Chapter 13

Phosphorothioate Analogues of Phosphatidylinositol and Inositol 1,2-Cyclic Phosphate

Application to the Mechanism of Phospholipase C

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The diastereomers of phosphorothioate analogues of dipalmitoylphosphatidylinositol (DPPsI) have been synthesized from protected optically active *myo*-inositol derivatives. Their configurations at phosphorus have been determined on the basis of stereospecific hydrolysis catalyzed by phospholipase A_2. The reactions catalyzed by phosphatidylinositol-specific phospholipases C (PI-PLC) from *Bacillus cereus* and guinea pig uterus have been shown to be stereospecific toward the R_p isomer of DPPsI. The configuration of one of the products, inositol cyclic-1,2-phosphorothioate (IcPs), was determined from proton and ^{31}P NMR data. The results indicate that the conversion of DPPsI to IcPs, catalyzed by PI-PLC from both sources, proceeds with inversion of configuration at phosphorus, which suggests a direct displacement mechanism.

Phosphorothioate analogues of biophosphates have proven a valuable tool in investigating the mechanisms of action of various phosphohydrolase and phosphotransferase enzymes. Following reports on the synthesis, configurational analysis and biochemical applications of oxygen-labeled and phosphorothioate analogs of nucleotides (*1-5*), we have extended the concept of P-chiral phosphates to phospholipids and used this approach to study the mechanism of phospholipases and other phospholipid-metabolizing enzymes (*6*). This approach has generated two types of information regarding elementary steps in enzymatic reactions: stereospecificity toward one of the two diastereomers of chiral thiophospholipids, and steric course of the reaction involving cleavage of a P-O bond. With the exception of lecithin-cholesterol acyltransferase (*11*), we have found a remarkable stereospecificity of these enzymes toward one of the two diastereomeric thiophospholipids (*6-13*).

The possible fundamental mechanisms of phosphotransfer reactions have been described elsewhere (*14*). The steric course of enzyme-catalyzed phosphotransfer

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reactions is usually interpreted in terms of the number of steps involving a P-O bond cleavage during the entire catalytic process, since in enzymatic reactions a single nucleophilic displacement at a phosphorus atom usually proceeds with inversion of configuration (*1-14*). Thus an overall inversion of configuration at phosphorus suggests that the reaction proceeds by a single-step mechanism involving an in-line arrangement of the incoming nucleophile and the departing leaving group in the transition state. In very unusual situations the overall inversion could also result from a three-step mechanism. An overall retention of configuration suggests formation of an enzyme-substrate intermediate. In such cases two consecutive steps, each occurring by a single-displacement mechanism (with an inversion of configuration at phosphorus), are necessary to convert a substrate into a product.

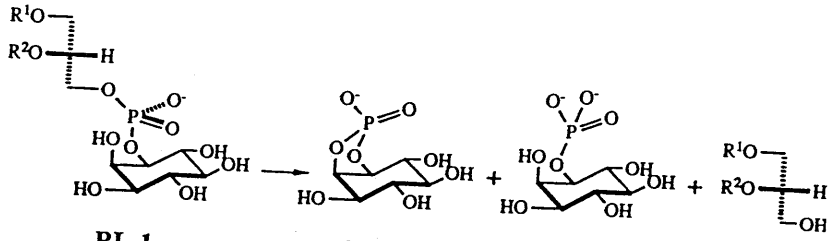
The stereochemical information of phospholipases is of particular significance since it is usually difficult to perform detailed kinetic studies due to the surface specificities of these enzymes. The crucial step in determining the steric course of the enzymatic reaction is the configurational assignment of both the substrate and the product. In principle, determination of configurations of P-chiral phosphorothioate analogues of phospholipids can be made on the basis of the above mentioned stereoselectivity of phospholipases A_2 and C with different substrates. For a more comprehensive discussion on the stereochemistry of phospholipases the reader is referred to a recent review (*6*).

Phosphatidylinositides-specific phospholipase C (PI-PLC, E.C.3.1.4.10) is an enzyme catalyzing hydrolysis of phosphatidylinositol (PI, **1**) (or its phosphorylated derivatives) to produce three products: *myo*-inositol-1-phosphate (IP, **2**), *myo*-inositol cyclic-1-phosphate (IcP, **3**), and diacylglycerol (*15,16*) (Scheme 1). This is a critical step in the transduction of the extracellular hormone stimulus into an intracellular physiological signal (*17-19*). The PI-PLC from bacterial and mammalian sources differ greatly in their protein sizes, amino acid sequences and metal-ion requirements, and in the products of their reactions (*15,16,20,21*). Mammalian enzymes usually require Ca^{2+} for their activity and produce a mixture of IcP and IP, whereas *Bacillus cereus* PI-PLC is metal ion independent and forms only IcP. For mammalian enzymes the ratio IcP/IP varies somewhat depending on the enzyme source and reaction conditions.

This chapter describes synthesis and configurational assignment of the phosphorothioate analogues of DPPI (dipalmitoyl-PI) and IcP (abbreviated as DPPsI and IcPs, respectively) and the results of the stereochemistry of the reactions catalyzed by PI-PLC from *B. cereus* and two isozymes (I and II) from guinea pig uterus (*22-24*).

Synthesis of DPPsI. Three methods have been developed to synthesize DPPsI. In the first method (Scheme 2) D-2,3:5,6-di-*O*-cyclohexylidene-4-*O*-methoxymethyl-*myo*-inositol (**4**) was phosphitylated with chloro-(*N*,*N*-diisopropylamino)methoxyphosphine (**5**) (*25*) in the presence of triethylamine in dichloromethane (*22,23*). The resulting phosphoramidate **6** was subjected to the reaction with 1,2-dipalmitoyl-*sn*-glycerol under tetrazole catalysis in THF-acetonitrile, and subsequently the sulfur addition. The mixture of the diastereomeric phosphorothionates (**7a,b**) could not be resolved by chromatographic techniques and was further subjected to deprotection steps with 80% aqueous acetic acid (to remove

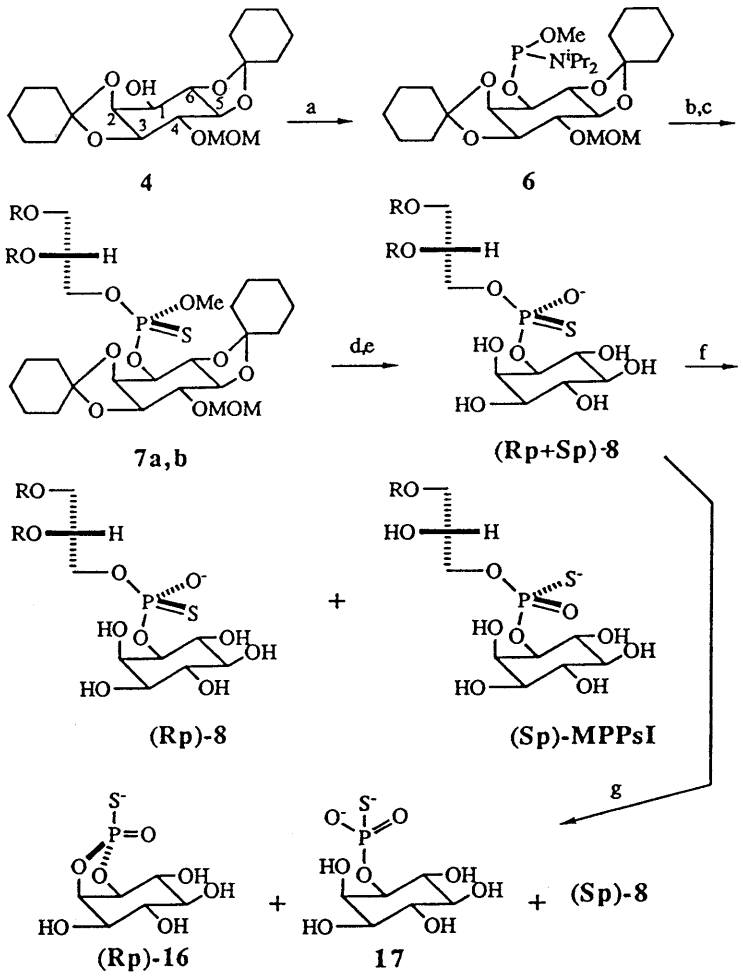
Scheme 1



PI, 1 **IP, 2** **IcP, 3**

R^1 = stearoyl; R^2 = arachidonoyl

Scheme 2



a) $ClP(OMe)N^iPr_2$ (5), Et_3N; b) 1,2-dipalmitoyl-*sn*-glycerol, tetrazole; c) S_8; d) 80% AcOH; e) NMe_3, f) phospholipase A_2; g) PI-PLC; R = palmitoyl

acetal protective groups), followed with trimethylamine in toluene (for demethylation). The DPPsI **8** thus synthesized is a mixture of two diastereomers and gives two signals of equal intensity at 57.45 and 57.05 ppm in a ^{31}P NMR spectrum. Separation of diastereomers was effected enzymatically by taking advantage of the stereospecificity of phospholipase A_2 (PLA2) and PI-PLC, as described in the next section.

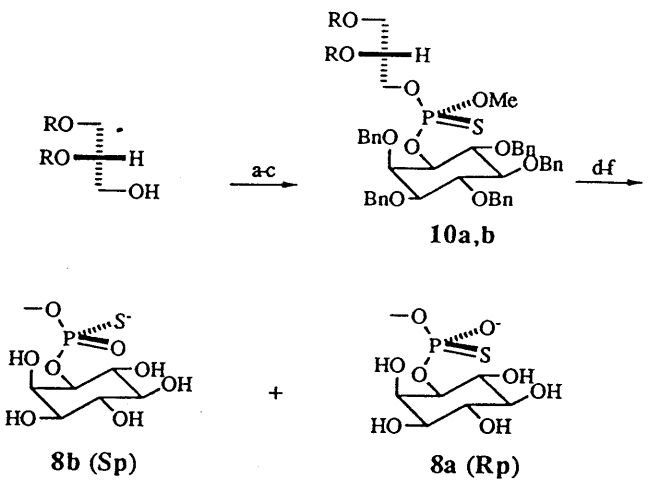
Both the second and the third methods give separate isomers of DPPsI through chromatographic separation of one of the intermediates. The second method (Scheme 3) employed the same phosphitylation steps as described in method 1, but used 1,2-dipalmitoyl-*sn*-glycerol as the starting material and L-1,2,4,5,6-pentabenzyl-*myo*-inositol (**9**) as the second hydroxyl component for the synthesis (*24*). The mixture of R_p and S_p triesters **10a,b** (at the ratio ca. 58:42) could be separated by column chromatography on silica gel using carbon tetrachloride-acetone (40:1) as an eluting solvent. Separated diasteromers **10a** and **10b** were demethylated with trimethylamine and debenzylated with BF_3-etherate/ethanethiol to produce DPPsI **8a** and **8b**, respectively.

The third method (Scheme 4) has been developed recently (Bruzik, K. S., Tsai, M.-D., unpublished results) and employs D-camphor-protected inositol derivative **11**, which was synthesized from *myo*-inositol and D-camphor dimethyl acetal in an one-pot procedure as described elsewhere (*26*). Treatment of **11** with *tert*-butyldiphenylsilyl (TBDPS) chloride resulted in a remarkable selective protection of the 1-hydroxyl group, and the resulting triol **12** was fully blocked with methoxymethylene (MOM) groups to give **13**. Deprotection by tetrabutylammonium fluoride yielded **14** as the starting substrate for phosphitylation. The synthesis of phosphorothionates **15a,b** was carried out in an analogous way as described above. The diastereomers **15a** and **15b** could be separated by silica gel chromatography (hexane-acetone/20:1), and were then subjected to demethylation with trimethylamine followed by acid-catalyzed cleavage of all acetal protective groups to give **8a** and **8b**, respectively.

All three procedures employ similar phosphorylating agents, and the yields are all in the range of 40-60% from the protected D-*myo*-inositol. The more difficult part of the synthesis is really in the synthesis of the protected D-*myo*-inositol (**4**, **9**, and **14** in methods 1, 2, and 3, respectively). The syntheses of **4** and **9** have not been shown in the schemes, but they are significantly lengthier than the synthesis of **14** and also involve camphor (method 2) or camphanic acid (method 1) derivatives in the separation of protected D- and L-*myo*-inositols. Method 3 offers a significant short-cut due to the one-pot synthesis of **11** in enantiomerically pure form and the remarkable selectivity of the derivatization of **11** with the sterically bulky *tert*-butyldiphenylsilyl function. Although the synthetic work reported here was primarily aimed at the synthesis of DPPsI, the improvements of the synthetic procedures can also be applied to the synthesis of natural PI and IP.

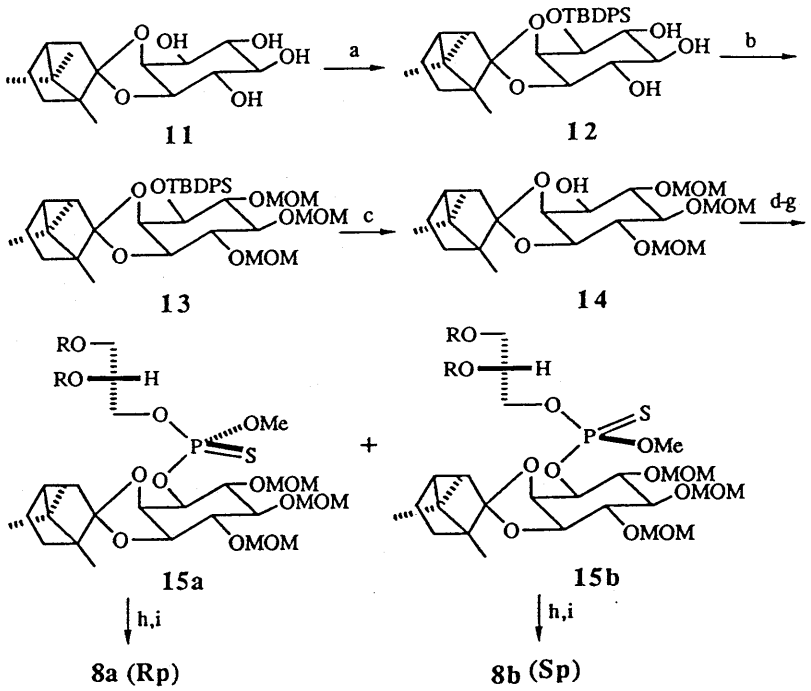
Configurational Assignment of DPPsI. It has been established previously that PLA2 from various sources hydrolyze specifically the R_p isomers of the phosphorothioate analogues of phosphatidylcholine (DPPsC) and phosphatidylethanolamine (DPPsE) (*6*, *7*, *10*, *27*). Since the phosphorus stereospecificity of PLA2 depends on the stereospecific interaction involving the

Scheme 3



a) **5**, Et_3N; b) L-1,2,4,5,6-pentabenzyl-*myo*-inositol (**9**), tetrazole; c) S_8; d) separation; e) NMe_3; f) BF_3·Et_2O-EtSH

Scheme 4



a) $^{t}BuPh_2SiCl$/ imidazole, DMF; b) $ClCH_2OMe$, $^{i}Pr_2EtN$; c) Bu_4N^+, F^-; d) 5, Et_3N; e) 1,2-dipalmitoyl-*sn*-glycerol, tetrazole; f) S_8; g) separation; h) Me_3N; i) 80% AcOH

phosphate group with Ca^{2+} (*10*) and possibly other residues at the active site of the enzyme, and since PLA2 is relatively nonspecific to the structure of the head group of phospholipids, the DPPsI isomer of the same chirality should be the preferred substrate for PLA2. However, due to a reversal of the relative priority of phosphorus substituents in DPPsI relative to DPPsC or DPPsE, R_p and S_p isomers of DPPsI correspond to S_p and R_p isomers, respectively, of DPPsC and DPPsE, in terms of the stereochemical structure of the phosphorothioate group.

As shown in Figure 1, treatment of (R_p+S_p)-DPPsI (spectrum a) in its micellar form with PLA2 resulted in the hydrolysis of the isomer corresponding to the lower field signal in ^{31}P NMR as illustrated by the gradual decrease in its intensity (spectra b-d). The chemical shift of the product (S_p)-MPPsI (Scheme 2, MP stands for 1-monopalmitoyl) nearly coincides with that of the unhydrolyzed (R_p)-DPPsI (isomer of the higher field signal), but they can be resolved in the expanded spectrum e. It was thus concluded that the configurations of isomers giving rise to the signals at δ 57.05 and 57.45 ppm (**8a** and **8b**, respectively) are R_p and S_p, respectively.

(R_p+S_p)-DPPsI as a Substrate of PI-PLC. In the case of *B. cereus* PI-PLC the reaction with (R_p+S_p)-DPPsI as a substrate leads to the formation of inositol cyclic-1,2-phosphorothioate (IcPs) (**16**) (Scheme 2), which gives rise to a ^{31}P NMR signal at 71.4 ppm (Figure 2, spectrum b). At the same time the resonance of the R_p isomer decreases. Both isozymes I and II of PI-PLC from guinea pig uterus are also specific to the R_p isomer of DPPsI and produce the same cyclic product **16**. In addition, formation of the acyclic *myo*-inositol 1-phosphorothioate (**17**, IPs) was observed at the ratio IcPs/IPs ca. 2 for isozyme I and 0.5 for isozyme II. These values were close to IcP/IP ratios observed for natural PI as substrate (1 and 0.5 for isozymes I and II, respectively). These results indicate that all three PI-PLC show the same stereospecificity (prefer the R_p isomer of DPPsI) as the non-PI-specific PLC (prefers the S_p isomers of DPPsC and DPPsE) (*6*). In order to elucidate the steric course of the formation of the IcPs it is necessary to determine the phosphorus configuration of the product **16**.

Synthesis and Configurational Analysis of *cis* and *trans* IcPs. In order to assign the configuration of **16** both isomers of IcPs were synthesized independently as outlined in Scheme 5, starting from DL-1,4,5,6-tetrabenzyl-*myo*-inositol (**18**). Treatment of **18** with **5** in the presence of diisopropylethylamine and subsequently with tetrazole resulted in the formation of a mixture of diastereomeric cyclic phosphites **19** to which elemental sulfur was added in the next step. The resulting phosphorothionates **20** were separated by chromatography and deprotected with lithium in THF-ammonia at -78° to give two diastereomers **16a** and **16b** resonating at δ 71.4 and 70.5 ppm, respectively, in ^{31}P NMR. The configurational assignment of IcPs isomers was made indirectly based on the following findings:

a) The yields of **16a** and its precursors **20a** and **19a** are much lower than those of **16b**, **20b** and **19b**, respectively. On the basis of related studies of a series of 4,5-disubstituted 1,3,2-dioxaphospholanes (*28*), the more stable and predominant isomer **19b** should have the *trans* geometry as indicated in Scheme 5. The oxidation and the

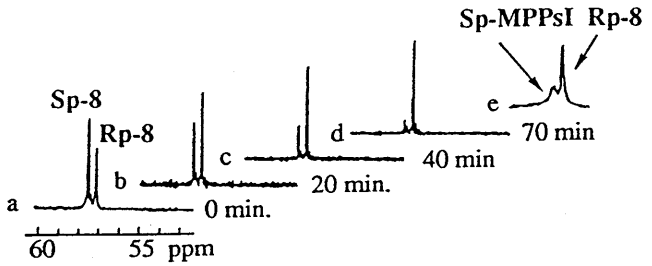


Figure 1. ^{31}P NMR spectra showing a time course of the hydrolysis of (R_p+S_p) DPPsI (5 mM, pH 7.2, 4% Triton X-100) in the presence of phospholipase A_2 (bee venom) at 310 K; spectrum e shows an expanded upfield signal from spectrum d. (Adapted with revision from reference 24)

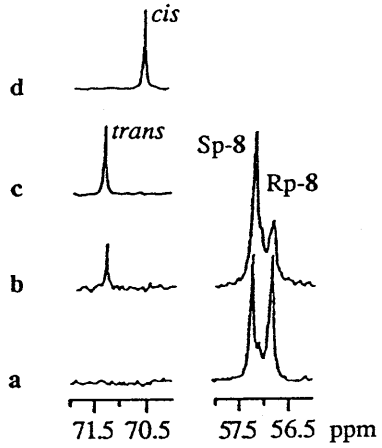
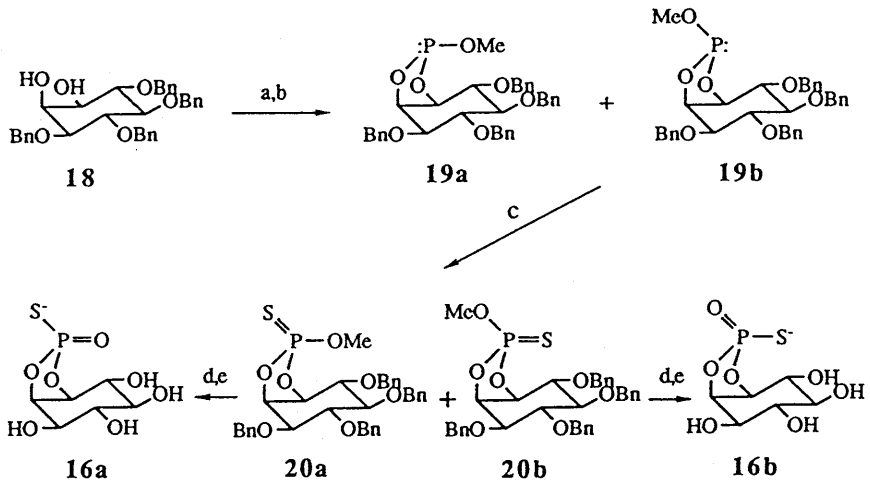


Figure 2. ^{31}P NMR spectra showing stereospecificity of PI-PLC (*B. cereus*) catalyzed hydrolysis of (R_p+S_p)-DPPsI (sample conditions analogous to Figure 1). a) DPPsI prior to adding PI-PLC; b) 72 h after adding 2 mg of PI-PLC; c) *trans*-IcPs (**16a**); d) *cis*-IcPs (**16b**). (Adapted with revision from reference 22)

Scheme 5



a) 5, $^{i}Pr_2EtN$; b) tetrazole; c) S_8; d) separation; e) Li/THF-NH_3

sulfur addition to cyclic P(III) compounds (such as **19**) usually proceeds with a retention of configuration, and the isomeric ratio is generally preserved (*28*). Thus **20a** and **20b** should be *trans* and *cis*, respectively. The *cis/trans* notations are based on the geometric relationship between the inositol ring and the other larger substituent of phosphorus (which is the OMe group in the case of **19**, but the sulfur atom in the case of **20**).

b) Comparison of model compounds (*28*) suggest that the ^{31}P chemical shift of the *cis* isomer **20b** should be 0.5-2.5 ppm more upfield than that of the corresponding *trans*-isomer **20a**. The observed chemical shifts **20a** and **20b** are 85.9 and 84.2 ppm, respectively.

c) The ^{1}H-^{31}P vicinal coupling constants (between P and H-1 of the inositol ring) of 18.4 and 9.7 Hz for **20a** and **20b**, respectively, correlate well with the values calculated from the Karplus equation (*29*) (18.3 and 9.8 Hz, respectively) for MM-2 optimized conformations of these isomers.

d) The ^{1}H 2D-NOESY spectra show a through-space interactions between the *O*-methyl group and H-4 of inositol in **20a** but not in **20b**. On the other hand, interactions between *O*-methyl group and H-2 are observed in **20b** but not in **20a** (*23*).

We therefore conclude that **20a** has a *trans* and **20b** a *cis* geometry. The same are true for isomers **16a** and **16b**, respectively, since the cleavage of methyl ester in **20** does not involve bond breaking around phosphorus. The absolute configuration at phosphorus of **20a** and **16a** as shown in Scheme 5 should be R_p according to the Cahn-Ingold-Prelog rules (30). Since the synthesis of IcPs starts with DL-**18**, **20a** and **16a** are actually racemic mixtures (D-R_p+L-S_p), so are **20b** and **16b** (D-S_p+L-R_p).

Steric Course of the Formation of IcP Catalyzed by PI-PLC. The results presented above establish that the IcPs produced from (R_p)-DPPsI is the *trans* isomer **16a**. This statement is a corollary to the conclusion that *PI-PLC catalyzed conversion of PI to IcP proceeds with an inversion of configuration* at phosphorus, most likely via direct, in-line attack at phosphorus by the 2-hydroxyl group of the inositol ring.

Implications on the Mechanism of PI-PLC. Figure 3 outlines six possible mechanisms of mammalian PI-PLC. Mechanisms E and F can be excluded because no incorporation of ^{18}O label into IcP was found when the reaction was carried out in [^{18}O]water (*31, 32*). Mechanisms B and D involve an enzyme-phosphoinositol intermediate and predict retention of configuration for the formation of IcP or IcPs. Thus mechanisms B and D can be ruled out based on the observed inversion of configuration at phosphorus. The two remaining possible mechanisms are A and C. Mechanism C resembles the mechanism of ribonuclease A (*33*) in that a cyclic intermediate is formed as the precursor of the hydrolytic product, but also differs in the sense that the cyclic intermediate is not released from ribonuclease A under normal catalytic conditions. Mechanism A represents a unique mechanism in that two reaction pathways proceed in parallel from a common substrate.

Mechanism A is the favored mechanism for mammalian PI-PLC on the basis of the following evidence: (i) there is no detectable conversion of IcP to IP catalyzed by the enzyme (*15, 20, 34, 35*); (ii) mechanism C would predict an increase of the proportion

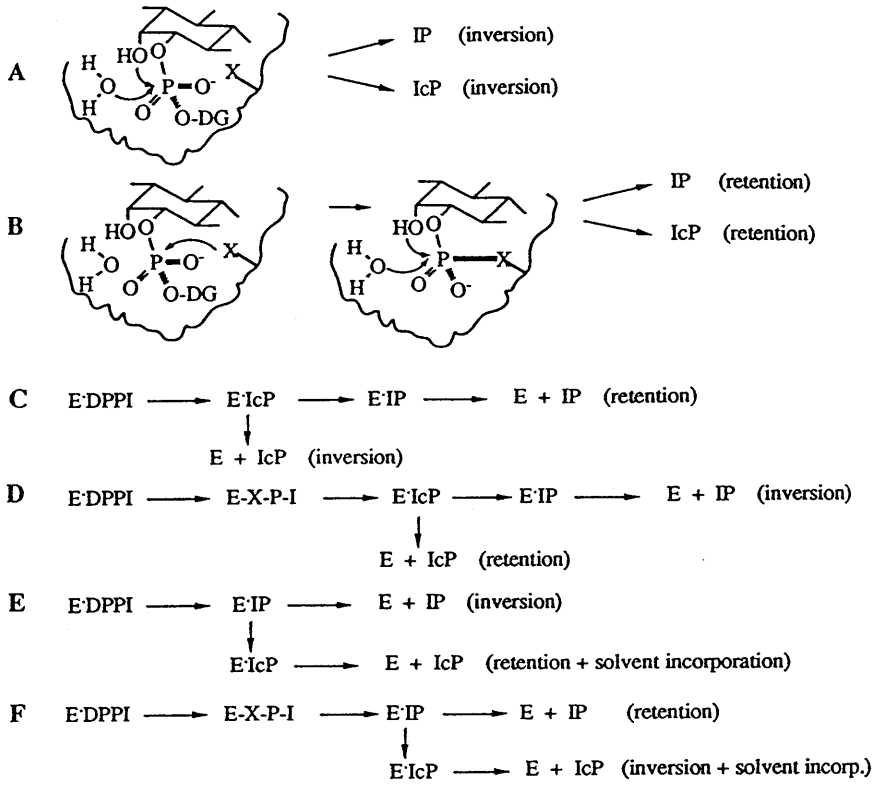


Figure 3. Possible mechanisms of mammalian PI-PLC: A) parallel reactions, direct displacement; B) parallel reactions, covalent enzyme-substrate intermediate; C) consecutive reactions (IcP → IP), direct displacement; D) consecutive reactions (IcP → IP), covalent enzyme-substrate intermediate; E) consecutive reactions (IP → IcP), direct displacement; F) consecutive reactions (IP → IcP), covalent enzyme-substrate intermediate. DG stands for the diacylglyceryl moiety of DPPsI. (Adapted with revision from reference 23)

of the cyclic product in the case of the phosphorothioate substrate since sulfur substitution would most likely slow down the enzyme-mediated hydrolysis of IcPs, but we have observed that the ratio IPs/IcPs is comparable to the ratio IP/IcP, as mentioned in a previous section. However, it should be noted that neither evidence is absolute. The kinetic competence of an intermediate in enzyme catalysis depends on specific rate constants and equilibrium constants (*36*). The ratio IPs/IcPs could also be fortuitously similar to the ratio IP/IcP in mechanism C.

Recently PI-PLC from *B. cereus* has been shown to exhibit a thousand-fold lower cyclic phosphodiesterase activity (i.e., conversion of IcP to IP) in addition to the main phosphotransferase activity (*37*). This observation strongly suggests that mechanism C is the actual mechanism for PI-PLC from *B. cereus*. Since the specific activity of mammalian PI-PLC is usually thousand-fold lower than that of *B. cereus* PI-PLC, it is possible that mammalian and bacterial enzymes both utilize mechanism C, but bacterial enzymes have evolved to greatly improve the first step and the release of the cyclic intermediate. Alternatively, mammalian and bacterial enzymes could have evolved to different mechanisms (parallel and sequential pathways, respectively). There is at least one closely related precedent: phosphatidylserine synthase from yeast catalyzes the reaction via a sequential mechanism, whereas that from *Escherichia coli* uses a ping-pong mechanism (*13*). In both cases one of the differences between the enzymes from lower and higher organisms is requirement for divalent metal ions in the latter. The unequivocal distinction between mechanisms A and C for mammalian PI-PLC is awaiting elucidation of the steric course of the formation of IP from PI.

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