Nonessential Activation and Competitive Inhibition of Bacterial Phosphatidylinositol-Specific Phospholipase C by Short-Chain Phospholipids and Analogues †

ARTICLE *in* BIOCHEMISTRY · DECEMBER 1998

Impact Factor: 3.02 · DOI: 10.1021/bi980601r · Source: PubMed

2 AUTHORS:

Qing Zhou
Huazhong University of Science and Technol…
3 PUBLICATIONS **101** CITATIONS
SEE PROFILE

Mary F Roberts
Chestnut Hill College
252 PUBLICATIONS **6,217** CITATIONS
SEE PROFILE

Nonessential Activation and Competitive Inhibition of Bacterial Phosphatidylinositol-Specific Phospholipase C by Short-Chain Phospholipids and Analogues[†]

Chun Zhou and Mary F. Roberts*

Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02167

Received March 17, 1998; Revised Manuscript Received September 22, 1998

ABSTRACT: Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* is an allosteric enzyme with both a phospholipid activator site and an active site. The activation of PI-PLC enzyme is optimal with phosphatidylcholine (PC) binding to the activator site and anchoring the enzyme to the interface [Zhou, C., et al. (1997) *Biochemistry 36*, 347−355; Zhou, C., et al. (1997) *Biochemistry 36*, 10089−10091]. In contrast to PC, anionic short-chain phospholipids with smaller headgroups [phosphatidylmethanol (PMe) and phosphatidic acid (PA)] as well as phosphatidylglycerol (PG) can bind to both sites playing dual roles: nonessential activation and competitive inhibition of cyclic-(1,2)-inositol phosphate hydrolysis. PG is also a substrate, albeit a poor one, for PI-PLC, and is cleaved slowly to form α-glycerol phosphate. Analysis of enzyme kinetics using cIP as the substrate coupled with effects of different short-chain phospholipids on enzyme intrinsic fluorescence indicates that anionic phospholipids with small headgroups bind to the two sites with different affinities. If no interface is present, all dihexanoylphospholipids bind to the activator site more strongly than to the active site. When the activator site is occupied, it is likely that the enzyme undergoes a conformational change that allows phospholipids to bind easily to the active site. Such behavior is consistent with the observation that enzyme activation is detected at low short-chain anionic phospholipid concentrations with inhibition observed at higher concentrations, and that only inhibition is seen with these phospholipids added as monomers in the presence of a PC interface that optimally activates the PI-PLC. A kinetic model is used to extract the affinity of short-chain lipids for the active site from experimental data.

A number of bacteria secrete a soluble phosphatidylinositol-specific phospholipase C (PI-PLC)[1] that is specific for nonphosphorylated PI (*1*−*8*) and also cleaves GPI anchors often at a rate 10-fold higher than the rate for cleaving PI (*9*). These bacterial PI-PLC enzymes share significant sequence homology with one another, but little homology with mammalian intracellular PI-PLC enzymes (*5*). PI-PLC enzymes from *Bacillus* sp. catalyze the cleavage of the glycerophosphate linkage of PI in a stereospecific (*10*) and Ca^{2+}-independent manner in two steps (*5*, *11*): (i) an intramolecular phosphotransfer reaction to form inositol cyclic 1,2-monophosphate (cIP) and (ii) hydrolysis of the cIP to produce inositol 1-phosphate. The second step is considerably less efficient than the phosphotransferase reaction since the enzyme has a much higher K_m for cIP and a much lower V_{max} (*12*).

Bacillus thuringiensis PI-PLC exhibits a 5−6-fold kinetic preference for micellar PI compared to monomeric substrate ["interfacial activation" (*13*)]. However, one of the more unusual features of this secreted PI-PLC is a novel type of interfacial activation of the enzyme toward cIP, a water-soluble substrate with no tendency to partition into interfaces. PC (or PE) micelles (as well as bilayers) activate the enzyme significantly by binding to the enzyme at a discrete site and allosterically affecting both K_m and V_{max} (*12*, *14*, *15*). In the presence of diC_7PC micelles, K_m decreased (e.g., from 90 to 29 mM with diC_7PC micelles added), while V_{max} increased almost 7-fold. The enzyme efficiency (V_{max}/K_m) in the presence of diC_7PC micelles increased more than 21-fold, although it was still 20-fold lower than the initial phosphotransferase activity for monomeric diC_6PI. It has been suggested that this type of phospholipid activation serves to anchor the enzyme to the aggregate surface in a more active form that enhances catalysis of water-soluble as well as interfacial substrates (*15*). PC activation is quite striking, in part, because that molecule appears to have no affinity for the enzyme active site. Micelles of phospholipids with other headgroups exhibit little activation (*12*), but that could reflect active site inhibition of PI-PLC.

To determine how a wide variety of phospholipids interact with *B. thuringiensis* PI-PLC, we have used kinetic, NMR, and fluorescence experiments to define activator and active site binding. A model is proposed that includes a nonessential activation of PI-PLC by an amphiphile binding to the activator site coupled with phospholipids with headgroups

[†] This work has been supported by NIH Grant GM 26762 (M.F.R.).

* To whom correspondence should be addressed. E-mail: mary.roberts@bc.edu. Fax: (617) 552-2705. Phone: (617) 552-3616.

[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; PLD, phospholipase D; CMC, critical micelle concentration; PI, phosphatidylinositol; PC, phosphatidylcholine; diC_n-, 1,2-diacyl phospholipid; PS, phosphatidylserine; PG, phosphatidylglycerol; PMe, phosphatidylmethanol; PEg, phosphatidylethylene glycol; PA, phosphatidic acid; cIP, myoinositol 1,2-(cyclic)-phosphate; MeOH, methanol.

10.1021/bi980601r CCC: $15.00
Published on Web 10/28/1998

smaller than PC binding to the active site as competitive inhibitors of cIP hydrolysis. This provides relative affinities of different lipids for both sites on PI-PLC.

MATERIALS AND METHODS

Materials. The short-chain phospholipids diC_6PC, diC_7-PC, diC_6PE, diC_6PG, and diC_8PG and long-chain phospholipids diC_{18}PG, diC_{16}PMe, and 1-palmitoyl-2-oleoyl-PA (1-C_{16}-2-$C_{18:1}$PA) were purchased from Avanti and used without further purification. diC_7PI with L-inositol stereochemistry was synthesized and purified as described previously (*16*). D_2O (99.9% enriched) and Triton X-100 detergent were obtained from Sigma. NaOD, DCl, and Tris-d_{11} were purchased from Cambridge Isotope Laboratories.

Isolation of PI-PLC and Preparation of the cIP Substrate. A recombinant strain of *Bacillus subtilis* transfected with the *B. thuringiensis* PI-PLC gene for overproduction of the PI-PLC enzyme was obtained from M. G. Low (Columbia University, New York). The enzyme was isolated from culture supernatants and purified as described previously (*12*, *17*). cIP was enzymatically synthesized from crude phosphatidylinositol (50% PI) purchased from Sigma by the action of PI-PLC on PI/TX-100 mixed micelles (*12*, *14*). After PI was completely converted to cIP (as monitored by ^{31}P NMR spectroscopy), the aqueous phase was chromatographed on an AG1-X8 anion exchange column to purify the cIP.

Enzymatic Synthesis and Purification of diC_nPMe and diC_nPEg. PLD from *Streptomyces* VII with high transferase activity was purchased from Sigma. Fifty milligrams of diC_6-PC (or diC_7PC) was dissolved in 2 mL of 50 mM acetic acid/sodium acetate buffer (pH 6.0). For PMe synthesis, 50% MeOH (2 mL of MeOH) was added along with 30 μg of PLD. The reaction progress was monitored by ^{31}P NMR spectroscopy. After the reaction was complete (100% transfer took several hours), the solvent was evaporated and the sample was lyophilized overnight. The dry residue was mixed with $CHCl_3$ to solubilize phospholipids. After the undissolved salt was filtered, the chloroform solution was applied to a silica gel column and eluted with MeOH/$CHCl_3$ (2:3) saturated with H_2O. About 35 mg of diC_6PMe (or diC_7-PMe), characterized by a ^{31}P chemical shift of 2 ppm and the phosphorus-coupled methoxy resonance (J_{H-P} = 10 Hz) at 3.5 ppm in the ^{1}H spectrum [in 50 mM HEPES (pH 7.5)], was obtained in this fashion. For the preparation of diC_6-PEg or diC_7PEg, 300 μL of ethylene glycol in 50% ether (2 mL) was used in the PLD transferase reaction. The diC_n-PEg purification procedure was the same as that for diC_6-PMe.

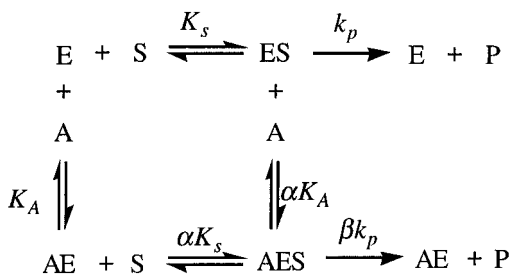
Enzymatic Synthesis and Purification of Short-Chain PA. PLD from *Streptomyces* purchased from Sigma was also used to generate short-chain PA. The reaction was carried out with 0.1 mM Ca^{2+} in 50 mM Tris-HCl (pH 7.0). After the hydrolysis reaction was complete, the PA was extracted with $CHCl_3$/MeOH (4:1). The extract solution was washed twice with H_2O. The solvent was evaporated, and the sample was redissolved in D_2O or water with 50 mM HEPES (pH 7.5). The chemical shift of diC_6PA was 4.2 ppm at pH 7.5. diC_7-PA and diC_8PA were prepared from the corresponding PC in a similar fashion.

^{31}P NMR Assay of PI-PLC Activity. The cyclic phosphodiesterase activity of PI-PLC was monitored by ^{31}P NMR spectroscopy. The buffer used in all PI-PLC assays was 50 mM HEPES (pH 7.5). All stock solutions were prepared in D_2O, and the pH was adjusted to a meter reading of 7.5. The cIP concentration was 8 mM unless otherwise noted; this is well below the K_m for this substrate of PI-PLC (*12*). The ^{31}P NMR parameters were optimized on the basis of those initially used by Griffith and co-workers (*11*) as modified by Zhou et al. (*12*). The reaction, initiated by addition of the appropriate amount of PI-PLC, was monitored for 1−2 h at 30 °C. The rate of cIP hydrolysis (micromoles per minute per milligram) was calculated from the integrated intensity of the I-1-P (increasing) or cIP (decreasing).

Fluorescence Measurements. PI-PLC used for intrinsic fluorescence measurements was 1−2 μM in HEPES at pH 7.5. Steady-state fluorescence measurements were taken with a Shimadzu RF 5000 V spectrofluorimeter (with a xenon light source) at 23 °C. The excitation wavelength was 290 nm, with both excitation and emission slit widths set at 3 nm. The emission was scanned from 295 to 500 nm.

Preparation of Samples for TRNOE Measurements. The purified PI-PLC enzyme was dialyzed overnight against 50 mM NaCl (pH 7.0) and then frozen and lyophilized. The sample was rehydrated in D_2O containing 20 mM Tris-d_{11} buffer (pH 7.5). A typical sample used for TRNOE measurements contained 1.2 mg of enzyme in 350 μL. Two-dimensional NMR experiments were carried out on a Varian UNITY 500 NMR spectrometer using an indirect probe. TRNOE spectra were acquired using the two-dimensional NOESY pulse sequence in the phase-sensitive mode by collecting hypercomplex data. Residual water suppression was achieved by selectively saturating the water resonance during the relaxation delay (1.5 s) and the mixing time (0.1−0.2 ms) of the NOESY sequence. Spectra were obtained at 30 °C without spinning the sample. There were 2048 points in F2 and 256 points in F1 dimensions. Zero filling was employed, and final spectra contained 2K × 2K real points. A Gaussian weighting function was used in both dimensions.

Kinetic Treatment for the Nonessential Activation and Competitive Inhibition. Bacterial PI-PLC has an allosteric activator site as well as an active site (*12*, *14*, *15*), and a given phospholipid can bind to either site or both sites. In the following scheme, E represents enzyme, A is an activator or inhibitor, and S is the water-soluble substrate cIP. The notation AE represents ligand bound to the activator site; EA or ES indicates occupation of the active site by A or S. {A} represents activator lipid associated with an interface. Binding constants and rate constants for the activated enzyme are assumed to be proportional to those for the unactivated enzyme with proportionality constants α (for K_s) and β (for $k_p = k_{cat}$).



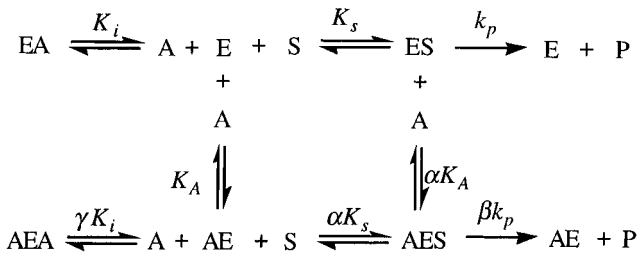
Enzyme velocity is given by $v = k_p[ES] + \beta k_p[AES]$.

$$v = \frac{V_{max}\frac{[S]}{K_s} + \beta V_{max}\frac{[A][S]}{\alpha K_A K_s}}{1 + \frac{[S]}{K_s} + \frac{[A]}{K_A} + \frac{[A][S]}{\alpha K_A K_s}}$$

$$v = \frac{V_{max}[S]}{K_s\frac{\left(1 + \frac{[A]}{K_A}\right)}{\left(1 + \frac{\beta[A]}{\alpha K_A}\right)} + [S]\frac{\left(1 + \frac{[A]}{\alpha K_A}\right)}{\left(1 + \frac{\beta[A]}{\alpha K_A}\right)}} \quad (1)$$

Since PI-PLC activity toward cIP is detected without any activating PC, the activation produced by PC micelles can be treated as a "nonessential" activation. V_{max} and K_s for cIP were measured previously (*12*); $V_{max} = 20$ μmol min^{-1} mg^{-1}, and $K_s = 90$ mM. The parameters α and β change with activator concentration, making this part of the curve hard to fit. With diC$_7$PC as an activator, $\alpha = 0.3$ and $\beta = 7$ when the activator site is saturated by the PC interface.

In contrast to PC, phospholipids with small headgroups can bind to the active site as competitive inhibitors; they can also have an affinity for the activator site which could differ for monomeric and micellar activators. They have dual roles in affecting PI-PLC catalysis: nonessential activation and competitive inhibition.

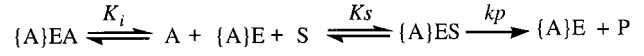


If only competitive inhibition is possible (because the activator site is saturated with a high-affinity ligand such as PC), eq 2 describes the effect of the inhibitor on enzyme activity.

$$v = \frac{V_{max}[S]}{K_s\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (2)$$

In this system, V_{max} and K_s vary with the concentration of the phospholipid activator. K_i, like K_s, can also change with lipid concentration [in fact, a nonhydrolyzable cIP analogue was shown to have its K_i substantially lowered in the presence of an interface (*14*)].

If the activator site is saturated by the presence of a PC interface, binding to the active site of another amphiphile (e.g., PMe, PG, etc.) will inhibit PI-PLC. The inhibition due to binding of a ligand at the active site gives rise to a measurable K_i.



The velocity equation becomes $v = k_p[\{A\}ES]$.

Extraction of this K_i value is straightforward if the inhibitor is water-soluble and binds only to the active site. If it aggregates and that affects the affinity for the active site, that complicates the interpretation of the extracted numbers. Also, if it has a higher affinity for the activator site when it is micellar, that may affect which lipid (PC or PMe, etc.) occupies the active site. Because of these complications, we chose to use the term "apparent K_i" for describing the extracted value that represents the inhibitory behavior of PMe, PG, and PA toward PI-PLC hydrolysis of cIP when diC$_7$PC is added to activate the enzyme. The K_i may be different for monomeric and for micellar ligands, and this could also be factored into the equation, although the data for a given lipid appear to be well fit by a single K_i (vide infra). For the enzyme activated by the diC$_7$PC interface, $V_{max} = 136$ μmol min^{-1} mg^{-1} and $K_s = 30$ mM.

RESULTS

Effect of Short-Chain Lipids on PI-PLC Activity toward cIP Hydrolysis. Hydrolysis of cIP, the second step of PI-PLC catalysis, is much slower than PI cleavage. As a water-soluble substrate with no measurable affinity for an interface, cIP is ideal for studying allosteric interfacial activation of PI-PLC. Using cIP eliminates changes in the interfacial concentration of substrate caused by an amphiphilic, surface active substrate. A previous study (*12*) of cIP hydrolysis by PI-PLC showed that many detergents and short-chain lipids at 8 mM activated the enzyme about 2-fold, with PC species yielding the largest increases in PI-PLC specific activity. PI-PLC specific activity toward 8 mM cIP (well below the K_m for this substrate), examined as a function of the concentration of TX-100, diC$_8$PC, diC$_7$PC, and diC$_6$PC, reached a maximum around the CMC of the detergent. For the PC lipids, enzyme activity remained relatively constant as the detergent concentration increased further above the PC CMC (diC$_6$PC activation of PI-PLC hydrolysis of cIP is shown in Figure 1 A). This behavior indicated that the enzyme was saturated by the activator molecule and/or surface. PI-PLC specific activity toward cIP as a function of the concentration of other added lipids (PG, PMe, and PA) exhibited markedly different behavior as shown in panels B−D of Figure 1. All of these anionic short-chain phospholipids exhibited similar behavior; at low lipid concentrations, PI-PLC activity toward cIP increased to a maximum, and then decreased at higher lipid concentrations. The concentration of lipid that led to the maximum PI-PLC activity toward 8 mM cIP depended on the identity of the phospholipid headgroup and on the acyl chain length. The maximum PI-PLC specific activity occurred at 16 mM diC$_6$-PG, 8.4 mM diC$_6$PMe, and 3.4 mM diC$_6$PA. The maximum increases in PI-PLC activity under these conditions were 9-, 6-, and 4-fold for diC$_6$PG, diC$_6$PMe, and diC$_6$PA, respectively. Both diC$_8$PG and diC$_7$PA were examined to determine the influence of acyl chain length on cIP hydrolysis. The maximum PI-PLC specific activity occurred at lower diC$_8$PG or diC$_7$PA concentrations compared to that of the diC$_6$ analogues, and the enhancement in activity was much smaller (panels B and D of Figure 1). diC$_6$PEg exhibited behavior similar to that of DiC$_6$PG; the slightly more hydrophobic diC$_7$PEg exhibited the same behavior as the

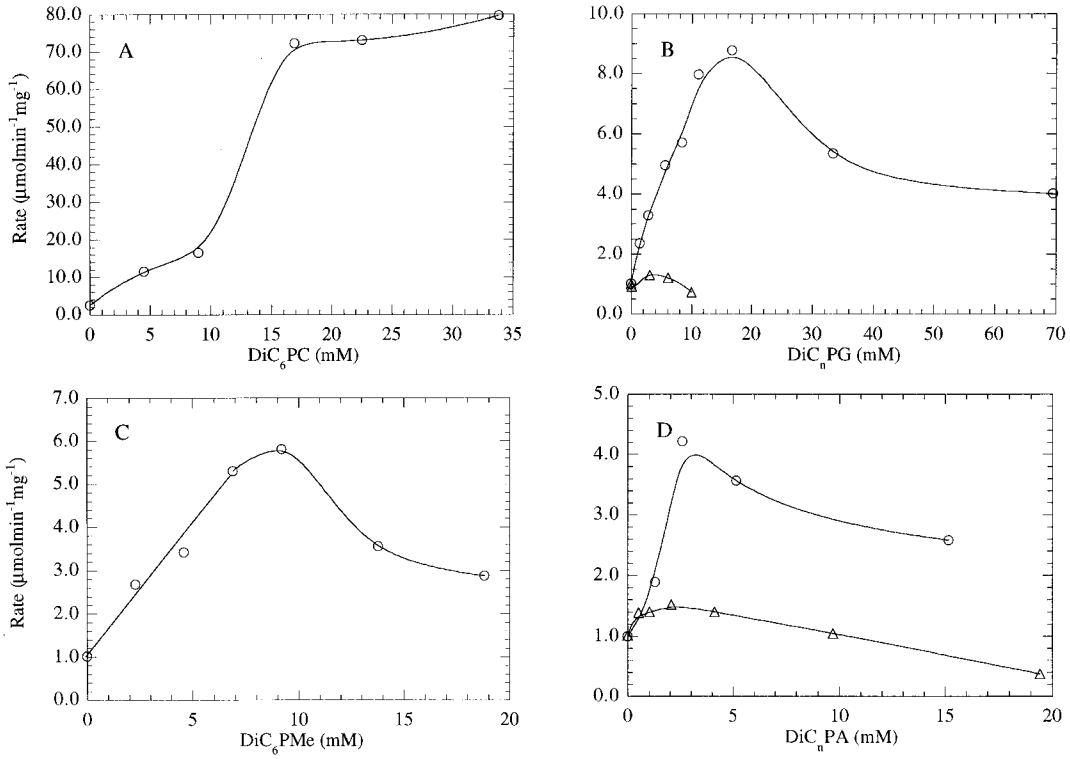


FIGURE 1: PI-PLC activity toward cIP as a function of the concentration of added (A) diC_6PC, (B) diC_6PG (○) and diC_8PG (△), (C) diC_6PMe, and (D) diC_6PA (○) and diC_7PA (△). Assay conditions included 50 mM HEPES (pH 7.5), 8 mM cIP, and 2.2 μg of enzyme (A) and 10 μg of enzyme (B−D). The data shown in panel A are from ref *12*.

diC_6PG/diC_8PG pair, a decreased maximum increase in PI-PLC activity toward cIP occurring at a lower diC_7PEg concentration. The onset of PI-PLC inhibition follows the CMC of the short-chain phospholipid. Therefore, these observations suggest that inhibition, i.e., binding of the amphiphile to the active site, is enhanced when the phospholipid is aggregated.

Not all short-chain lipids examined had significant affinity for the activator or the active site. As shown previously, PS has at best a very weak affinity for the activator site since no evidence of binding was detected in TRNOE or ^{31}P line width studies with diC_6PS (*15*), and no inhibition of cIP hydrolysis was observed at high concentrations of this lipid. Significant activation (~2-fold) only occurred above the diC_6-PS CMC (*15*). The affinity of substrate PI for the activator site is hard to assess because it is cleaved quickly by the enzyme. A previous study (*13*) showed that diC_7P-(L)I, whose CMC is 1.4 mM, was neither a substrate nor a good inhibitor of the correct isomer. The activator site of the enzyme is not specific and should not be stereoselective, so some binding to the activator site should occur. This can be measured by examining the effect of diC_7P(L-I) on cIP cleavage (Figure 2). The activation of PI-PLC toward 8 mM cIP was 2-fold, similar to that caused by TX-100 and PS. Activation occurred below the CMC of diC_7P(L-I), suggesting the molecule binds to the activator site as a monomer or the enzyme significantly lowers the CMC of this lipid. If the hyperbolic activation curve is used to extract a value of K_A for diC_7P(L-I) binding, a value of 0.3 mM is obtained. Interestingly, this is comparable to the values for diC_7PC and diC_6PC binding as monomers to the activator site of PI-PLC (*15*). This may suggest that diC_7P(L-I) binds to the enzyme (not at the active site), but not very effectively as an activator compared to PC or PE. It is likely that diC_7P-(D-I) behaves in a fashion similar to that of diC_7P(L-I); it may also bind at a site other than the active site, but such an interaction does not lead to the appropriate conformation of PI-PLC necessary for optimal activity.

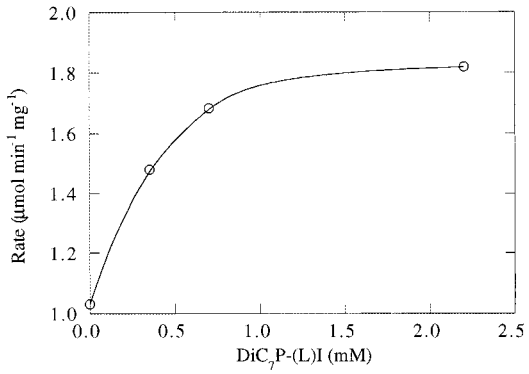


FIGURE 2: PI-PLC activity toward cIP as a function of the concentration of added diC_7P-(L)I.

Intrinsic Fluorescence of PI-PLC in the Presence of Short-Chain Lipids. PI analogues bound to the active site of PI-PLC cause a decrease in the protein intrinsic fluorescence (*18*). In contrast, a small increase in the PI-PLC intrinsic fluorescence occurs when monomeric PC binds to the activator site. This is followed by a much larger increase when the PC aggregates bind (*15*). The fluorescence of PI-PLC as a function of the concentration of diC_6PC is shown in Figure 3A. The large increase in fluorescence intensity correlates with the micellization of diC_6PC and anchoring of PI-PLC to a micelle via the bound monomeric PC (*15*). In contrast to PC, the other phospholipids examined led to inhibition of PI-PLC activity at high lipid concentrations. If these lipids were bound to the active site, the intrinsic fluorescence of PI-PLC should decrease. diC_6PG, diC_6PMe,

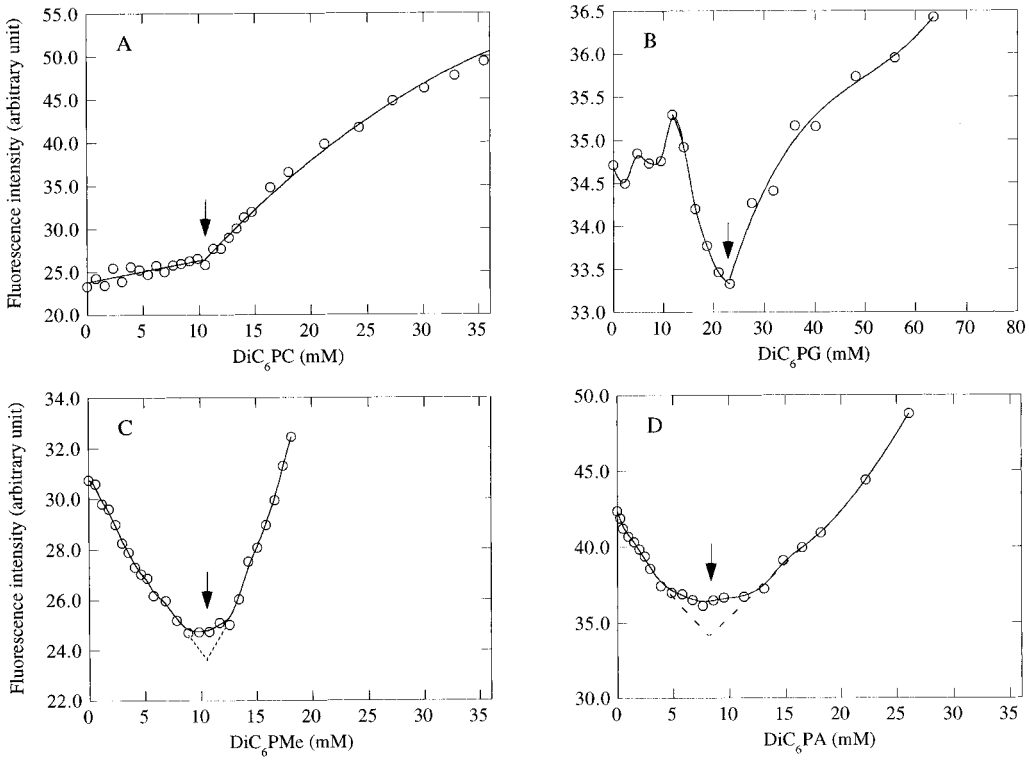


FIGURE 3: PI-PLC (2 μM) intrinsic fluorescence intensity as a function of the concentration of added (A) diC_6PC, (B) diC_6PG, (C) diC_6-PMe, and (D) diC_6PA. The data in panel A are from ref *15*.

and diC_6PA were examined for their effects on PI-PLC fluorescence. diC_6PG has a fairly high CMC, ~22 mM; the CMC of diC_6PMe is ~12 mM (*19*), and that of diC_6PA is ~10−14 mM depending on buffer conditions (*27*). The response of PI-PLC fluorescence to the presence of diC_6PG was complex (Figure 3 B) and exhibited three distinct phases. At low diC_6PG concentrations, there was a small increase in enzyme fluorescence that reflects occupation of the activator site by a monomeric lipid. Above 10 mM diC_6PG (but significantly below the CMC for this lipid), the decrease in fluorescence indicated that monomeric diC_6PG binding to the active site was significant and dominated the intrinsic fluorescence behavior of the protein. The decreasing PI-PLC fluorescence corresponding to active site binding of PG correlated with a decreased PI-PLC activation level and, in the vicinity of the CMC, inhibition of the enzyme activity toward cIP (Figure 1B). Above 25 mM diC_6PG, the protein fluorescence increased again, consistent with anchoring the enzyme to the PG interface.

The fluorescence of PI-PLC in the presence of diC_6PMe is shown in Figure 3C. The kinetic data in Figure 1C (notably, the decrease in activity at a lipid concentration 5 mM above that producing the maximum activity) suggest that diC_6PMe is a more potent inhibitor than diC_6PG. Rather than appearing triphasic as the fluorescence of PI-PLC with diC_6PG, the PI-PLC fluorescence decreased at diC_6PMe concentrations below the CMC and then increased as interface was present (above 10 mM) and served to anchor the enzyme. Since low concentrations of PMe activated PI-PLC, the decrease in fluorescence must include both the binding to the activator site and the binding to the active site. Because the affinity of diC_6PMe for the active site is higher than that of diC_6PG, the fluorescence change due to the binding to the activator site is canceled by the binding to the active site. diC_6PA affected PI-PLC intrinsic fluorescence in much the same way as the PMe compound. The fluorescence intensity first decreased, and then increased once micellar PA species were formed. However, for this lipid the kinetic inhibition after activation occurs well below the CMC of the lipid, indicating that unlike the other short-chain lipids, PA has a high affinity for the active site.

An added bonus of the PI-PLC fluorescence data is that the CMCs of the lipids in the presence of the enzyme can be extrapolated from the intersection of the linear decrease in fluorescence with the increase caused by the presence of micelles. For diC_6PG, diC_6PMe, and diC_6PA, these values are 22, 10.5, and 8 mM, respectively.

Affinity of Phospholipids for the PI-PLC Active Site. Anionic phospholipids with small (PA or PMe) or moderate size (PG) headgroups have affinity for both the activator site and the active site of PI-PLC. If we can saturate the activator site, the affinity for the active site can be measured under these conditions. diC_7PC was chosen to saturate the activator site since it has high affinity (~0.2 mM) for the enzyme and since it has a low CMC (*15*). As shown above, the added anionic lipids have weaker affinities for the activator site and compete poorly with aggregated PC at the activator site. However, they may partition to the PC interface and decrease the PC surface concentration which would in turn cause inhibition of PI-PLC. This surface dilution effect was examined using TX-100 (0.26 mM CMC) as shown in Figure 4A. If it is assumed that micelles of TX-100 and diC_7PC mix ideally [an assumption reasonably well-justified by fluorescence studies of mixing of pyrene-labeled PC with the nonionic detergent (*20*)], the presence of 8 mM TX-100 dilutes the diC_7PC (8 mM) surface 2-fold. However, PI-PLC activity toward cIP decreased only 15% as the PC surface concentration was decreased 2-fold, indicating that

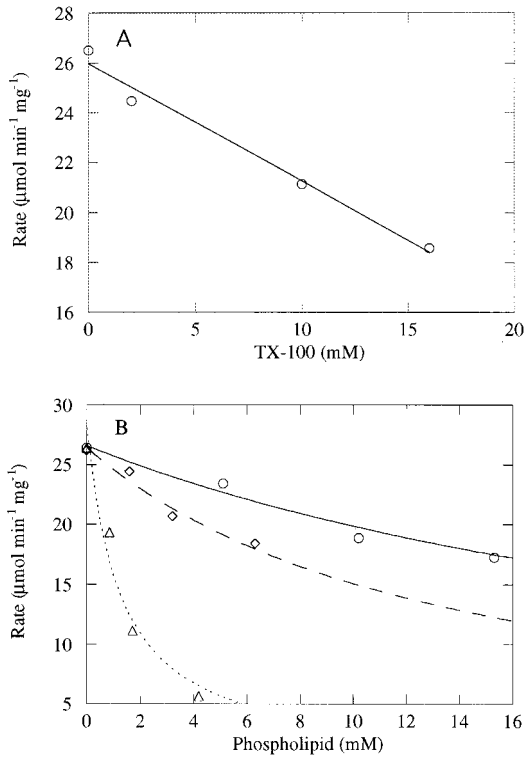


FIGURE 4: (A) TX-100 surface dilution of 8 mM diC$_7$PC activation of PI-PLC toward cIP and (B) inhibition of diC$_7$PC (8 mM)-activated PI-PLC hydrolysis of cIP by (○) diC$_6$PG, (◇) diC$_6$PMe, and (△) diC$_6$PA.

the PC has a higher affinity for the activator site than TX-100. The decrease in PI-PLC activity was proportional to the concentration of TX-100. If PG, PMe, and PA dilute the PC surface in a fashion similar to that of TX-100 and do not bind significantly to the activator or to the active site, this is the magnitude of the inhibition of PI-PLC activity toward cIP that would be expected. The three lipids diC$_6$-PG, diC$_6$PMe, and diC$_6$PA in the monomer concentration region were examined for their effects on diC$_7$PC-activated PI-PLC hydrolysis of cIP (Figure 4B). Under these conditions, the distribution of anionic lipids in the PC interface should be very small and the surface dilution effect (e.g., reduction of the effective concentration of PC at the activator site or replacing it with a less effective anionic lipid) can be ignored. Of the three anionic lipids, diC$_6$PA was clearly the most potent inhibitor; diC$_6$PG was the weakest of the three.

As mentioned earlier, the extent of competitive inhibition of an additive with cIP can be estimated when the PI-PLC activator site is saturated with PC. The K_i value extracted is fairly quantitative for a monomeric additive, but its interpretation of the value is more complicated if the additive partitions into the PC micelles and is a strong competitor for PC binding to the activator site, and once bound does not induce the same extent of activation. The curves in Figure 4B represent the fits to eq 2 which assumes competitive inhibition by the anionic lipids. The K_i values were 23, 10, and 1 mM for diC$_6$PG, diC$_6$PMe, and diC$_6$PA, respectively. All the anionic phospholipids with small headgroups have a higher affinity for the active site than cIP ($K_m \sim 30$ mM). PA is a potent inhibitor when it is monomeric, since 2 mM PA added to a system with 8 mM diC$_7$PC decreased the extent of cIP hydrolysis by more than 50%. This dianionic lipid clearly has a high affinity for the

Table 1: Apparent K_i Values for Phospholipids Binding to PI-PLC and Inhibiting cIP Hydrolysis in the Presence of diC$_7$PC Causing Saturation of the Activator Site[a]

phospholipid	apparent K_i (mM)[b]	phospholipid	apparent K_i (mM)[b]
diC$_6$PA	1.0	diC$_7$PMe	2.4
diC$_7$PA	1.2	diC$_{16}$PMe	1−2
1-C$_{16}$-2-C$_{18:1}$PA	0.7	diC$_6$PG	23
diC$_6$PMe	10	diC$_{18}$PG	1.1

[a] Assay conditions include 8 mM cIP and 8 mM diC$_7$PC, in 50 mM HEPES (pH 7.5). [b] Apparent K_i values of the added phospholipid determined from fitting PC-activated PI-PLC activity toward cIP in the presence of diC$_n$PX as a competitive inhibitor.

active site of PI-PLC. For the other two anionic lipids, the K_i values correspond to the CMC values for diC$_6$PG and diC$_6$PMe. This strongly suggests that micellar PG and PMe have a higher affinity for the active site than the corresponding monomeric lipids. Interestingly, the K_i values estimated for diC$_7$PMe and diC$_7$PA are 2.4 and 1.2 mM, respectively (Table 1). The value for diC$_7$PA is essentially the same as that for diC$_6$PA, while the K_i for PMe follows its CMC. A comparison of longer-chain phospholipids (Table 1) indicates that once an inhibitory molecule is in interface, the apparent K_i values are all similar and in the 1−2 mM range; i.e., there is no dependence on chain length for molecules binding to the active site. Alternatively, the inhibition data indicate that a mole fraction of 0.1−0.2 PA, PMe, or PG in the PC surface is sufficient to occupy 50% of the active sites and inhibit cIP hydrolysis. This is consistent with what has been extrapolated from the crystal structure of the enzyme with inositol bound (*28*). Chain interactions of the substrate with the enzyme are predicted to be relatively nonspecific.

Affinity of Different Phospholipids for the PI-PLC Activator Site. Previous studies using TRNOE and line shape analysis derived a dissociation constant of 0.2−0.5 mM for binding of PC to the activator site (*15*). For the other lipids with smaller headgroups, binding to both activator and active sites makes an analysis of phospholipid line shape intractable. However, the TRNOE pattern can provide a qualitative analysis of monomeric lipid binding to PI-PLC. Monomer concentrations of diC$_6$PMe and diC$_6$PG exhibited a few positive NOEs, similar to monomeric concentrations of diC$_6$-PC. In the presence of PI-PLC (0.1 mM), the cross-peaks due to cross relaxation of diC$_6$PMe or diC$_6$PG (4 mM) changed to the same phase as diagonal peaks, indicating NOEs were now negative. The number and identity of cross-peaks observed depended on the TRNOE mixing time as shown in Figure 5. At a mixing time of 0.1 s, negative NOEs were observed between different acyl chain protons or between different backbone protons. When the mixing time was increased to 0.2 s, all resonances were correlated (i.e., cross-peaks between all resonances were detected). This latter pattern is similar to that for diC$_6$PC in the presence of PI-PLC at a mixing time of 0.1 s (*15*). The apparent K_i values representing binding of these lipids to the active site when the activator site was saturated with 8 mM diC$_7$PC were 10 mM for diC$_6$PMe and 23 mM for diC$_6$PG. Since the K_m for cIP and K_i for cICH$_2$P, a substrate analogue (*14*), are at least 3-fold higher when the activator site is not saturated by PC, it is likely that affinities of PG and PMe for the active site are poorer and the K_i values higher than those mentioned above. Since the TRNOE experiments were

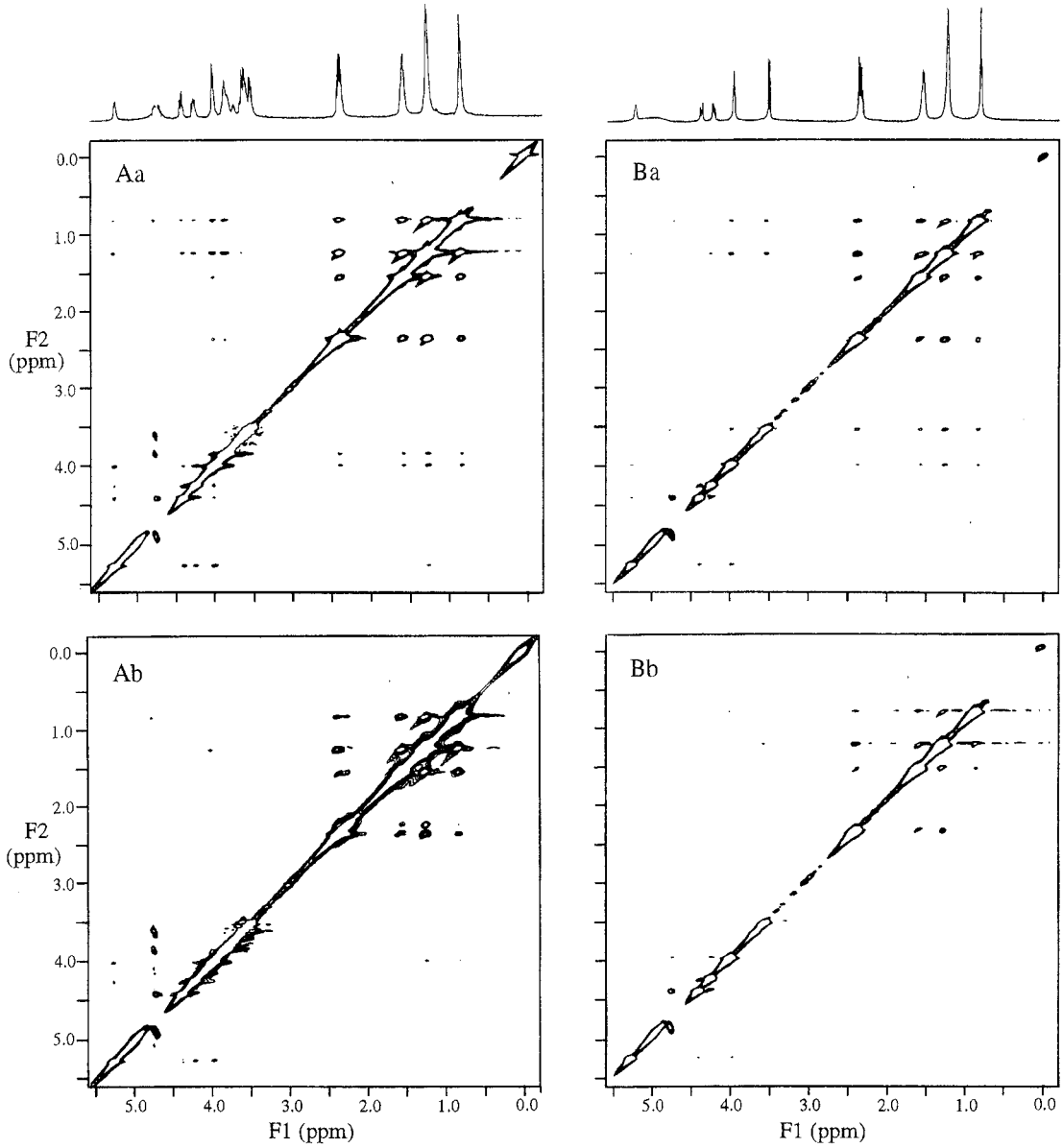


FIGURE 5: NOESY contour plots for 4 mM diC_6PG (A) and 4 mM diC_6PMe (B) in the presence of 0.1 mM PI-PLC. The mixing time is 0.2 s in panels a and 0.1 s in panels b. All cross-peaks have the same phase as diagonal and indicate negative NOEs.

carried out without PC, the K_i values for PG and PMe binding to the active site are no lower than 10 mM for PMe and 23 mM for PG and are probably higher. Thus, very little of these species should be bound to the active site of the enzyme, and active site-bound phospholipid should represent a minor fraction of the lipid at the 4 mM diC_6PG and diC_6-PMe concentrations used in the TRNOE experiments. Under these conditions, the bulk of the lipid interacts with PI-PLC through the activator site. The TRNOE data indicated that PMe or PG affinities for the activator site are weaker than that of PC [but much stronger than that of PS (*15*)].

A striking observation in the TRNOE spectrum (0.1 s mixing time) for diC_6PG is that there are extra peaks in the acyl chain region (Figure 5Ab), suggesting another species is present in the sample (this experiment was carried out after the TRNOE with a 0.2 s mixing time). The ^{31}P NMR spectrum (Figure 6) of this sample showed a new resonance corresponding to α-glycerophosphate. Thus, under these conditions where monomeric PG can bind to the activator site, sufficient PG must also bind to the PI-PLC active site so that cleavage of diC_6PG is detected. The headgroup of PG has a hydroxyl group at the β-position, similar to the 2′-OH of inositol. Presumably when PG binds to the active site, this hydroxyl group can be oriented for an intramolecular phosphotransferase reaction to form a cyclic intermediate which is then released. Such a cyclic glycerophosphate would not be stable and would quickly be hydrolyzed in water to form α-glycerophosphate. The PI-PLC specific activity toward 4 mM diC_6PG (e.g., monomeric PG) was estimated to be 10^{-3} μmol min^{-1} mg^{-1}, about 250-fold lower than with a comparable concentration of cIP as a substrate. The observation that PG is a substrate supports the hypothesis that it (and PMe and PA) is a competitive inhibitor of cIP hydrolysis by PI-PLC.

The activation curves in Figure 1 are hard to fit using eq 1 because, as discussed previously, parameters α and β are not constant but a function of lipid concentration. In a simpler model, the contribution to the observed PI-PLC activity from short-chain phospholipid binding to the active site as a competitive inhibitor can be estimated. This will

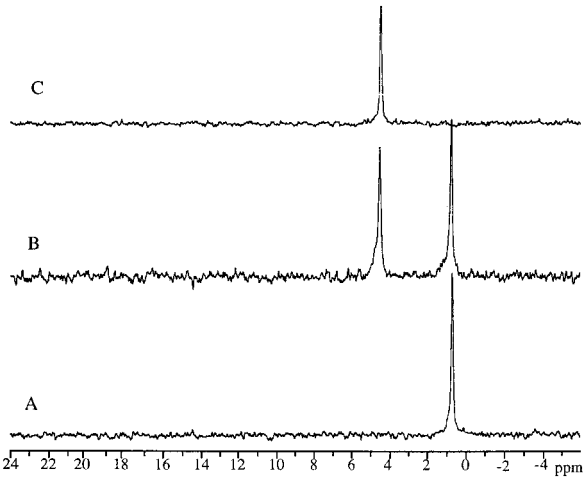


FIGURE 6: ^{31}P NMR spectra of diC$_6$PG (A) alone or (B) after incubation with 0.1 mM PI-PLC for 30 h at 30 °C and (C) authentic α-glycerophosphate.

produce a new curve that reflects only lipid activation of PI-PLC toward cIP. There are two limits for approximating the activation. (i) The activator site is not occupied; the K_i values for monomeric diC$_6$PG and diC$_6$PMe are probably at least 69 and 30 mM (3 times higher than the values determined for these lipids in the presence of diC$_7$PC), while the K_s for cIP is 90 mM. (ii) The activator site is saturated with the anionic phospholipid interface; K_i values for monomeric diC$_6$PG and diC$_6$PMe are 23 and 10 mM, and the K_s for cIP is 30 mM. According to eq 2, the competitive inhibition can then be corrected with the factor $[[S] + K_s(1 + [I]/K_i)]/([S] + K_s)$. The two limits for correcting for active site binding of PG and PMe lead to the dotted and dashed lines in Figure 7. These calculated curves now have specific activities considerably higher than the actual data (open circles). The true course of PI-PLC activation toward cIP induced by diC$_6$PG and diC$_6$PMe will be somewhere between the two calculated lines since saturation of the enzyme activator site by PG and PMe may not induce the same extent of activation of PI-PLC toward cIP as PC (i.e., these anionic phospholipids behave more like TX-100 and not like PCs). A more complicated model could have the high K_i and K_m values for monomeric lipid and the 3-fold lower values once the added phospholipid forms micelles. However, the effect of the anionic interface may not be the same as that of a PC interface. If upon binding to the PI-PLC activator site it behaves more like TX-100, then relative affinities of ligands for the active site are weaker than when the enzyme has PC bound to the activator site. Given these complications, we chose to use a single K_i (optimized for PC-activated PI-PLC) for each specific anionic lipid; for diC$_6$-phospholipid, the K_i in the presence of a PC interface is dominated by monomer behavior, and for the diC$_7$-lipid, the K_i in the same PC-activated PI-PLC system is dominated by the effect of the aggregated phospholipid. The point of these calculations is that with corrections for PG and PMe competitive inhibition, the maximum PI-PLC activity (13 μmol min^{-1} mg^{-1} for diC$_6$-PG and 10 μmol min^{-1} mg^{-1} for diC$_6$PMe) induced by the anionic phospholipids begins to approach that for cIP hydrolysis in the presence of PC micelles (70−75 μmol min^{-1} mg^{-1}). It is, however, still lower, strongly suggesting that occupation of the activator site by the anionic lipids does not stabilize the optimal PI-PLC conformation.

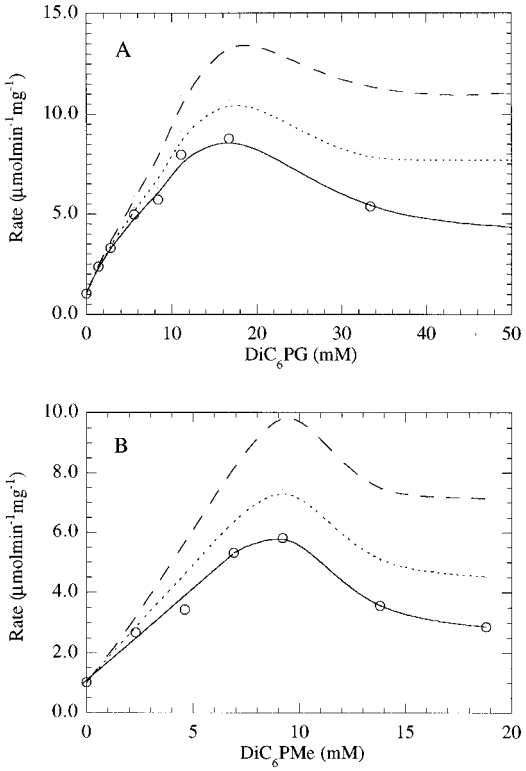


FIGURE 7: Approximation of (A) diC$_6$PG- and (B) diC$_6$PMe-induced activation of PI-PLC toward cIP by removing the effect of competitive inhibition by the same short-chain lipids. The experimental dependence of the extent of PI-PLC hydrolysis of cIP on short-chain lipid concentration, which contains both activation and competitive inhibition, is shown with the open circles. Two models are used to estimate activity vs lipid concentration without inhibition due to lipid binding at the active site. (i) The activator site is not occupied; the K_i values for monomeric diC$_6$PG and diC$_6$-PMe are 3 times higher than the values determined for these lipids in the presence of diC$_7$PC, while the K_s for cIP is 90 mM (dotted line). (ii) The activator site is saturated with the PG or PMe interface; K_i values for diC$_6$PG and diC$_6$PMe are 23 and 10 mM, respectively, and the K_s for cIP is 30 mM (dashed line). It is likely that the true activation curve lies between the dotted and dashed lines.

Even when monomeric lipids are examined, diC$_6$PC induces significantly higher PI-PLC activity than diC$_6$PG or diC$_6$PMe. The constant for binding of diC$_6$PC to the activator site is 0.2−0.5 mM (*15*); however, the extent of kinetic activation of PI-PLC toward cIP is linear in the range of 1−10 mM PC. A reasonable interpretation of this is that the increase in PI-PLC specific activity with an increasing amount of "monomeric" PC arises from local aggregate formation around the tightly bound monomeric lipid that optimally activates some of the PI-PLC−diC$_6$PC complexes. Once the CMC is reached, diC$_6$PC enhances PI-PLC activity dramatically because now all the PI-PLC−PC complexes are saturated with interfaces. On the basis of the comparisons of the TRNOE profiles, the affinities of monomeric diC$_6$PG and diC$_6$PMe for the PI-PLC activator site are lower than that of diC$_6$PC; yet they must be sufficiently strong such that an enzyme−monomeric phospholipid complex is formed in the millimolar region. Similar to what is proposed for PC, the linear increase in PI-PLC specific activity with millimolar monomeric PG or PMe may also represent local aggregate formation about a PI-PLC−diC$_6$PMe or PLC−diC$_6$PG complex that stabilizes a more active conformation of PI-PLC. However, the lack of a very strong enhancement

Table 2: Effect of Different Short-Chain Phospholipids on the Specific Activity of PI-PLC toward cIP

phospholipid	CMC[a] (mM)	[diC$_n$PX] (mM) for SA_{max}	SA_{max}[b]/SA_o	apparent K_i[c] (mM)
diC$_6$PC	14	>15	30−50	ni[d]
diC$_7$PC	1.5	>4	30−50	ni[d]
diC$_6$PG	21.5 ± 1.5[e]	16	8.5	23
diC$_8$PG	<4	2	1.4	−[f]
diC$_6$PA	14 ± 1[g]	3.4	4.0	1.0
diC$_7$PA	1.4[g]	2	1.4	1−2
diC$_6$PMe	12	8.4	5.7	10

[a] Determined in 50 mM HEPES (pH 7.5). [b] Ratio of maximum PI-PLC activity induced by added short-chain phospholipid to enzyme activity in the absence of phospholipid. [c] Apparent K_i of the added phospholipid (diC$_n$PX) determined from fitting PC-activated PI-PLC activity toward cIP in the presence of diC$_n$PX. [d] No inhibition of PI-PLC-catalyzed hydrolysis of cIP. [e] Determined by surface tension in 50 mM Tris acetate (pH 8.0); from ref *19*. [f] K_i not determined. [g] Data for lipids in 0.05 M Tris-HCl and 0.1 M NaCl (pH 8.5) (*27*).

in specific activity around the CMC of the anionic lipids suggests that this active conformation is not optimal. In other words, the ligand at the activator site does exhibit specificity in promoting the allosteric change of PI-PLC to its most active conformation. PC and PE [as shown previously (*12*)] are the most effective ligands, leading to a 70-fold increase in PI-PLC specific activity toward 8 mM cIP (the result of a lowered K_m and a loss of cooperativity and increased V_{max}). Other phospholipids are not as effective; anionic lipids with small headgroups can produce at most a 10−15-fold enhancement (as extrapolated above) under these conditions, while PS and L-PI, anionic lipids with relatively larger headgroups, lead to an only 2-fold enhancement of PI-PLC cyclic phosphodiesterase activity.

Kinetic Model for PI-PLC Activation and Inhibition by Phospholipids. Although bacterial PI-PLC is a small protein (33 kDa), it is an allosteric enzyme with an activator site as well as an active site, although whether they are spatially as well as functionally distinct is a point yet to be resolved. Binding of a ligand to the activator site can induce a conformational change in the enzyme that increases k_{cat} and decreases K_m. However, the most active conformation of the enzyme is stabilized when the enzyme is anchored to an interface through the activator site (*15*). A number of phospholipids with relatively small headgroups have affinity as monomers for both the activator site and the active site (for a summary of activation and inhibition by these different species, see Table 2). For the diC$_6$-lipids examined, the affinity of the monomers for the activator site is higher than the affinity for the active site and binding to the active site can be ignored (except for that with diC$_6$PA). Once a micelle interface is available, these same lipids have decreased K_i values, indicating a stronger affinity for the active site. A kinetic model incorporating this behavior is shown in Figure 8. The top of the figure illustrates that activation of PI-PLC by a phospholipid molecule is not absolutely essential for hydrolysis of cIP. Occupation of the activator site by a monomeric phospholipid leads to allosteric activation of the enzyme (right side of the figure), although optimal activation requires an interface and a bulky headgroup that precludes binding of the ligand to the active site (e.g., PC). In contrast to PC, PS, and L-PI, phospholipids with smaller headgroups also compete with cIP for the enzyme active site. This competitive inhibition can be added to the model for PI-PLC (left side of the figure). The bottom of the figure shows that when the enzyme is anchored to the interface it exists in the most active form.

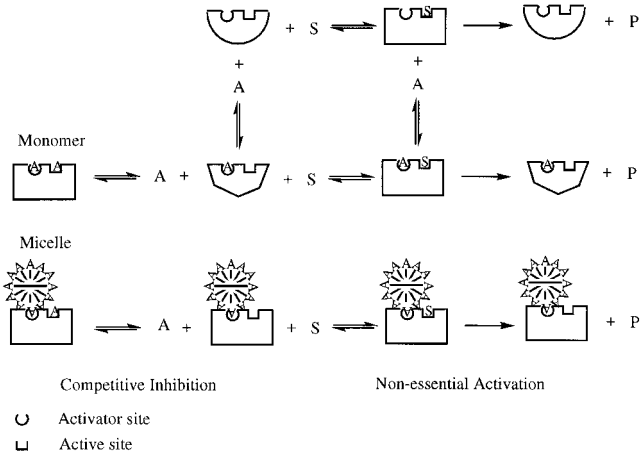


FIGURE 8: Kinetic model for phospholipid activation and inhibition of PI-PLC enzyme. The lipid attached to the left side of E represents binding to the activator site, while the species (nonsubstrate lipid or substrate) on the right side of E indicates occupation of the active site.

DISCUSSIONS

The kinetic data presented in this work indicate that all short-chain phospholipids have some affinity for the PI-PLC activator site, and except for the affinity of PA, these affinities are higher than those for the active site. PS and PI with L-inositol stereochemistry were less effective in binding to the allosteric site and had virtually no affinity for the enzyme active site. Monomeric diC$_6$PS binding to PI-PLC could not be detected by TRNOE, nor was there an increase in PI-PLC activity toward cIP when this lipid at 4−8 mM was present with cIP. A less than 2-fold activation above the CMC of diC$_6$PS indicated the weak affinity of PS for the activator site which required an interface to enhance this lipid binding. For diC$_7$P(L-)I, monomeric lipid appeared to bind to PI-PLC; however, it was not effective in promoting the optimal conformational change of the protein since no more than a 2-fold increase in specific activity was observed. Substrate diC$_7$P(D-)I will likely have the same affinity for the activator site as the L-PI. This is consistent with earlier observations where activation of PI-PLC toward PI in mixed diC$_7$PC/PI micelles required a PC:PI ratio of ∼6, a value considerably greater than the amount of PC needed to solubilize the PI (PC:PI ∼ 2). In that mixed micelle assay system, the PC was important not only in solubilizing the PI but also in saturating the activator site with the more potent effector. Interestingly, when short-chain PI was used as a substrate, a 5−6-fold interfacial activation of PI-PLC was observed (*13*) upon PI micellization. This activation is much smaller than that observed for PC (activation of PI-PLC for cIP or PI hydrolysis). The PI interfacial activation can be broken down into a 2-fold interfacial activation similar to that produced by PS, other detergents, and most notably diC$_7$P(L-)I and a 2−3-fold change due to other parameters (e.g., processive catalysis). The latter enhancement is similar in magnitude to what is observed for the nonspecific PLC (*21*).

The effectiveness of lipids binding to the activator site may be related to the conformation of the short-chain lipid

in solution. Lipid conformation includes chain stacking and headgroup orientation. Both PC and PE have an sn-2 chain kinked at C-2 of the acyl chain, while PG and PA have the sn-1 chain kinked in crystal structures of these lipids (*22*, *23*). Since all of these phospholipids can activate PI-PLC, the orientation of the chains is not critical. The headgroup orientation is more likely to be important. In bilayers, the headgroups of PC, PE, or PG are perpendicular to the chain-packing direction and parallel to the interface. There are reports that PS is different from PC (*24*, *25*). The orientation of the PS headgroup relative to the chain-packing direction is pH-dependent (*25*). Under our assay conditions, the headgroup of PS should be parallel to the chain-packing direction. The inositol ring in PI is also parallel to the chain-packing direction (*26*). PA and PMe have small headgroups, which may enhance binding to the active site (these are the most potent inhibitors), but may have little effect on binding in the optimal conformation to the activator site. Efficient binding to the activator site may require the phospholipid headgroup to be perpendicular to the chain-packing direction; there may also be specific interactions with charged or polar moieties of the headgroup. The net result is that the phospholipid headgroup appears to be the most critical factor in lipids (either as monomers or in the presence of local aggregates) binding to the activator site and inducing the optimal enzyme conformation.

What controls inhibitory potency is significantly different. Aggregation of all but PC, PS, and diC_7P(L-)I lipid led to strong inhibition of cIP hydrolysis by PI-PLC. The noninhibitory lipids all have moderately large headgroups and may not fit into the PI-PLC active site. More importantly, the point of maximum PI-PLC activity toward cIP correlated with the lipid CMC. diC_6PG has a high CMC (∼22 mM), and PI-PLC reached an activity maximum with 16 mM PG; diC_6PMe with a CMC of 12 mM led to the maximum PI-PLC activity at 8.4 mM. diC_6PA was an exception, inhibiting PI-PLC well below its CMC. For PG, PMe, and PA effects on cIP hydrolysis, as soon as the lipid began to form micelles, inhibition of the enzyme was enhanced. Since PG was a substrate for PI-PLC, it can bind to the active site. The simplest model has the PG and small headgroup lipids, PA and PMe, binding to the active site with this leading to competitive inhibition of PI-PLC. In this model, a key effect of an interface is enhancing binding of molecules (cIP or small headgroup phospholipids) to the active site. Similarly, increasing the chain length of the phospholipid decreased its ability to activate the enzyme and increased its potency as an inhibitor. Such behavior is even more pronounced when phospholipids packed in vesicles are added to cIP assay mixtures. Small unilamellar vesicles of PMe or PA dramatically inhibit PI-PLC activity toward cIP (*12*). Under these conditions, the vesicle lipid effectively occupies both the activator site and active site and prevents cIP from binding to the enzyme.

An alternate model for explaining the inhibition by PG, PMe, and PA holds that these anionic lipids prevent hydrolysis of cIP by binding the enzyme, not necessarily at the active site, in such a way that it can no longer have access to and bind cIP. Micellar PC, PE, PS, and L-PI also bind to the enzyme but do not alter the accessibility of the active site to cIP. Therefore, if the second model is to be accepted, the interaction with zwitterionic PC and PE and anionic PS and L-PI surfaces must be different from that with PA, PG, and PMe surfaces. To be consistent with the data, occlusion of the enzyme active site must exhibit a specificity that is not related to lipid charge but related to headgroup structure. The simplest explanation is, again, that PG, PMe, and PA inhibitory lipids bind to the active site.

REFERENCES

1. Camilli, A., Goldfine, H., and Portnoy, D. A. (1991) *J. Exp. Med. 173*, 751−754.
2. Mengaud, J., Braun-Breton, C., and Cossart, P. (1991) *Mol. Microbiol. 5*, 367−372.
3. Leimeister-Wachter, M., Domann, E., and Chakraborty, T. (1991) *Mol. Microbiol. 5*, 361−366.
4. Taguchi, R., and Ikezawa, H. (1978) *Arch. Biochem. Biophys. 186*, 196−201.
5. Griffith, O. H., Volwerk, J. J., and Kuppe, A. (1991) *Methods Enzymol. 197*, 493−502.
6. Ikezawa, H., and Taguchi, R. (1981) *Methods Enzymol. 71*, 731−741.
7. Artursson, E., and Puu, G. (1992) *Can. J. Microbiol. 38*, 1334−1337.
8. Jager, K., Stieger, S., and Brodbeck, U. (1991) *Biochim. Biophys. Acta 1074*, 45−51.
9. Ferguson, M. A. J., Low, M. G., and Cross, G. A. M. (1985) *J. Biol. Chem. 260*, 14547−14555.
10. Lin, G., Bennett, F., and Tsai, M.-D. (1990) *Biochemistry 29*, 2747−2757.
11. Volwerk, J. J., Shashidhar, M. S., Kuppe, A., and Griffith, H. (1990) *Biochemistry 29*, 8056−8062.
12. Zhou, C., Wu, Y., and Roberts, M. F. (1997) *Biochemistry 36*, 347−355.
13. Lewis, K. A., Garigapati, V. R., Zhou, C., and Roberts, M. F. (1993) *Biochemistry 32*, 8836−8841.
14. Wu, Y., Zhou, C., and Roberts, M. F. (1997) *Biochemistry 36*, 356−363.
15. Zhou, C., Qian, X., and Roberts, M. F. (1997) *Biochemistry 36*, 10089−10091.
16. Garigapati, V. R., and Roberts, M. F. (1993) *Tetrahedron Lett. 34*, 769−772.
17. Low, M. G., Stiernberg, J., Waneck, G. L., Flavell, R. A., and Kincade, P. W. (1988) *J. Immunol. Methods 113*, 101−111.
18. Volwerk, J. J., Filthuth, E., Griffith, O. H., and Jain, M. K. (1994) *Biochemistry 33*, 3464−3474.
19. Tan, C., Clifford, J., and Roberts, M. F. (1998) *Biochim. Biophys. Acta*, (submitted for publication).
20. Soltys, C., and Roberts, M. F. (1994) *Biochemistry 33*, 11608−11617.
21. El-Sayed, M., DeBose, C. D., Coury, L. A., and Roberts, M. F. (1985) *Biochim. Biophys. Acta 837*, 325−335.
22. Hauser, H., Pascher, I., and Sundell, S. (1988) *Biochemistry 27*, 9166−9174.
23. Pascher, I., Lundmark, M., Nyholm, P.-G., and Sundell, S. (1992) *Biochim. Biophys. Acta 1113*, 339−373.
24. Huber, W., Mantsch, H. H., Paltauf, F., and Hauser, H. (1994) *Biochemistry 33*, 320−326.
25. Sanson, A., Monck, M. A., and Neumann, J.-M. (1995) *Biochemistry 34*, 5938−5944.
26. Zhou, C., Garigapati, R., and Roberts, M. F. (1997) *Biochemistry 36*, 15925−15931.
27. Garigapati, V., Bian, J., and Roberts, M. F. (1995) *J. Colloid Interface Sci. 169*, 486−492.
28. Heinz, D. W., Ryan, M., Bullock, T. L., and Griffith, O. H. (1995) *EMBO J. 14*, 3855−3863.

BI980601R