Action of Phosphatidylinositol-specific Phospholipase Cγ1 on Soluble and Micellar Substrates

SEPARATING EFFECTS ON CATALYSIS FROM MODULATION OF THE SURFACE*

(Received for publication, September 29, 1998, and in revised form, November 10, 1998)

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The kinetics of PI-PLCγ1 toward a water-soluble substrate (inositol 1,2-cyclic phosphate, cIP) and phosphatidylinositol (PI) in detergent mixed micelles were monitored by 31P NMR spectroscopy. That cIP is also a substrate (Km = ~15 mM) implies a two-step mechanism (intramolecular phosphotransferase reaction to form cIP followed by cyclic phosphodiesterase activity to form inositol-1-phosphate (1-I-P)). PI is cleaved by PI-PLCγ1 to form cIP and 1-IP with the enzyme specific activity and ratio of products (cIP/1-I-P) regulated by assay temperature, pH, Ca²⁺, and other amphiphilic additives. Cleavage of both cIP and PI by the enzyme is optimal at pH 5. The effect of Ca²⁺ on PI-PLCγ1 activity is unique compared with other isozymes: Ca²⁺ is necessary for the activity and low Ca²⁺ activates the enzyme; however, high Ca²⁺ inhibits PI-PLCγ1 hydrolysis of phosphoinositides (but not cIP) with the extent of inhibition dependent on pH, substrate identity (cIP or PI), substrate presentation (e.g. detergent matrix), and substrate surface concentration. This inhibition of PI-PLCγ1 by high Ca²⁺ is proposed to derive from the divalent metal ion-inducing clustering of the PI and reducing its accessibility to the enzyme. Amphiphilic additives such as phosphatidic acid, fatty acid, and sodium dodecylsulfate enhance PI cleavage in micelles at pH 7.5 but not at pH 5.0; they have no effect on cIP hydrolysis at either pH value. These different kinetic patterns are used to propose a model for regulation of the enzyme. A key hypothesis is that there is a pH-dependent conformational change in the enzyme that controls accessibility of the active site to both water-soluble cIP and interfacially organized PI. The low activity enzyme at pH 7.5 can be activated by PA (or phosphorylation by tyrosine kinase). However, this activation requires lipophilic substrate (PI) present because cIP hydrolysis is not enhanced in the presence of PA.

Mammalian phosphoinositide-specific phospholipase C (PI-PLC) enzymes are key components of PI-mediated signaling cascades in vivo (1, 2). These enzymes are subdivided into three main classes, PI-PLC-β, γ, and δ, that share three conserved regions (3): (i) a N-terminal pleckstrin homology (PH) domain (these domains often have a high affinity for the phosphoinositide PIP₂), (ii) the X domain (~170 amino acids), and (iii) the Y domain (~260 amino acids). A C2 or Ca²⁺-lipid binding domain is also present at the C-terminal end of PI-PLC-δ and -δ. In all cases, the X and Y domains are necessary for catalysis (4) with the other domains involved in regulating activity. For example, in the case of PI-PLC-δ, the PH domain plays an allosteric role in binding the protein to a bilayer (5). PH domains also bind βγ G proteins very tightly (6), and PI-PLC-β activity is regulated by interactions with G proteins (7, 8).

PI-PLCγ isozymes are the largest of PI-PLC isozymes and are abundant in many tissues and cell types. The biological significance of the PI-PLCγ1 enzyme has been documented (2). When PI-PLCγ1 was “knocked out” of mice by targeted gene disruption (9), the embryos failed to develop beyond 9 days. The lethality was not due to a specific organ failure but to a general lack of growth in all parts of the embryo. Thus, in an intact mammal PI-PLCγ1 is indispensable for cell proliferation, and the requirement is not compensated for by other signal transduction pathways or other PI-PLC isozymes. The enzymes are activated by tyrosine kinase growth factor receptors (10, 11). The phosphorylation enhances translocation of protein from cytosol to plasma membrane (10). A unique feature of PI-PLCγ isozymes that distinguishes them from the β and δ classes is that the extended sequence between the X and Y domains is homologous to Src domains. These SH2 and SH3 domains can interact with protein tyrosine kinases and activate the enzyme (10–13). Flanking these Src homology domains is a split PH domain as well. Deletion of SH2 and SH3 domains produces an enzyme that still has catalytic activity but altered regulation (14). Expression of the X and Y domains in tandem leads to a folded structure that has a 20-fold higher activity toward PI and now has optimum activity at pH 7 (15).

Given the structural complexity of this protein, kinetic studies with well defined substrates can shed light on the roles and importance of the different domains for the actual catalytic cleavage of PI. Although purified PI-PLCγ1 exhibits a moderate preference for PIP₂ as substrate (3), it can hydrolyze PI efficiently yielding both cIP and 1-IP as products. In the present work, we have focused on characterizing the activity of PI-PLCγ1 toward the water-soluble substrate cIP as well as on PI/detergent mixed micelles. As was observed for PI-PLCδ1 (16), cIP is a substrate for PI-PLCγ1, and examining how nonsubstrate amphiphiles affect kinetic parameters is useful in assessing how interfacial modulation of the protein affects this

* This work has been supported by National Institutes of Health Grants GM 26762 (to M. F. R.) and CA75195 (to G. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PI-PLC, phosphatidylinositol-specific phospholipase C; PA, phosphatidic acid; PC, phosphatidylcholine; dCPC, diacylphosphatidylcholine with n carbons in each acyl chain; POPA, 1-palmitoyl-2-oleoylphosphatidic acid; PI, phosphatidylinositol; PIP₂, phosphatidylinositol-4,5-bisphosphate; FPMMe, 1-palmitoyl-2-myristoylphosphatidylmethanol; POPS, 1-palmitoyl-2-oleoylphosphatidylyserine; cIP, d-myo-inositol 1,2-cyclic phosphate; 1-IP, d-myo-inositol-1-phosphate; TX-100, Triton X-100; PH, pleckstrin homology domain.
catalytic step. Although PI-PLCγ1 kinetics show some similarity with PI-PLCδ1, there are critical differences. In particular, the observation of higher specific activity at pH 5 toward both water-soluble cIP and PI, inhibition of PI cleavage by Ca\(^{2+}\) concentrations in excess of a critical threshold, the lack of surface or organic solvent activation of the enzyme toward cIP, PA activation toward PI (but not cIP) at pH >6, and changes in the ratio of product cIP to I-1-P by temperature, pH, Ca\(^{2+}\), or other additives provide insights into how this enzyme could be regulated in vivo.

**MATERIALS AND METHODS**

**Chemicals—**DIC-PC, POPC, POPA, LPA, POPS, PMPMe, and PI were obtained from Avanti and used without further purification. Triton X-100, SDS, oleic acid, GPI, GPIP, GPPIP, PIP, and crude soybean PI were purchased from Sigma. Crude soybean PI (50% PI) was used for the enzymatic generation (using bacterial PI-PLC) of cIP as described previously (17).

**Enzymes—**A rat cDNA encoding PI-PLCγ1 was expressed as a histidine-tagged fusion protein that was purified by Ni\(^{2+}\) agarose affinity chromatography to homogeneity (18).

**Preparation of Assay Solution—**The buffers used were 50 mM sodium acetate/acetic acid (pH 5.0), 50 mM sodium citric/citric acid (pH 6.0), or 50 mM HEPES (pH 7.0, 7.5, or 8.0). A stock solution of cIP (200 mM) was prepared by dissolving cIP in D\(_2\)O and adjusting the pH to 6.0. In the cIP assays, 8 mM dicPC, 8 mM TX-100, and 24% organic solvent (Me\(_2\)SO, dimethylformamide, and isopropanol) were added to examine the interfacial activation and organic solvent effect at different pH values. Mixed micelles of PI solubilized in TX-100 or dicPC were also used as substrates for PI-PLCγ1. The ratio of PI to TX-100 or dicPC was varied as indicated. The addition of PA, oleic acid, SDS, and PMPMe to TX-100/PI mixed micelles was also examined. The optimum Ca\(^{2+}\) concentration for cIP or PI hydrolysis appears to be much higher than with phosphorylated water-soluble and amphiphilic substrates; hence it is not necessary to buffer Ca\(^{2+}\) in the assay solution. EGTA is not a good chelator for Ca\(^{2+}\) than with phosphorylated water-soluble and amphiphilic substrates; thus, it was quite different from the dependence of activity on Ca\(^{2+}\) in our assay solutions. Instead, the free Ca\(^{2+}\) concentration was measured using the probe Fluo-3 (19). The background of Ca\(^{2+}\) in the assay solution was around 10–20 μM. Additional Ca\(^{2+}\), ranging from 0.1 to 6 mM, was added to the assay solution. The total volume of each assay was fixed to 350 μl.

**31P NMR Assay of PI-PLCγ1 Activity—**

**31P** spectra were acquired at 202.7 Hz on a Varian Unity 500 spectrometer using a 5-mm broadband probe. **31P** parameters were based on those used previously (17). **31P** chemical shifts were referenced to phosphoric acid (5%) as an external standard. For all kinetic runs, a control spectrum (0 min) for cIP, the substrate for the cyclic phosphodiesterase step (17). In contrast, mammalian PI-PLCs generate both cyclic and acyclic inositol phosphate products simultaneously. If cIP is a substrate, the catalytic mechanism for the mammalian enzyme must be sequential. As shown in Table I, cIP is indeed a substrate for PI-PLCγ1, and the pH profile for the cyclic phosphodiesterase reaction is consistent with that reported for PIP\(_2\) cleavage (15), with the highest enzyme activity observed at pH 5.0. Although Ca\(^{2+}\) was absolutely required for cIP hydrolysis, the dependence of activity on Ca\(^{2+}\) was quite different from that reported for other substrates such as PIP\(_2\) and PIP. The maximum activity obtained toward cIP occurred with 2 mM Ca\(^{2+}\); higher Ca\(^{2+}\) led to a slight decrease in activity (Fig. 1A). In contrast, with PIP\(_2\) or PIP as the substrate, the Ca\(^{2+}\) requirement was much lower (100 μM), and the PI-PLC specific activity reached a maximum and then decreased with further increases in Ca\(^{2+}\) (20).

**RESULTS**

**Water-soluble cIP Is a Substrate of PI-PLCγ1—** Bacterial PI-PLC catalyzes the two discrete steps of PI cleavage sequentially with accumulation of cIP occurring before conversion to I-1-P. This is the result of facile product release and a high Km (~90 μM without interfaces, as low as 25 μM with interfaces present) and low Vmax (20 μmol min\(^{-1}\) mg\(^{-1}\)) for cIP, the substrate for the cyclic phosphodiesterase step (17). In contrast, mammalian PI-PLCs generate both cyclic and acyclic inositol phosphate products simultaneously. If cIP is a substrate, the catalytic mechanism for the mammalian enzyme must be sequential. As shown in Table I, cIP is indeed a substrate for PI-PLCγ1, and the pH profile for the cyclic phosphodiesterase reaction is consistent with that reported for PIP\(_2\) cleavage (15), with the highest enzyme activity observed at pH 5.0. Although Ca\(^{2+}\) was absolutely required for cIP hydrolysis, the dependence of activity on Ca\(^{2+}\) was quite different from that reported for other substrates such as PIP\(_2\) and PIP. The maximum activity obtained toward cIP occurred with 2 mM Ca\(^{2+}\); higher Ca\(^{2+}\) led to a slight decrease in activity (Fig. 1A). In contrast, with PIP\(_2\) or PIP as the substrate, the Ca\(^{2+}\) re-

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**Table I**

| Additive | Specific activity (μmol min\(^{-1}\) mg\(^{-1}\)) |
|----------|------------------------------------------|
| None     | 2.0                                      |
| None     | 0.2                                      |
| diC-PC (8 mM) | 1.4                                      |
| Triton X-100 (8 mM) | 2.4                                      |
| Isopropanol (24%) | <0.1\( ^a \)                            |
| Dimethylformamide (24%) | <0.1\( ^b \)                            |
| Dimethylsulfoxide (24%) | 1.15                                     |
| diCIP/PA (1 mM) | 0.27\( ^c \)                            |
| AMP (1 mM) | 0.33                                     |

\( ^a \) Activity measured in 50 mM 50 mM HEPES, 1 mM Ca\(^{2+}\), pH 7.5.   
\( ^b \) No observable reaction was detected overnight.   
\( ^c \) PA also significantly inhibits the cIP hydrolysis reaction at pH 7.5.

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**Fig. 1.** A, effect of Ca\(^{2+}\) concentration on PI-PLCγ1 activity toward 8 mM cIP in 50 mM acetate, pH 5.0, and 30 °C. B, specific activity of PI-PLCγ1 as a function of cIP concentration in 50 mM acetate, pH 5.0, in the presence of 2 mM Ca\(^{2+}\); the curve is the fit to the Michaelis-Menten equation with K\(_m\) = 15 mM and V\(_{max}\) = 2.7 μmol min\(^{-1}\) mg\(^{-1}\).

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The dependence of enzyme activity on cIP concentration at pH 5.0 (Fig. 1B) is hyperbolic with a K\(_m\) of 15 mM and a V\(_{max}\) of 2.7 μmol min\(^{-1}\) mg\(^{-1}\) obtained by fitting the data with the Michaelis-Menten equation. The activity of the PI-PLCγ1 preparation decreased slowly even when stored at −20 °C so that this value for V\(_{max}\) could be higher. The K\(_m\) of PI-PLCγ1 for cIP was about half that for full-length PI-PLCδ1 (16). With a K\(_m\) of 15 mM (and a lower V\(_{max}\) than PI cleavage), cIP levels would be expected to accumulate as PI is cleaved and then decrease as I-1-P is generated as long as the release of cIP into the solution is a facile event.
Effect of Interfaces and Solvent on cIP Kinetics—A kinetic analysis of cIP hydrolysis by PI-PLC in the absence and presence of interfacially active additives allows one to determine whether the interfaces interact directly with the enzyme. In particular, if interfaces enhance cIP hydrolysis, that interfacial activation must be due to an allosteric transition of the enzyme rather than a change in the substrate properties because cIP is water-soluble and has no measurable tendency to associate with interfaces (17). This approach was used to show that bacterial PI-PLC is allosterically activated by a variety of PC aggregates (micelles and bilayers) (17) and that a similar PC activation of PI-PLCγ1 requires an intact PH domain (16). In contrast to the large activation observed for bacterial PI-PLC or PI-PLCγ1, 8 mM diC7PC micelles inhibited PI-PLCγ1-catalyzed hydrolysis of cIP (8 mM) by 33%, and 8 mM TX-100 micelles had only a small activating effect (15%) close to the error limit of the assays, both at pH 5 (Table I) and at pH 7.5 (data not shown). Whatever the role of the PH, SH2, and SH3 domains, any interactions they have with interfaces do not appreciably affect the binding or hydrolysis of cIP.

PA has been shown to enhance PI-PLCγ1 activity toward PIP2 (solubilized in TX-100 micelles) at pH 7.5 (21). However, there was no enhancement of cIP hydrolysis in the presence of short chain monomeric or micellar PA at either pH 5.0 or 7.5. Rather, inhibition of the cyclic phosphodiesterase activity was observed at both pH values (Table I). The presence of low concentrations of PIP2 (1 mM) more than sufficient to enhance PI-PLCγ1 hydrolysis of cIP (22) also failed to activate PI-PLCγ1 toward 8 mM cIP.

Water-miscible organic solvents, such as 24% Me2SO, dimethylformamide, or isopropanol, likewise inhibited PI-PLCγ1 at both pH 5.0 and 7.5. Both dimethylformamide and isopropanol were extremely potent inhibitors of the enzyme. This is in marked contrast to the strong activating effect of these solvents on the cyclic phosphodiesterase activity of bacterial PI-PLC (23) and PI-PLCγ1 (16). For the latter PI-PLCs, the reduced solvent polarity is thought to activate the enzyme by stabilizing a more active conformation of the enzyme. The mixed solvents could mimic the polarity generated locally by interfaces such as PC bound to the enzyme. That neither PC nor solvent activated PI-PLCγ1 suggests that other factors are more critical for activity of this enzyme.

PI Cleavage in TX-100 Mixed Micelles—With naturally occurring PI, detergents such as TX-100 are often used to form mixed micelles that are good substrates for PI-PLC. Because TX-100 does not affect cIP hydrolysis by PI-PLCγ1, it is unlikely to have an allosteric effect on the enzyme. However, interactions of the detergent with PI and cofactor Ca2+ can have pronounced effects on PI-PLC kinetics by virtue of modifying the properties or accessibility of PI in the interface. The reaction rates for PI-PLCγ1 catalyzed hydrolysis of 8 mM PI were measured as a function of Ca2+ concentration at different ratios of TX-100 to PI at both pH 5.0 and pH 7.5 (Fig. 2A shows this dependence for TX-100/PI = 2). Similarly to the pH dependence of cIP hydrolysis, the rate of PI cleavage was about 20-fold higher at pH 5 than at pH 7.5. The optimal Ca2+ concentration also varied with pH. It decreased when the pH was increased with a value of 0.5 mM Ca2+ at pH 5.0 and 0.1 mM at pH 7.5.

Product distribution (cIP/1-P) also varied with pH. Both cIP and 1-P were observed at pH 5 with the ratio of products constant in a particular assay. This behavior is not consistent with rapid release of cIP from the enzyme because the $K_m$ for cIP is 15 mM. It suggests that PI-PLCγ1, like PI-PLCγ1, does not readily release cIP generated in situ but holds it for sufficient time so that the rate of attack by water is comparable with its release (16, 24). Interestingly, there was no observable cIP produced at pH 7.5; I-1-P was the only product detected from PI cleavage.

Inhibition of PI-PLC cleavage of PI by Ca2+ occurred above a threshold at each pH, 1 mM at pH 5 and 0.5 mM at pH 7.5, when the ratio of TX-100 to PI was 2. This inhibition by Ca2+ decreased when TX-100/PI = 6 (at fixed PI) where considerably more detergent was present (Fig. 2B). The PI hydrolysis rate and light scattering by TX-100/PI particles at 500 nm as a function of added TX-100 are shown in Fig. 3 (A and C). The maximum PI-PLC activity toward PI was reached at TX-100/PI = 4, although the PI was well solubilized by a 2-fold excess of TX-100 compared with PI (e.g. the reduced light scattering at TX-100/PI = 2). The requirement for increased TX-100 suggests that PI molecules need to be well separated in TX-100 mixed micelles to obtain the highest activity. The need for separation of PI molecules is balanced by “surface dilution” of the substrate that gives rise to an apparent inhibition (25, 26). In contrast to PI-PLCγ1, the bacterial PI-PLC acting on a fixed concentration of PI solubilized in TX micelles exhibited a maximum activity at TX/PI = 1.5 (17) corresponding to conversion of the bulk of the multilamellar vesicles to mixed micelles. As the amount of TX-100 was increased, bacterial PI-PLC activity decreased, presumably be-
cause the surface concentration of PI decreased.

The ratio of TX-100/PI was held constant and the concentration of PI was varied to obtain an apparent $K_m$ for PI solubilized in a TX-100 matrix. Although the apparent $K_m$ extracted from this approach is a combination of both a surface adsorption binding step and the true $K_s$ value, it is a useful parameter for comparing the effects of different interfaces on enzyme activity. For PI-PLCγ1, the apparent $K_m$ for PI decreased (from 4.3 to 2.8 mM) as the TX-100/PI ratio increased (Fig. 4, A and B), suggesting that substrate molecules in the more dilute PI surface (0.14 versus 0.33 mole fraction PI) were more accessible to the enzyme. The apparent $V_{max}$ also increased with the more dilute PI surface (from 2.6 to 6.4 μmol min$^{-1}$ mg$^{-1}$), confirming that PI was a better substrate when more dilute in TX micelles (e.g. at TX/PI = 6 or 0.14 mole fraction versus TX-100/PI = 2 or 0.33 mole fraction). The TX-100 enhanced activation must reflect a modification of substrate in the interface to which the enzyme is sensitive, because TX-100 had only minimal effects on cIP hydrolysis. Interestingly, cooperativity was observed for PI cleavage (but not for cIP hydrolysis) by PI-PLCγ1. The lack of cooperativity for cIP hydrolysis strongly suggests that cooperativity is not due to enzyme dimerization or conformational change; rather it is related to substrate aggregation and interface behavior.

Insight into the inhibition by high concentrations of Ca$^{2+}$ was obtained by examining PI aggregation behavior, monitored by the $^{31}$P line width of PI in TX-100 micelles. If Ca$^{2+}$ induces aggregation of PI in TX-100 micelles (not particle growth but clustering of PI), the line width of PI should increase because the mobility of the clustered PI has been reduced. The $^{31}$P line widths for PI solubilized in TX-100 at different ratios of PI to TX-100 (and mole fractions of PI) and Ca$^{2+}$ concentration are shown in Fig. 4C. The line width increased at Ca$^{2+}$ concentrations greater than 1 mM for a TX-100/PI ratio of 2; however, the $^{31}$P line width did not change much with TX-100/PI = 6. The increased line width for the higher mole fraction PI indicated a lower mobility for PI consistent with a Ca$^{2+}$-induced aggregation. The trend of increased aggregation of PI with increasing Ca$^{2+}$ paralleled the
decreased PI hydrolysis rate. For TX-100/PI of >4, the lack of effect of Ca\(^{2+}\) on line width indicated that the PI molecules are well separated and not as susceptible to clustering/aggregation by Ca\(^{2+}\). Thus, a more dilute PI surface may be a better substrate for PI-PLC\(\gamma\)1 because it is less susceptible to Ca\(^{2+}\)-induced clustering.

**PI Cleavage in diC\(_7\)PC Mixed Micelles—**An excess of diC\(_7\)PC micelles inhibited PI-PLC\(\gamma\)1 activity toward cIP, suggesting that some interaction of this lipid with the enzyme does occur. Nonetheless, these short chain PC micelles can be used as a matrix to solubilize PI for comparison to TX-100/PI mixed micelles. The Ca\(^{2+}\) requirement for PI-PLC\(\gamma\)1 cleavage of PI in diC\(_7\)PC mixed micelles is dramatically different from that in TX mixed micelle as shown in Fig. 2C. PI solubilized in the diC\(_7\)PC matrix abolished the inhibition of PI-PLC\(\gamma\)1 by high concentrations of Ca\(^{2+}\) at both pH 5.0 and pH 7.5; the cIP/I-1-P ratio was also significantly increased (Table II). A likely explanation is that the zwiterionic PC matrix prevents PI from Ca\(^{2+}\)-induced clustering, possibly by interacting with the Ca\(^{2+}\). The activity of PI-PLC\(\gamma\)1 toward PI solubilized in diC\(_7\)PC micelles was also much greater than toward PI in TX-100 micelles at pH 5.0. This apparent activation by PC is different from that occurring with bacterial PI-PLC and PI-PLC\(\delta\) because the PC interface has no activating effect on cIP hydrolysis; rather there is a modest inhibition at this level of PC. The enzyme-catalyzed hydrolysis rate of a fixed concentration of PI as a function of diC\(_7\)PC was also different from that for PI solubilized in TX-100 micelles (Fig. 3B). The faster rate of PI hydrolysis in diC\(_7\)PC micelles and lower requirement for PC to solubilize the PI indicate that in the assay mixture PI is better solubilized in a PC micelle than in a TX-100 mixed micelle. The kinetic effect is consistent with the Ca\(^{2+}\)-induced PI clustering, a physical effect associated with reduced enzyme activity that is reduced in diC\(_7\)PC micelles.

**The Effect of POPA and Other Interfacial Additives—**Previously, it has been reported that PI-PLC\(\gamma\)1 can be activated by PA with the PA acting as an allosteric modifier in reducing KS\(_{\text{m}}\) (21). The water-soluble phosphoester AMP inhibited PI-PLC\(\gamma\)1 activity (21). The water-soluble phosphoester AMP inhibited PI-PLC\(\gamma\)1 activity (21). The water-soluble phosphoester AMP inhibited PI-PLC\(\gamma\)1 activity (21). The water-soluble phosphoester AMP inhibited PI-PLC\(\gamma\)1 activity (21). The water-soluble phosphoester AMP inhibited PI-PLC\(\gamma\)1 activity (21). The water-soluble phosphoester AMP inhibited PI-PLC\(\gamma\)1 activity (21).

**Factors That Control cIP/I-1-P Partitioning—**Throughout the time course of an individual assay, the ratio of PI-PLC\(\gamma\)1 products (cIP/I-1-P) generated by PI-PLC\(\gamma\)1 catalyzed cleavage of PI under different conditions (Table II). However, cIP/I-1-P can be altered by Ca\(^{2+}\) concentration, temperature, pH, or other additives (e.g., PA). The cIP/I-1-P ratio decreased with increasing Ca\(^{2+}\) concentration when PI was solubilized in TX-100 at TX-100/PI = 2 (Fig. 7A). However, increased Ca\(^{2+}\) had a much smaller effect on cIP/I-1-P when the PI concentration was fixed. The ratio of cIP to I-1-P was higher for TX-100/PI = 2 (Fig. 7A). However, increased Ca\(^{2+}\) had a much smaller effect on cIP/I-1-P when the mixed micelles contained a more dilute surface concentration of PI (e.g., TX-100/PI = 6). The ratio of cIP to I-1-P was higher for TX-100/PI = 6 compared with TX-100/PI = 2; the more dilute PI surface favored release of cIP into the solution compared with attack by water to form I-1-P. The cIP/I-1-P product ratio was also higher for PI solubilized in diC\(_7\)PC micelles (2.1 PC/PI). Because the ratio of cIP to I-1-P is likely to depend on the residence time of the EcIP complex produced in situ (16, 22), any factors that alter the residence time of EcIP will affect the ratio of cIP to I-1-P. Interfaces can do this by altering substrate or product exchange or partitioning in the surface as well as by interacting directly with the protein.

**Assay temperature can also affect EcIP stability (16, 22).** The specific activity of PI-PLC\(\gamma\)1 was examined at several temperatures (Fig. 7B). Because the apparent KS\(_{\text{m}}\) for PI in TX-100 micelles at TX-100/PI = 2 was 2.8 mM, the PI concentration was fixed at 8 mM in the assay. At this substrate concentration the activity was near its maximum value, and

| Table II | Kinetics of PI-PLC\(\gamma\)1 catalyzed cleavage of PI under different conditions |
| --- | --- |
| | Assays carried out at 30 °C unless otherwise noted. |
| Detergent | Detergent/PI | Ca\(^{2+}\) Additive | cIP/I-1-P (pH 5) | cIP/I-1-P (pH 7) |
| --- | --- | --- | --- | --- |
| TX-100 | 2:1 | 1 | None | 1.25 | <0.05 |
| (30 °C) | 6:1 | 1 | None | 0.85 | <0.05 |
| (10 °C) | 2:1 | 0.1 | None | 2.9 | <0.05 |
| 2:1 | 1 | None | 1.25 | <0.05 |
| 2:1 | 5 | None | 0.6 | <0.05 |
| 5:1 | 0.3 | None | 0.7 | <0.05 |
| 5:1 | 0.3 | PA\(^{a}\) | 3.5 | 0.5 |
| 5:1 | 0.3 | PC | 0.85 | <0.05 |
| diC\(_7\)PC | 2:1 | 1 | None | 0.35 |
| 2:1 | 1 | PA\(^{a}\) | 0.24 |

\(^{a}\) Ratio of PA to PI is 0.5.

**FIG. 5. Effect of anionic amphiphilic additives on PI-PLC\(\gamma\)1 catalyzed cleavage of PI in TX-100 micelles with 0.1 mM Ca\(^{2+}\).** pH 7.5. The ratio of TX-100/PI/additives was 4.5:1:0.5.

POPA, lyso-PA, oleic acid, and SDS enhanced PI hydrolysis to different extents, whereas PMPMe and POPS had little effect. The PA-induced activation of PI-PLC\(\gamma\)1 activity occurred in both TX-100/PI and diC\(_7\)PC/PI mixed micelles to about the same extent. Because PA activation was the largest, it was examined as a function of Ca\(^{2+}\) concentration (from 0.02 to 0.5 mM) and pH; the surface mole fraction of PA was 0.08, and the surface concentration of substrate PI was 0.16. The maximum PI-PLC\(\gamma\)1 activity was reached at 0.1 mM Ca\(^{2+}\); there was no significant change in activity above 0.1 mM Ca\(^{2+}\) (data not shown). Under these conditions, the presence of PA abolished the high Ca\(^{2+}\) inhibition of PI hydrolysis. The pH profile of PI-PLC\(\gamma\)1 toward PI was also different in the presence and absence of POPA at 0.3 mM Ca\(^{2+}\) (Fig. 6A). PA had little effect at pH 5.0 and enhanced PI-PLC activity only above pH 6.0. The specific activity of PI-PLC\(\gamma\)1 toward PI in the presence of POPA at pH 7 was within a factor of two of the specific activity at pH 5.0 in the absence of PA. This represents a very large enhancement of PI-PLC\(\gamma\)1 activity.

**Factors That Control cIP/I-1-P Partitioning—**Throughout the course of an individual assay, the ratio of PI-PLC\(\gamma\)1 products (cIP/I-1-P) generated by PI-PLC\(\gamma\)1 catalyzed cleavage of PI under different conditions (Table II). However, cIP/I-1-P can be altered by Ca\(^{2+}\) concentration, temperature, pH, or other additives (e.g., PA). The cIP/I-1-P ratio decreased with increasing Ca\(^{2+}\) concentration when PI was solubilized in TX-100 at TX-100/PI = 2 (Fig. 7A). However, increased Ca\(^{2+}\) had a much smaller effect on cIP/I-1-P when the mixed micelles contained a more dilute surface concentration of PI (e.g., TX-100/PI = 6). The ratio of cIP to I-1-P was higher for TX-100/PI = 6 compared with TX-100/PI = 2; the more dilute PI surface favored release of cIP into the solution compared with attack by water to form I-1-P. The cIP/I-1-P product ratio was also higher for PI solubilized in diC\(_7\)PC micelles (2.1 PC/PI). Because the ratio of cIP to I-1-P is likely to depend on the residence time of the EcIP complex produced in situ (16, 22), any factors that alter the residence time of EcIP will affect the ratio of cIP to I-1-P. Interfaces can do this by altering substrate or product exchange or partitioning in the surface as well as by interacting directly with the protein.

Assay temperature can also affect EcIP stability (16, 22). The specific activity of PI-PLC\(\gamma\)1 was examined at several temperatures (Fig. 7B). Because the apparent KS\(_{\text{m}}\) for PI in TX-100 micelles at TX-100/PI = 2 was 2.8 mM, the PI concentration was fixed at 8 mM in the assay. At this substrate concentration the activity was near its maximum value, and
specific activity approximated \( k_{\text{cat}} \). The generation of I-1-P was less temperature-dependent than cIP production from 10 to 30 °C, leading to an increase in cIP/I-1-P with temperature (Fig. 7C); above 40 °C the enzyme began to lose activity. Transition state theory applied to data below 40 °C (\( \ln (k_{\text{cat}}/T) \) versus \( 1/T \)) is linear in that region) indicated that the \( \Delta D^\ddagger \) for release of cIP from E\text{cIP} compared with hydrolysis to I-1-P is +32 kJ/mol, whereas the \( \Delta S^\ddagger \) is +0.1 kJ/K-mol higher. These values are very close to the \( \Delta D^\ddagger \) and \( \Delta S^\ddagger \) for PI-PLC\( d_1 \) (16).

Assay pH is another parameter that might also be expected to affect cIP/I-1-P because the cIP hydrolysis step releases a proton. The ratio of cIP/I-1-P decreased rapidly with increasing pH (Fig. 6B). The inclusion of PA in the surface also affected the product ratio. POPA not only increased the PI hydrolysis rate at pH values above 6.0 but also increased the ratio of cIP/I-1-P. PA in these micelles is a monoanion below pH 5.5 and a dianion above pH 7. Perhaps it acts as an interfacial buffer in facilitating proton release from the protein upon cIP hydrolysis.

DISCUSSION

Mammalian PI-PLC isoforms are multidomain enzymes with key roles in signal transduction by virtue of their generation of second messengers by substrate cleavage. A recurring theme is that their action can be modulated by interfaces interacting with several different domains (PH, C2, SH2, and SH3). Distinguishing kinetic enhancements caused by changes in surface properties from those caused by allosteric effects on \( k_{\text{cat}} \) is often difficult with a phospholipid substrate. However, hydrolysis of cIP, the water-soluble intermediate in the cleavage of PI, can be used as a probe of allosteric effects of interfaces on PI-PLC enzymes. The high \( K_m \) of several PI-PLC enzymes for cIP compared with PI suggests that an interfacial substrate binds more effectively to the enzyme. Coupled with water-miscible organic solvent activation in the case of bacterial PI-PLC and PI-PLC\( d_1 \), the high \( K_m \) for cIP is likely to reflect difficulties in the enzyme binding a well hydrated polar molecule. In striking contrast to those PLCs, PI-PLC\( \gamma_1 \) is more efficient in hydrolyzing cIP and does not exhibit solvent activation. Because cIP structure is the same, the lower \( K_m \) for cIP, lack of cooperativity in cIP binding, and higher \( V_{\text{max}} \) of PI-PLC\( \gamma_1 \) may reflect a local decreased polarity at the active site.

Another key kinetic difference involves PH domains. The N-terminal PH domain of PI-PLC\( d_1 \) was shown to interact allosterically with amphiphiles (PC or PIP\(_2\)), causing a 2-fold increase in \( k_{\text{cat}} \) toward cIP (16, 22). PI-PLC\( \gamma_1 \) has both an N-terminal domain and a split PH domain between the X and Y domains. Although there is significant sequence homology between the PH domains of \( \delta_1 \) and \( \gamma_1 \) isoforms, functional
similarity is less obvious because there was no effect of PC or other interfaces (including PIP2 solubilized in TX-100 or diC7PC micelles) on the cyclic phosphodiesterase reaction of PI-PLCγ1. The PH domain of PI-PLCγ1 could still aid in anchoring the enzyme to an interface; however, such interactions do not affect the catalytic machinery.

PI cleavage by PI-PLCγ1 generates both cIP and I-1-P in parallel. Given the kinetic parameters for cIP hydrolysis (which predict a build-up of cIP prior to I-1-P generation), PI-PLCγ1 is similar to the δ1 isozyme where the E-cIP complex has a relatively long lifetime. Because PI-PLCγ1 also requires Ca2+ for activity, the cIP may have a binding orientation at the active site similar to that for a nonhydrolysable cIP analog (27) binding to PI-PLCδ1 (24), where the phosphonate is a bidentate ligand of the active site Ca2+. This arrangement would stabilize E-cIP. However, Ca2+ has another more complex effect on the phosphotransferase activity of PI-PLCγ1 toward PI. Above a threshold concentration, Ca2+ inhibits the first step of the reaction; the cyclic phosphodiesterase step is not inhibited by high Ca2+. The lack of effect on water-soluble cIP is consistent with the Ca2+ affecting properties of the lipid substrate (e.g. Ca2+-induced PI clustering) and not the enzyme. Alternatively, if one invokes a secondary Ca2+ site on the enzyme, then its occupation affects the phosphotransferase step but not the cIP hydrolysis step. However, because additional nonsubstrate interfacial molecules (that do not affect cIP hydrolysis) reduce the high Ca2+ inhibition, the most reasonable explanation is that high Ca2+ alters substrate-Ca2+ interactions, and these changes inhibit PI-PLCγ1.

The pH dependence for PI-PLCγ1 action on PI and cIP (roughly a 20-fold higher activity at pH 5 than 7) is also unusual and has mechanistic implications. One or more key residues must be protonated for optimal activity of both phosphotransferase and cyclic phosphodiesterase activities of the full-length enzyme. Interestingly, expression of X and Y domains without the SH2 or SH3 inserted domains yields a highly active enzyme whose pH optimum is shifted to 7 (15). A possible model for the pH dependence of PI-PLCγ1 (Fig. 8) has the active site of PI-PLCγ1 covered or occluded by the SH2-SH2-SH3 domains at pH 7; this “lid” or cover would be displaced at pH 5 by protonation of key residues (15). At pH 5, this SH2-SH2-SH3 lid is “open” and PI-PLCγ1 activity is high; at pH 7, the lid is “closed” and the enzyme has much lower activity. Consistent with this model is the previous observation that protease V8 cleavage activates the enzyme at pH 7; and the cleavage occurs within the lid between the SH3 and Y domains (28). Active enzyme at pH 5 can be inhibited by excess Ca2+ sequestering the substrate PI; this kinetic effect depends on the detergent matrix used to solubilize PI as well as on the Ca2+ concentration. This Ca2+ modulation of the TX-100-PI interface also occurs at pH 7.

The addition of anionic lipids (PA, LPA, SDS, and oleic acid) also affects the pH profile, enhancing enzyme activity at pH 7.5. The mechanism for the altered hydrolysis of PI could be due to the amphiphiles interacting with either the enzyme or the substrate. One possible explanation is that anionic lipids bind to the enzyme and open the SH2-SH2-SH3 lid. Their
presence should enhance both PI and cIP cleavage at pH 7.5. Because only activity toward PI is enhanced, substrate PI must also be needed to facilitate lid opening at pH 7. Alternatively, activation by PA and similar lipids could be the result of altered substrate properties. The anionic amphiphiles could compete with PI and lessen Ca\(^{2+}\)--induced aggregation of substrate. However, that is unlikely to account for (i) the very large activation observed at pH 7.5 and (ii) the observation of the same magnitude of PA activation with PI solubilized in diC\(_7\)PC and TX-100 micelles (the TX-100 surface is much more susceptible to Ca\(^{2+}\)--induced clustering than the PC/PI surface, hence one would expect a difference in the behavior of these two interfaces with PA added). The pK\(_a\) of micellar PA or LPA (either short chain PA or long chain PA in TX-100 micelles) is ~7 (29). The significant activation of PI-PLC by this value in the presence of PA suggests that the PA dianion is critical for this effect. PI-PLC is regulated in vivo by phosphorylation (30, 31). Phosphorylation might be another switch that alters the interaction of the SH domains with the rest of the protein leading to activation of PI-PLC at pH 7. Precedence for such interactions can be seen with the SHP-2 structure (32). This offers the possibility of proteins modulating PI-PLC activity by interacting with the SH domains and opening the (hypothetical) lid.

In summary, a model consistent with unusual kinetics of PI-PLC toward both water-soluble cIP and interfacially organized PI has been proposed. It includes specific enzyme conformational changes due to pH or nonsubstrate ligands as well as Ca\(^{2+}\)--induced modulation of interfacial substrate properties as ways to alter enzyme specific activity. The model shown in Fig. 8 also makes several predictions that can be tested in the future: (i) there should be a large conformational change (presumably involving the SH domains) in the enzyme between pH 5 and 7 and (ii) because phosphonate esters have pK\(_a\) values shifted from phosphate esters, a phosphono-PA analog would be expected to activate the enzyme but alter the pH profile of PI hydrolysis in the presence of PA.

Acknowledgment—We thank Xiaoqing Qian for the initial work on cIP kinetics.

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