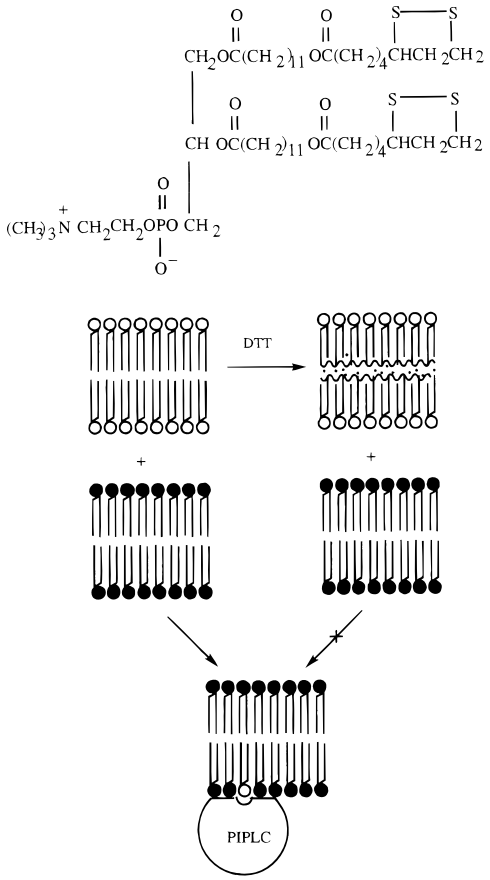


FIGURE 6: Chemical structures of BLPC and the scheme for BLPC cross-linking. Cross-linking prevents the enzyme from exchange/transfer to the PI bilayer.

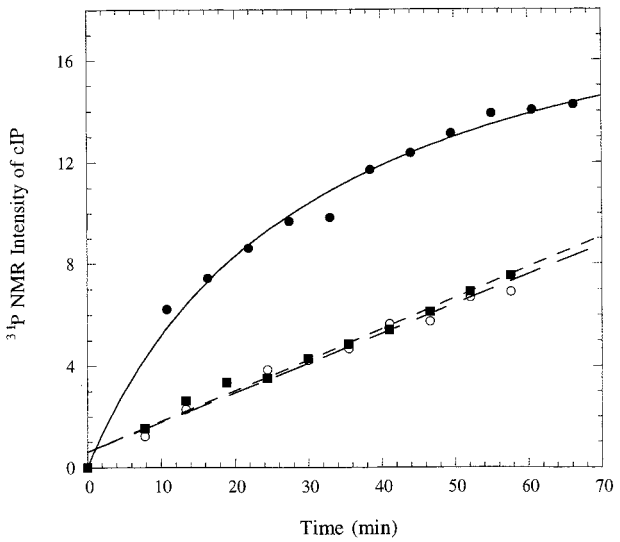


FIGURE 7: Reaction progress curves for PI-PLC toward PI vesicles: (○) 10 mM PI vesicles; (■) 10 mM PI vesicles in the presence of 10 mM cross-linked BLPC vesicles; (●) cosonicated vesicles of 10 mM PI and 2.5 mM cross-linked BLPC.

$\mu$mol·min$^{-1}$ mg$^{-1}$). The initial rate for PI-PLC hydrolyzing PI vesicles in the presence of 10 mM DMPC vesicles (360 $\mu$mol·min$^{-1}$ mg$^{-1}$) was 3-fold higher than that for PI vesicles alone. Thus, the polymerized BLPC vesicles cannot activate PI-PLC toward PI vesicles. If a mixture of 10 mM PI vesicles and 2.5 mM BLPC vesicles was cosonicated, the PI-PLC initial rate was increased to 560 $\mu$mol·min$^{-1}$ mg$^{-1}$, about 5-fold higher than that for PI vesicles alone. This indicates that the BLPC molecule can activate the enzyme PI-PLC toward PI packed in the same vesicles. In the BLPC and PI covesicles, a patch of cross-linked BLPC will be near a patch of PI. Under these conditions, the enzyme can bind a BLPC molecule and still hydrolyze the nearby PI molecules. Therefore, for activation with separate vesicles, it is necessary for PC molecules to be transferred to the PI substrate pool.

*PI-PLC Partitioning with Phospholipid Vesicles.* The kinetic analyses showed that some phospholipid vesicles activated the enzyme PI-PLC while other phospholipid vesicles inhibited the enzyme. Differences in PI-PLC binding affinity for these nonsubstrate vesicles might be related to the differential kinetic effects. Several methods, including fluorescence, a lipid-coated SDVB-bead binding assay, and gel filtration, were used to estimate the $K_d$ for PI-PLC binding to PC SUVs.

The fluorescence emission of PI-PLC is dominated by seven tryptophan residues. Previous fluorescence studies (*16*) of PI-PLC from *B. cereus* showed that the protein intrinsic fluorescence emission intensity increased in the presence of POPC vesicles. PI-PLC binding to DMPC vesicles also showed an increase in protein fluorescence. The maximum change in the fluorescence intensity of PI-PLC upon binding to DMPC vesicles was only 15−20%, an increase much smaller than the increase in protein fluorescence when the protein bound di$C_7$PC micelles where 100% increase was detected (*15*). Nonetheless, an analysis of the change in the fluorescence behavior of the enzyme as a function of added DMPC yielded an apparent dissociation constant of 0.08 mM, comparable to the 0.2 mM detected for POPC previously (*16*). While this method is useful for tight binding ligands, it is problematic for phospholipids with lower affinity or whose binding has a smaller effect on protein fluorescence.

An alternative binding assay uses a stable monomolecular phospholipid film on the surface of hydrophobic SDVB beads (*17*) as the interfacial ligand. The coated beads can provide direct evidence for PI-PLC binding to phospholipids. Various concentrations of PI-PLC were incubated with the POPC-coated beads and then bound and unbound enzyme separated by centrifugation (free enzyme is in the supernatant while the bound enzyme pellets with the beads). The amount of PI-PLC in the supernatant ($E_f$) was measured and is plotted against $E_b$, the enzyme bound to the PC−coated beads, in Figure 8. The apparent dissociation constant was estimated as 22 $\mu$M. For SPM small unilamellar vesicles, the binding profile was nearly the same, generating a $K_d$ also in the micromolar range. However, this binding assay was unsuccessful for other phospholipid vesicles. For example, POPA-coated beads showed no evidence of enzyme binding using this concentration of phospholipid and range of enzyme concentrations, while the kinetic study definitely showed inhibition of PI-PLC-catalyzed hydrolysis of PI vesicles by the POPA vesicles. There are several explanations for the apparent lack of PI-PLC binding to the phospholipid-coated beads. (i) The POPA may not coat the beads very well. (ii) In the SDVB bead binding assay, the bulk concentration of phospholipid is about 60 $\mu$M. If the apparent dissociation constant for POPA is much larger than that, the PI-PLC bound to the beads would be too small to be detected accurately.

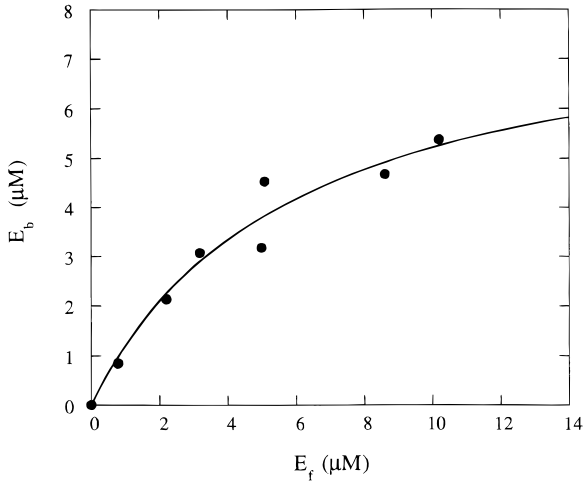


FIGURE 8: Binding of PI-PLC to POPC-coated styrene-divinylbenzene beads as monitored by the dependence of bound PI-PLC, $E_b$, on free enzyme in solution, $E_f$. The free enzyme concentration was determined by its activity measured using 4 mM PI solubilized in 24 mM di$C_7$PC, 50 mM HEPES, pH 7.5. $E_b$ was calcuated as $E_t - E_b$, where $E_t$ is the total amount of enzyme. The solid line represents the theoretical fit using the equation $[E_b] = [L_t]/(n + K_d/[E_f])$, with the total PC ligand concentration $[L_t]$, $K_d$, and $n$ values determined by fitting.

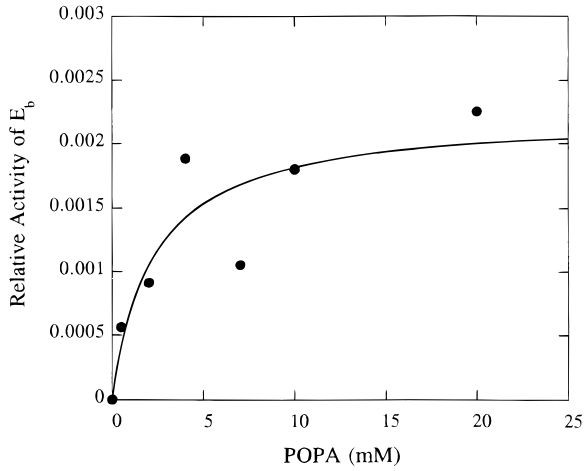


FIGURE 9: Gel filtration analysis of PI-PLC binding to POPA vesicles. The bound PI-PLC, as measured by enzyme activity (A) toward 4 mM PI solubilized in 24 mM di$C_7$PC, 50 mM HEPES, pH 7.5, is shown as a function of bulk POPA concentration, $[L_t]$. The curve indicates the best fit for $A = A_o L_t/(K_d + [L_t])$, where $A_o$ is the maximum activity of PI-PLC observed (when $[E_b] = [E_t]$).

Gel filtration separates particles according to their sizes. The relatively large (compared to enzyme) phospholipid vesicles with bound PI-PLC elute in the column void volume and can be well separated from the free enzyme. By varying the incubation concentration of lipid, the dependence of $E_b$ (bound enzyme) on total phospholipid can be obtained. With this technique, the total concentration of the enzyme was kept at 17 $\mu$M, and PA concentration was varied from 0 to 20 mM. From the dependence of bound enzyme on total phospholipid (Figure 9), the $K_d$ for the POPA binding to PI-PLC was estimated as 2 mM (such a value could not be measured within the constraints of the SDVB-coated bead binding assay). The $K_d$ for POPA vesicles is several orders of magnitude weaker than that for POPC; however, it is comparable to the apparent $K_m$ for PI SUVs. Under PI-PLC assay conditions, a good fraction of the enzyme would be bound to the PA vesicles, but not all of it. For 10 mM PA and 10 mM PI cosonicated, essentially all of the PI-PLC activity is inhibited. Thus, the affinity of a single PA molecule for PI-PLC when the latter is anchored to the interface must also be important. If PA also can be transferred to PI vesicles via collisions, and if PA monomers have a high affinity for the PI-PLC active site (as suggested by the lack of activity detected in 1:1 cosonicated PI/PA vesicles), then PI-PLC activity toward separate vesicles would also be strongly inhibited. This is indeed the case as seen in Table 1. A 1:1 mixture of POPA/PI vesicles shows a drop to 17% of the activity toward PI vesicles alone. This probably represents some partitioning of PI-PLC to PA vesicles, but also PA in largely PI vesicles binding to the enzyme active site.

## DISCUSSION

Although bacterial PI-PLC is much smaller than the intracellular PI-PLCs with roles in signal transduction, it has some sequence and structural homology to the X-domains of these mammalian PI-PLC enzymes. Hence, detailed studies of its interactions with phospholipids can provide detailed structure and mechanistic information on how the mammalian enzymes work. The catalytic mechanisms of phospholipases are more complicated than soluble enzymes since there is an additional step, the binding of enzyme to the aggregated surface, that occurs prior to optimal binding and hydrolysis of substrate at the active site. Modulation of this association of the enzymes with their aggregated substrate is one way of regulating phospholipid turnover. Interfacial catalysis on biomembranes has attracted considerable attention because such processes are believed to be responsible for many cellular regulatory mechanisms. The purpose of the present work was to (i) determine the kinetic characteristics for PI-PLC from *Bacillus thuringiensis* acting toward PI vesicles in the presence of separate PC vesicles and toward PI in PI/PC mixed vesicles, (ii) investigate the mechanism of PC vesicle activation of PI-PLC toward separate PI vesicles, and (iii) measure the affinity of PI-PLC binding to different phospholipid vesicles.

Without any additives and for the fixed amount of enzyme (400 ng of PI-PLC), enzyme phosphotransferase specific activity was constant over the range 6−14 mM PI. In this concentration range, there are many more small unilamellar vesicles than enzyme molecules in the kinetic system. The reaction progress curves showed that, when the activity leveled off, not all the substrate was hydrolyzed. Such results are consistent with (but not proof) PI-PLC functioning in a scooting mode, in which each enzyme can bind to one vesicle and hydrolyze all the outer layer PI molecules. They are also consistent with the enzyme-generated DAG acting as a product inhibitor and/or causing a phase change in the substrate vesicles that inhibits PI-PLC. Therefore, the extent and the rate of hydrolysis should be determined by the amounts of the enzyme in this concentration range.

The addition of nonsubstrate phospholipid vesicles has been shown to alter the specific activity of PI-PLC. Notably, PC vesicles activate PI-PLC while anionic lipid vesicles tend to inhibit it. Micellar PC was found to increase the phosphotransferase activity of PI-PLC toward long-chain PI in a mixed micelle system (*11*). Given that result, it is not

surprising that PC also activates PI-PLC toward the PI in mixed phospholipid vesicles at low mole fractions of PC (maximum activation occurs at 20 mol % PC). The more intriguing question is how this activation occurs when the two molecules, substrate PI and activator PC, exist in two different vesicles. The fluorescence energy transfer results showed that in the absence of $Ca^{2+}$ (not a cofactor in the bacterial PI-PLC reaction), there is exchange of outer layer components between PI and PC SUVs such that significant amounts of PC are available in the PI interface. How much PC is transferred clearly depends on the concentration of the PC SUVs—the higher the concentration, the more PC is transferred, either by collisional exchange or by limited fusion that, while scrambling the lipids, does not leak CF. The instantaneous (i.e., no lag phase) activation of PI-PLC toward PI vesicles by PC molecules in separate vesicles must be due to phospholipid exchange/vesicle fusion and incorporation of PC into the PI vesicle. The extent of this clearly depends on the identity of the PC (the longer the chains, the less likely collisional exchange will occur rapidly), as well as the ratio of PC to PI.

The phospholipid-coated SDVB beads provide evidence that PI-PLC has a high affinity for POPC vesicles, with an apparent $K_d$ of 22 $\mu$M. The intrinsic fluorescence emission of PI-PLC increased in the presence of DMPC (*15*) and POPC vesicles (*16*), which also indicated POPC molecule(s) bound to the enzyme. Several modes of PI-PLC interaction with mixtures of vesicles can be envisioned: (i) the enzyme can bind to the POPC vesicle surface (most of it would be partitioned to PC vesicles over PI vesicles given the much higher affinity for PC surfaces) and once anchored on that surface interact with PI in vesicles via collisions; (ii) upon binding to a PC vesicle, the PI-PLC binds a monomer molecule tightly, and in this activated state, it can dissociate from the PC vesicle and bind to a substrate PI vesicle for activated catalysis; and (iii) PI-PLC partitions among the different vesicles, but now has a higher affinity for the PI vesicles since the predominantly PI vesicle surface has some PC incorporated (by lipid exchange or fusion) into it. The lack of activation by cross-linked BLPC in separate vesicles suggests the activator PC must be present in the substrate interface; otherwise no activation can occur, eliminating the first scenario. Cross-linked lipid molecules also have a pronounced damping effect on two types of lipid motions: (i) lateral diffusion and (ii) the motion of the molecules along the normal of the vesicle surface. Thus, a comparison of phospholipase activity toward PI in vesicles cosonicated with normal PC or cross-linked PC vesicles probes the importance of vertical and lateral motions in activating the enzyme as well as lipid removal and exchange. The observation that patches of cross-linked BLPC activated the enzyme when present in the same aggregate as PI indicates that these vertical and lateral motions are not critical to the PC binding to the activator site.

Binding of PI-PLC to PC vesicles and extraction of a single PC molecule are energetically very costly. The free energy for the removal of phospholipids from a bilayer exceeds +20 kcal/mol. While such behavior is possible, it would imply that PI-PLC has significant PC phospholipid transferase activity. At least in the systems examined in this work, no such activity was detected (making the second mechanism unlikely), although the fluorescent probes used to detect lipid exchange/fusion could be too big and bulky to bind to the PI-PLC activator site. Perhaps the most reasonable explanation is that collisional exchange/limited fusion of phospholipid components can in fact occur when separate PI and PC vesicles are mixed. Not much PC must be transferred to the PI vesicles to activate the enzyme. For the 3-fold increase in PI-PLC activity detected with a 1:1 mixture of PI and DMPC separate vesicles, only about 5 mol % PC would be needed in the PI vesicle surface. The fluorescence resonance energy transfer experiments indicate that such PC transfer (either by collisional transfer of monomers or by limited fusion) could certainly occur, although it is difficult to quantify the exact extent of the PC transfer in those experiments. This behavior also provides an explanation for why PC vesicle activation toward separate PI vesicles requires a much higher ratio of PC to PI than PC activation toward PI in the same vesicle or in a PC/PI mixed micelle system. Collisional transfer or limited fusion of PC to a PI vesicle will introduce only small amounts of PC into the PI surface, and the extent of transfer will depend strongly on the concentration of PC. Not all zwitterionic lipids in separate vesicles activated PI-PLC toward PI vesicles. Separate SPM vesicles (1:1 with PC) inhibited PI-PLC activity. However, the activation by SPM in the same vesicle as PI was similar to POPC and DMPC (Table 1). The hydroxyl group in the sphingosine backbone of SPM may be part of a hydrogen bond network between SPM molecules packed in the vesicle. The energy needed to disrupt this hydrogen bonding may disfavor exchange of SPM molecules upon collisions with PI vesicles. The slight inhibition seen may reflect PI-PLC partitioning among SPM and PI vesicles.

In the SDVB bead/PC binding assay, the PI-PLC adheres to the beads quite tightly. The $K_d$ for PC binding is much lower than the apparent $K_m$ for PI vesicle hydrolysis. While the latter does not solely reflect PI vesicle binding (it is a combination of interfacial binding as well as active site binding), it does suggest that at the higher ratios of PC/PI separate vesicles, the binding to PC vesicles does not tightly anchor the enzyme to those substrate-less (or at least substrate-poor) vesicles. If such tight binding did occur, significant PI-PLC inhibition should be detected. In the kinetic assays with separate PI and PC vesicles, the enzyme must be able to hop among the mostly PC and the mostly PI vesicles. Partitioning of PI-PLC among PI and other phospholipid vesicles may also be complex. If some amount of PC lipid exchange/fusion occurs, many of the other phospholipids in separate vesicle experiments should be able to exchange with PI in the outer monolayer of PI vesicles. If the other phospholipid is an inhibitor of PI-PLC [e.g., PMe (*16*)], then one should eventually detect significant inhibition of PI-PLC toward the PI vesicles, but at a higher ratio of inhibitor phospholipid to PI than for cosonicated vesicles of the two species. PA and PG are potent inhibitors of PI-PLC, and mixing two separate vesicles pools of these lipids inhibits PI-PLC phosphotransferase activity. No detectable PI-PLC activity is observed when 1:1 PI/PG vesicles are mixed or when a 1:1 mixture is cosonicated. For PA, however, some PI-PLC activity is still detected when 10 mM PI SUVs are mixed with 10 mM POPA SUVs. If the same vesicle mixture is cosonicated, no PI-PLC hydrolysis of PI is detected within 2 h (indicating a specific activity $<1$ $\mu$mol·min$^{-1}$·mg$^{-1}$ in the $^{31}$P NMR assay). Since the $K_d$ for

PI-PLC interacting with the PA vesicles was 2 mM (in the same range as the $K_m$ for PI vesicles), one would have expected some PI-PLC activity toward the separate PI vesicles if the activity were governed by PI-PLC partitioning between the two types of vesicles. However, if these phospholipids are transferred to the PI vesicle surface, more significant inhibition of PI-PLC may be observed (depending on the affinity of PA for the catalytic site as well as its affinity for surface binding). Separate PS vesicles act as a much weaker inhibitor than PA or PG. Consistent with this being caused by lipid exchange/limited fusion, PS was much more inhibitory when cosonicated with PI. Previous work with short-chain PS molecules showed that PI-PLC did not have a high affinity for monomeric PS as detected by the lack of $^{31}P$ broadening or TRNOE cross-peaks in the presence of PI-PLC (*15*).

In summary, bacterial PI-PLC activity toward PI packed in vesicles is enhanced significantly by the presence of PC (also PE or SPM) molecules in the vesicle surface. In cosonicated binary vesicles, the maximum PI-PLC activity corresponds to a surface concentration of 0.2 mole fraction PC. When separate vesicles of PI and PC are mixed, collisional transfer/exchange of lipids and/or some limited fusion yields modified PI vesicles with enough PC incorporated to activate the enzyme. Since so little PC is necessary to activate PI-PLC, this may be a way to regulate enzyme activity toward substrate.

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