Stereocontrolled Syntheses of Water-Soluble Inhibitors of Phosphatidylinositol-Specific Phospholipase C: Inhibition Enhanced by an Interface†

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ABSTRACT: Three inositol 1,2-(cyclic)-phosphate analogs, inositol cyclic phosphonates with different stereochemistry at the C-2 position of the inositol ring, have been synthesized as water-soluble inhibitors of phosphatidylinositol-specific phospholipase C (PI-PLC). Their inhibition of both phosphotransferase and cyclic phosphodiesterase activities has been studied in the absence and presence of an interface. Key results include the following. (i) Only the analog with the same stereochemistry at the C-2 position of the inositol ring as the natural substrate, *myo*-inositol 1,2-(cyclic)-phosphate (cIP), exhibits effective inhibition of PI-PLC. (ii) The inhibition of the PI-PLC cyclic phosphodiesterase activity by this cIP analog is enhanced by the presence of an interface (Triton X-100 or diC_7PC micelles). This is the first observation of detergent enhancing the effectiveness of a water-soluble inhibitor competing with a water-soluble substrate. (iii) For the cyclic phosphodiesterase activity measured in the presence of 8 mM of the best (e.g., most activating) interface, diC_7PC, *myo*-inositol 1,2-(cyclic)-2-methylenephosphonate (cICH_2P) was shown to be a competitive inhibitor with a K_i of 12.3 mM. (iv) The IC_{50} obtained for the same compound inhibiting the PI-PLC hydrolysis of PI dispersed in diC_7PC micelles was consistent with a K_i ∼10 mM for the phosphotransferase activity. The similarity of K_i for both PI and cIP processing by PI-PLC suggests both reactions occur at the same site on the enzyme.

Phosphatidylinositol-specific phospholipase C (EC 3.1.4.11) enzymes are very important in cell signaling. They exist both intracellularly and extracellularly in a wide variety of tissues and organisms. Extracellular PI-PLCs[1] have been isolated from the culture media of several microorganisms (Ikezawa & Taguchi, 1981; Low, 1981), while intracellular PI-PLC isozymes are prevalent in mammalian cells (Rhee et al., 1989; Takenawa & Nagai, 1981; Homma et al., 1988; Ryu et al., 1986, 1987; Bennett & Crooke, 1987; Banno et al., 1988) and are involved in second messenger metabolism (Berridge, 1984, 1986). The intracellular enzymes hydrolyze phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG), a membrane localized activator of protein kinase C, and inositol trisphosphate, a water-soluble second messenger involved in Ca^{2+} mobilization. The extracellular bacterial PI-PLC enzymes are water soluble and relatively specific for nonphosphorylated PI. They are involved in the release of proteins tethered to the plasma membrane by cleavage of their glycosylphosphatidylinositol anchors (Griffith et al., 1991). Bacterial PI-PLC catalyzes the hydrolysis of phosphatidylinositol (PI) to yield DAG and *myo*-inositol 1,2-(cyclic)-phosphate (cIP) (Figure 1). This same enzyme can also slowly catalyze the hydrolysis of cIP to D-*myo*-inositol 1-phosphate (I-1-P) (Volwerk et, al., 1990). Similar to many well-studied phospholipases, PI-PLC shows a preference for aggregated substrate over monomeric PI. This kinetic phenomenon is known as "interfacial activation" (Lewis et al., 1993; Volwerk et al., 1994). With short-chain PI, the kinetic effect is a 5−6 fold increase in apparent V_{max}. The same phenomena for extracellular phospholipase A_2 enzymes is characterized by a much larger increase in apparent V_{max}, and detailed characterization of the interfacial behavior of that enzyme has been reported (Jain & Berg, 1989; Berg et al., 1991; DeHaas, 1995). Recently, a crystal structure of PI-PLC from *Bacillus cereus* has been reported. The structure with *myo*-inositol bound to PI-PLC has led to a proposed catalytic mechanism which involves two histidines (Heinz et al., 1995). However, an explanation for the discrete two-step kinetic mechanism of PI-PLC where specific activities appear to be so different is not apparent.

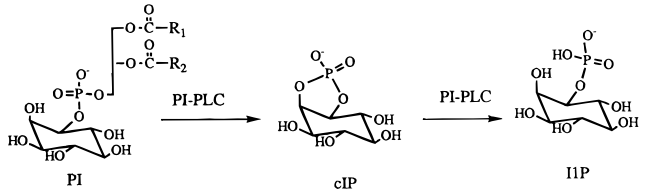

FIGURE 1: Two-step mechanism proposed for PI-PLC catalyzed hydrolysis of PI.

Several lipophilic inhibitors of PI-PLC have been synthesized (Potter & Lampe, 1995; Garigapati & Roberts, 1993; Vinod et al., 1994; Shashidhar et al., 1989) in attempts to gain an understanding of the catalytic mechanism of this interfacial enzyme. These inhibitors are all PI analogs and are generally effective inhibitors of PI-PLC hydrolysis of PI. A fundamental concern with lipophilic inhibitors is that they could generally change the surface properties of the substrate interface. Observed inhibition could be due to the

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[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; cIP, D-inositol 1,2-(cyclic)-phosphate; I-1-P, D-inositol 1-phosphate; diC_7PC, diheptanoylphosphatidylcholine; DAG, diacylglycerol; cICH_2P, *myo*-inositol 1,2-(cyclic)-2-methylenephosphonate; LDA, lithium diisopropylamide; DCC, dicyclohexylcarbodiimide; Bn, benzyl.

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PI analog competitively binding to the active site, or to the analog altering the initial binding of the enzyme to the surface or changing the surface structure. Micelle systems are complicated by surface dilution problems (the surface concentration of substrate depends on the detergent/substrate ratio, not on bulk substrate concentration), and quantitative determination of the kinetic parameters of these inhibitors can be quite complicated (for a recent review, see Carman et al., 1995). Vesicle systems may also prove complicated if phospholipids other than PI are used and these bind to the enzyme.

A water-soluble fluorinated cIP analog has been previously reported to be an inhibitor of PI-PLC (Campbell & Thatcher, 1991). Here we report the synthesis of three cIP analogs with different stereochemistry in the C-2 position of the inositol ring, and their inhibition of both the first-step (phosphotransferase) and second-step (cyclic phosphodiesterase) activities of PI-PLC in the absence and presence of an interface.

MATERIALS AND METHODS

Chemicals. Methylene chloride (CH_2Cl_2) and triethylamine (TEA) were distilled over calcium hydride. Tetrahydrofuran (THF) was distilled from benzophenone under argon. Dimethylformamide (DMF) was stored over 4 Å molecular sieves for at least 24 h before use. All other reagents were used as received. All moisture or oxygen sensitive reactions were carried out under an atmosphere of argon.

Preparation of 4,6-Di-O-benzyl-myo-inososе 1,3,5-O-Orthoformate (**2**). The method of Lee and Kishi (1985) was used for the synthesis of ketone **2** in 5 steps with an overall yield of 35%. Compound **2** has a low melting point and is a colorless solid. It was found to partially hydrate to form the *gem*-diol, and the hydrated water molecule could be removed by incubating the material with 4 Å molecular sieves in $CHCl_3$ overnight. The high reactivity of **2** may be due to the strain effects, resulting in the destabilization of sp^2 carbonyl carbon relative to sp^3 (Riley & Potter, 1995).

4,6-Di-O-benzyl-myo-inositol 1,3,5-O-Orthoformate 2-(Diethyl methinephosphonate) (**3**). Tetraethyl methylenediphosphonate (543 mL, 2.2 mmol) was added to 5 mL of THF at room temperature under argon and then cooled to −78 °C. LDA (1.0 mL of a 2 M solution in heptane/THF/ethylbenzene) was added dropwise under argon, and stirring was continued for 1 h. Compound **2** (340 mg, 1.0 mmol) in 5 mL of THF was cannula transferred (dropwise) to the reaction mixture. After 12 h, the reaction mixture was allowed to warm gradually to room temperature. About 50 mL of diethyl ether was added to the reaction mixture, and the resulting slurry was filtered through a short silica gel column by eluting with diethyl ether. The crude product was further purified by silica gel chromatography, eluting with 8% diethyl ether in methylene chloride to recover 80 mg (25%) of starting material **2**, followed by 15% diethyl ether in methylene chloride, to obtain 332 mg (70% yield) of **3**, a colorless, viscous liquid. ^{1}H NMR chemical shifts in $CDCl_3$ (ppm from TMS) include the following: 0.91 (t, 3H), 1.30 (t, 3H), 3.72 (m, 2H), 4.08 (m, 2H), 4.40 (m, 3H), 4.56 (m, 3H), 4.71 (d, 1H, J = 10 Hz), 4.87 (d, 1H, J = 10 Hz), 5.63 (s, 1H), 5.67 (d, 1H, J = 15 Hz), 6.04 (d, 1H, J = 2 Hz), 7.24 (m, 10H). The ^{31}P NMR chemical shift of the compound in $CDCl_3$ was 14.19 ppm.

Thermodynamically Controlled Catalytic Hydrogenation To Produce myo-Inositol 1,3,5-O-Orthoformate 2-(Diethyl methylenephosphonate) (**5T**). A 150 mg sample of 10% Pd/C was added to **3** (251 mg, 0.5 mmol) in 100 mL of EtOH under argon, and the reaction mixture was heated to 60 °C before a hydrogen balloon was applied and the mixture was stirred vigorously. The reaction was easily followed by TLC. After 5 h, a mixture of **5T** (60%) and **5T1** (40%) was obtained. After 10 h, the conversion from **3** to **5T** was completed. The reaction mixture was then cooled to room temperature, and the catalyst was removed by filtering through Celite. After flash chromatography (50% EtOAc/CH_2Cl_2), 160 mg of a white powder, **5T** (99% yield with greater than 90% stereoselectivity as judged by NMR), was obtained. Further separation of the two stereoisomers can be achieved by HPLC. ^{1}H NMR chemical shifts of **5T** in $CDCl_3$ (ppm from TMS) include the following: 1.35 (t, 6H, J = 7 Hz), 2.27 (dd, 2H), 2.75 (m, 1H), 3.50 (b, 2H, exchangeable), 4.10 (m, 6H), 4.28 (m, 1H), 4.45 (m, 2H), 5.48 (d, 1H, J = 2 Hz). ^{1}H NMR chemical shifts in $CDCl_3$ (ppm from TMS) for **5T1** include the following: 1.27 (t, 6H), 2.25 (dd, 2H), 2.83 (m, 1H), 4.10 (m, 4H), 4.26 (m, 4H), 4.49 (m, 1H), 4.63 (dd, 4H), 5.48 (s, 1H), 7.25 (s, 10H).

Kinetically Controlled Catalytic Hydrogenation To Produce 4,6-Di-O-benzyl-scyllo-inositol 1,3,5-O-Orthoformate 2-(Diethyl methylenephosphonate) (**5K**). Raney Ni (150 mg) was added to **3** (251 mg, 0.5 mmol) in 100 mL of EtOH under argon, and the reaction mixture was cooled to 0 °C before a hydrogen balloon was applied and the mixture stirred vigorously. The reaction was followed by TLC and was completed in 6 h. The catalyst was removed by filtering through Celite. After flash chromatography (15% EtOAc/CH_2Cl_2), 236 mg of a white powder, **5K**, (94% yield with greater than 95% stereoselectivity as judged by NMR) was obtained. This kinetically controlled reaction product can also be achieved by catalytic hydrogenation with 10% Pt/C at 0 °C, resulting in a slightly lower stereoselectivity (90%). ^{1}H NMR chemical shifts in $CDCl_3$ (ppm from TMS) include the following: 1.20 (t, 6H, J = 7 Hz), 2.43 (dd, 2H), 3.12 (m, 1H), 3.93 (m, 4H), 4.33 (m, 2H), 4.52 (m, 2H), 4.57 (m, 1H), 4.68 (s, 4H), 5.57 (s, 1H), 7.25 (s, 10H).

Deprotection and Cyclization of **3** *To Yield myo-Inositol 1,2-(Cyclic)-2-methinephosphonate* (**4**). TFA/H_2O (9:1; 100 mL) was added to 251 mg (0.5 mmol) of **3**, and the reaction mixture was heated to 65 °C. After 48 h, removal of the protecting groups was completed. Solvent was removed by vacuum, and the residue was further purified by elution from an anion exchange column. AG1-X8 resin (chloride form, 100−200 mesh) was purchased from Bio-Rad. For 100 mg of crude compound, about 5 g of resin was used. The resin was converted to its formate form by first rinsing with 20 column volumes of 1 N NaOH, washing with deionized water until the eluant pH was neutral, and elution of 2 bed volumes of 1 M formic acid, followed by washing with deionized water to a pH of 7. Samples applied to the column were eluted with 40 mM ammonium formate and 10 mM formic acid with a linear flow rate of about 0.5 cm/min. The fractions containing **4** were combined, and the pH was adjusted to 7.0 with NH_4OH. After lyophilizing, 127 mg of white powder of **4** (NH_4^+ salt) was obtained. ^{1}H NMR chemical shifts in D_2O include the following: 3.37 (m, 2H), 3.46 (m, 1H), 4.20 (m, 1H), 4.51 (m, 1H), 6.13 (d, 1H, J =

37.5 Hz). The ^{31}P NMR chemical shift of the compound in D_2O was 40.02 ppm. Compound **4** (ammonium salt) was further characterized by high resolution FAB mass spectrometry. The molecular weight for the species was experimentally determined to be 256.0585; the calculated molecular weight for the (M + H) ion was 256.0586, corresponding to $C_7H_{15}NO_7P$.

Deprotection of **5T** *To Yield myo-Inositol 2-Methylenephosphonate* (**6T**). TFA/H_2O (4:1; 100 mL) was added to 162 mg (0.5 mmol) of **5T**, and the reaction mixture was heated to 55 °C. After 48 h, removal of the protecting groups was complete. Solvent was removed by vacuum, and the residue was further purified by elution with 50 mM formic acid from an AG1-X8 column. A white powder that was the acid form of **6T** (127 mg, 99% yield) was obtained. ^{1}H NMR chemical shifts in D_2O include the following: 1.80 (dd, 2H), 2.50 (m, 1H), 3.05 (t, 1H), 3.27 (t, 2H), 3.51 (dd, 2H). The ^{31}P NMR chemical shift of the compound in D_2O was 31.22 ppm.

Deprotection of **5K** *To Yield scyllo-Inositol 2-Methylenephosphonate* (**6K**). TFA/H_2O (9:1; 100 mL) was added to 252 mg (0.5 mmol) of **5K,** and the reaction mixture was heated to 65 °C. After 48 h, removal of the protecting groups was complete. Solvent was removed by vacuum, and the residue was further purified by elution with 50 mM formic acid from an AG1-X8 column. Fractions containing **6K** were combined. After lyophilization, 128 mg (99% yield) of white powder of the acid form of **6K** was obtained. ^{1}H NMR chemical shifts in D_2O include the following: 1.72 (m, 1H), 2.06 (dd, 2H), 3.25 (m, 5H). The ^{31}P NMR chemical shift of the compound in D_2O was 30.10 ppm.

*Cyclization of Linear Inositol Phosphonates To Yield myo-Inositol 1,2-(Cyclic)-2-methylenephosphonate (***7***, Also Abbreviated as cICH$_2$P) and scyllo-Inositol 1,2-(Cyclic)-2-methylenephosphonate* (**8**). 1.0 M dicyclohexylcarbodiimide (700 mL) in methylene chloride solution (0.7 mmol) was added dropwise to **6T** or **6K** (acid form, 129 mg, 0.5 mmol) in 130 mL of DMF solution, and the reaction was monitored by ^{31}P NMR. After 12 h, the cyclization reaction was complete. Several drops of water were added to quench the reaction. After removing the solvent, the residue was extracted with water. The crude material was then purified on an AG1-X8 anion exchange column (formate form) eluting with 10 mM formic acid and 40 mM ammonium formate. Appropriate fractions were collected, and the pH was adjusted by NH_4OH. After lyophilization, 125 mg (97% yield) of white powder **7** (ammonium salt) or **8** (ammonium salt) was obtained. ^{1}H NMR chemical shifts in D_2O for **7** include the following: 1.77 (m, 1H), 3.00 (m, 1H), 3.15 (t, 1H), 3.45 (t, 1H), 3.52 (t, 1H), 3.69 (m, 1H), 4.05 (m, 1H); ^{31}P chemical shift (D_2O) 42.50 ppm. ^{1}H NMR chemical shifts for **8** in D_2O include: 1.50 (m, 1H), 1.98 (m, 2H), 3.31 (m, 3H), 3.52 (m, 2H). The ^{31}P NMR chemical shift of the compound in D_2O was 43.50 ppm. Compound **7** (ammonium salt) was further characterized by high resolution FAB mass spectrometry. The molecular weight for the species was experimentally determined to be 258.0744; the calculated molecular weight for the (M + H) ion was 258.0743, corresponding to $C_7H_{17}NO_7P$. Compound **8** (ammonium salt) was similarly characterized by high resolution FAB mass spectrometry. The experimental molecular weight for the (M + H) ion was 258.0741 as expected.

Enzymatic Synthesis of D*-myo-Inositol 1,2-(Cyclic)-phosphate.* Crude soybean PI was used for enzymatic generation of cIP. Crude PI (1.0 g) was dissolved (by sonication) in 15 mL of Tris buffer (pH 7.5) containing 4% Triton X-100. PI-PLC (20 μg) was added, and the reaction was monitored by ^{31}P NMR spectroscopy. The cleavage of PI to generate cIP was generally complete in 4 h at room temperature. Following extraction of the aqueous solution with chloroform−methanol (to remove PI and DAG), cIP was further purified by elution from an AG1-X8 anion exchange column (formate form) with 10 mM formic acid and 40 mM ammonium formate. About 110 mg of pure cIP could be made from 1.0 g of crude PI in this fashion.

Purification of PI-PLC. PI-PLC, isolated from culture supernatants of *Bacillus subtilis* (BG2320) transfected with the PI-PLC gene from *Bacillus thuringiensis*, was purified as described by Low et al. (1988) and modified by Zhou et al. (1997). The enzyme was stored at −20 °C in 50% glycerol and 20 mM Tris buffer, pH 8.5.

^{31}P NMR Assays of PI-PLC. For PI-PLC phosphotransferase kinetic studies, L-α-phosphatidylinositol, purchased from Avanti, was used as a substrate without further purification. A Triton X-100 mixed micelle stock solution (the ratio of substrate to Triton X-100 was fixed at 1:2 with 40 mM substrate) was prepared in 50 mM HEPES buffer, pH 7.5. A final concentration of 4 mM PI was used in these kinetic studies. For PI-PLC cyclic phosphodiesterase kinetic studies, cIP was used as a substrate. The stock solution of cIP (600 mM) was adjusted to pH 7.5 using NaOD. The range of cIP concentrations examined was 8−40 mM. Different detergents were also added to study the surface activation of the cyclic phosphodiesterase reaction. Stock inhibitor solutions (300 mM) were prepared in D_2O with the pH adjusted to 7.5 using NaOD. The range of inhibitor concentrations examined was 2−30 mM.

^{31}P NMR parameters were based on those previously used by Griffith and co-workers (Volwerk et al., 1990) as modified by Zhou et al. (1997). ^{31}P NMR (202.3Hz) spectra were acquired using a Varian Unity 500 spectrometer, 5 mm sample tubes, and chemical shifts were referenced to external 5% phosphoric acid. PI-PLC (freshly diluted from its concentrated stock solution with 20 mM Tris buffer, pH 8.5, containing 0.1% BSA) was added to initiate hydrolysis. The amount of enzyme added varied between 15 ng and 1 μg as determined by Bradford assay, depending on whether the phosphotransferase or the cyclic phosphodiesterase activity was being monitored. The hydrolysis rates were measured from the integrated intensity of the resonance corresponding to the phosphorylated product as a function of incubation time, typically 1−2 h, at 30 °C.

RESULTS AND DISCUSSION

Synthesis of Inositol 1,2-(Cyclic)-phosphate Analogs. Starting with *myo*-inositol, compounds **4**, **7**, and **8** have been synthesized as nonhydrolyzable analogs of inositol 1,2-(cyclic)-phosphate. The synthetic scheme is presented in Figure 2 and is based on previously published strategies (Lee & Kishi, 1985; Campbell & Thatcher, 1991). There are several key steps in this scheme. (i) The removal of benzyl protecting groups by TFA/H_2O generates compound **4**. The structure of **4** was confirmed by the observed coupling of ^{31}P to the C-1 proton of the inositol ring. Furthermore,

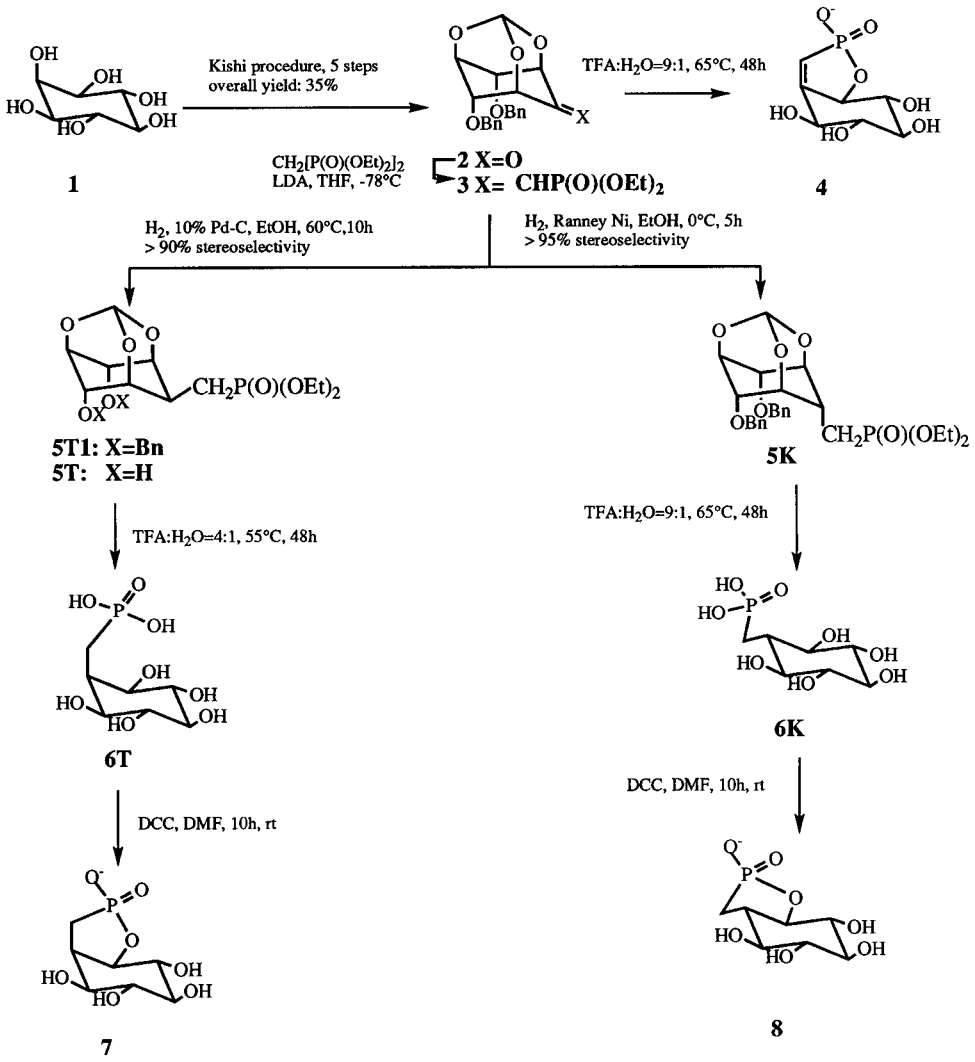

FIGURE 2: Synthetic scheme for the synthesis of cIP analogs **4**, **7**, and **8.** Compound **7,** abbreviated as $cICH_2P$, was shown to be a competitive inhibitor of PI-PLC.

compound **4** could be completely hydrolyzed with 1 M NaOH to generate the acyclic compound, identified by a characteristic ^{31}P NMR chemical shift of about 25 ppm at pH 7. (ii) Reduction of **4** with 10% Pd/C yields predominantly the *cis*-isomer, **7**; however, about 20% of the *trans*-isomer, **8**, is also formed. Carrying out the catalytic hydrogenation step on compound **3** can generate either the thermodynamic product **5T** or the kinetic product **5K** almost exclusively, depending on the conditions used. The structure of **5K** was further confirmed by the observed intramolecular NOE between the benzyl methylene protons and phosphonate methylene protons (spectrum not shown). In the thermodynamically controlled reaction, a mixture of **5T1** and **5T** is generated; these two compounds can be separated by flash chromatography. The structure of **5T1** was confirmed by the observed intramolecular NOE between the C-2 proton of the inositol ring and the protons on the benzyl methylene groups (spectrum not shown). For longer reaction times, **5T1** was completely converted to **5T.** The stereochemistry of **5T1** and **5T** at the C-2 position of the inositol ring is identical because each generates the same product, **6T,** after removing the protecting groups. Kinetic control can be achieved probably because in the presence of catalyst Raney Ni or 10% Pt/C, the addition of the two hydrogen atoms is concerted, and the catalyst can only bind to the substrate from the relatively less crowded face. Thermodynamic control can be achieved probably because, for catalyst 10% Pd/C, the addition of the two hydrogen atoms to the olefin is stepwise rather than concerted. After the addition of the first hydrogen atom, the double bond is partially destroyed and a rotation along that bond can occur at relatively high temperature. Therefore, the most thermodynamically stable product was finally obtained. (iii) The acyclic inositol phosphonates can be cyclized using DCC in DMF to generate the cyclic phosphonates **7** and **8**. The materials are racemic, but based on the earlier kinetic results with the L- and D-inositol isomers of PI (Lewis et al., 1993), only the D-inositol isomer should have any affinity for the enzyme. All inhibition parameters presented in this paper have been converted to reflect the D-isomer of the cyclic inositol phosphonates.

Inhibition Studies of PI-PLC. ^{31}P NMR spectroscopy was used to monitor hydrolysis rate of long-chain PI dispersed in Triton X-100 mixed micelles and the hydrolysis rate of cIP in the presence and absence of diC_7PC and Triton X-100 micelles. As shown in Figure 3, the resonances for PI, cIP, I-1-P, and the cyclic phosphonates are well separated, and their intensities are easily measured. In examining how the water-soluble phosphonates affect phosphotransferase activity, we have found that the analog with a double bond, **4**, and the *scyllo*-inositol derivative, **8**, were poor inhibitors of PI-PLC. Less than 10% inhibition was observed at 25 mM

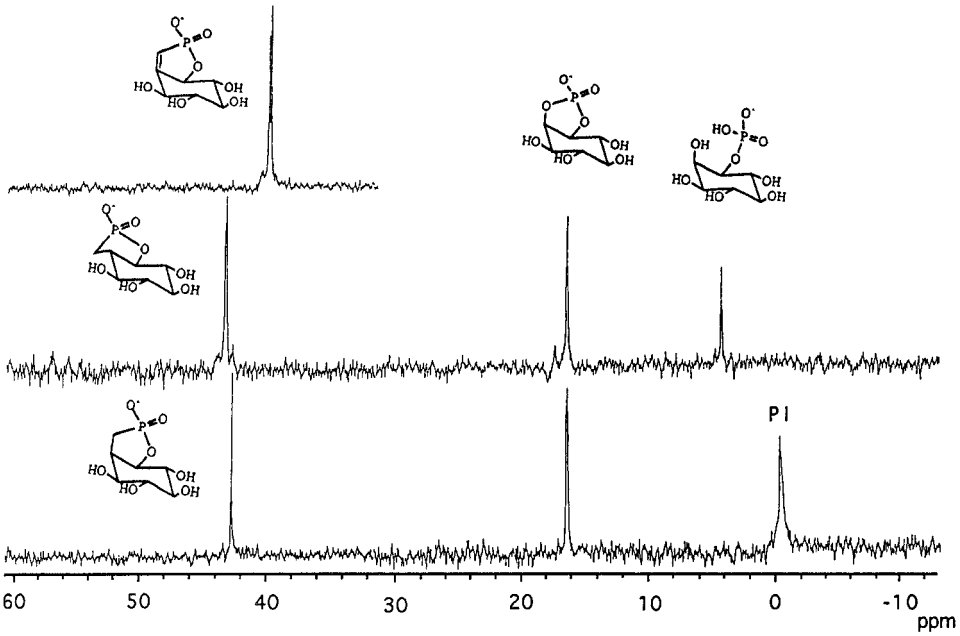

FIGURE 3: ^{31}P NMR spectra of the cIP analogs, reaction products (cIP, I-1-P), and substrates (PI, cIP) of PI-PLC. The chemical shifts are given in ppm referenced from external 5% phosphoric acid.

for these two compounds in an assay system with 4 mM PI, 8 mM Triton X-100, and 50 mM HEPES, pH 7.5. In contrast, $cICH_2P$, **7**, with stereochemistry at C-2 of the inositol ring comparable to that in PI, was moderately effective at inhibiting the enzymatic hydrolysis of PI in Triton X-100 mixed micelles ($IC_{50} = 8$ mM under these conditions). Compound **6T**, the hydrolysis product of $cICH_2P$, and an analog of inositol 2-phosphate, was also noninhibitory under these conditions. These results indicate the very stereospecific binding of $cICH_2P$ to PI-PLC.

myo-Inositol 1,2-(Cyclic)-2-methylenephosphonate ($cICH_2P$) Inhibition of cIP Hydrolysis in the Absence of an Interface. $cICH_2P$ was not an effective inhibitor of the second step of PI-PLC action on PI—the hydrolysis of cIP—in the absence of an interface. Concentrations of $cICH_2P$ up to 12 mM in aqueous solution (without detergent) were not inhibitory. Errors in determining cIP hydrolysis rates were less than 10%, and this could be used to get a crude estimate of the K_i for $cICH_2P$ in the absence of an interface. Assuming for the moment a 10% decrease in PI-PLC activity toward 13 mM cIP in the presence of 12 mM $cICH_2P$, a K_m for cIP of 90 mM under these conditions (Zhou et al., 1997), and competitive inhibition, K_i would be ~100 mM at best. Thus, the very high K_m (90 mM) for cIP in this assay system predicts very weak binding of $cICH_2P$ to PI-PLC. Lack of significant inhibition by $cICH_2P$ under these conditions could also be related to the cooperativity observed in the cyclic phosphodiesterase kinetics as well as the high K_m for cIP (Zhou et al., 1997). Thus, under these conditions, little or no inhibition by $cICH_2P$ would be observed.

$cICH_2P$ Inhibition of cIP Hydrolysis in the Presence of an Interface. Significant inhibition of cIP hydrolysis was observed if the assay was carried out in the presence of diC_7-PC micelles (Figure 4A). In the presence of this interface, the K_m for cIP was lowered significantly (Zhou et al., 1997). cIP has no affinity for either Triton X-100 or diC_7PC micelles (Zhou et al., 1997). Thus, this kinetic enhancement of cIP hydrolysis reflects an allosteric surface (e.g., diC_7PC) activation of PI-PLC toward the water-soluble cIP. The binding

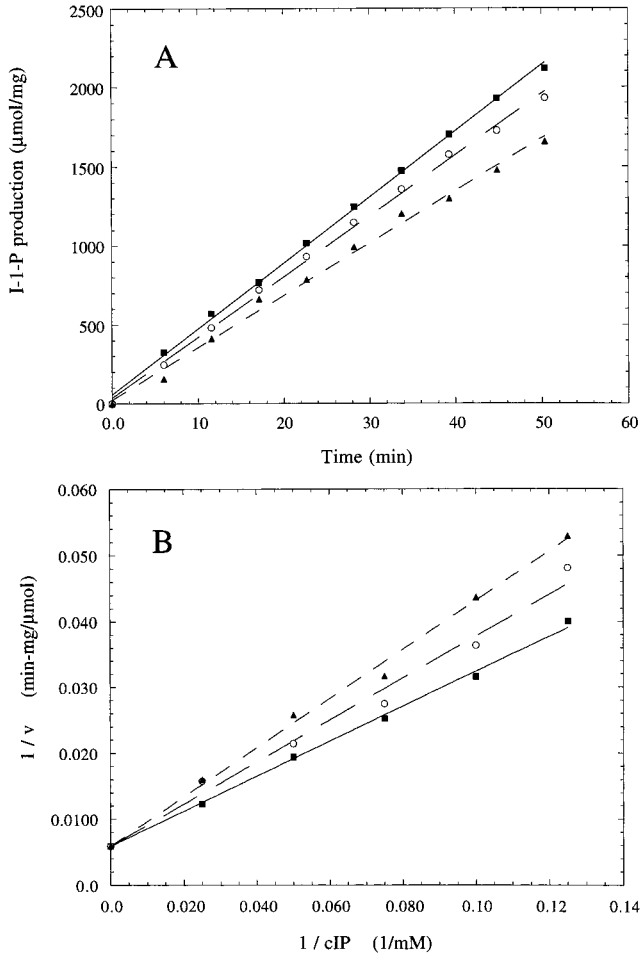

FIGURE 4: (A) A typical reaction progress curve for PI-PLC hydrolysis of cIP in the absence of $cICH_2P$ (n), or in the presence of 2.5 mM (m) or 5 mM (s) $cICH_2P$. The concentration of $cICH_2P$ has been converted to reflect the concentration of D-isomer. Reaction conditions include 8 mM diC_7PC (added as an interface), 13.3 mM cIP, 50 mM HEPES buffer, pH 7.5, and 1 μg of PI-PLC. (B) Lineweaver—Burk plot for PI-PLC cyclic phosphotransferase activity. The K_i determined from this plot is 12.3 mM.

of $cICH_2P$ to PI-PLC, like cIP, was enhanced in the presence of an interface since inhibition of PI-PLC activity was only observed when an activating detergent was added. Similar to cIP, $cICH_2P$ will not partition into diC_7PC (or Triton X-100) micelles; hence its increased inhibition potency reflects a higher binding affinity for the surface activated enzyme.

The cooperativity of PI-PLC cyclic phosphodiesterase kinetics complicates the quantitative determination of inhibition parameters (i.e., the Lineweaver−Burk plot is not linear if significant cooperativity exists). Fortunately, in the presence of the best (e.g., most activating) interface, diC_7PC micelles, the cooperativity was significantly reduced (the Hill coefficient was reduced from 1.8 to 1.3 if we fit the kinetic data with the Hill equation, but the data were also well fit by a simple Michaelis−Menten equation (Zhou et al., 1997)). Under these conditions, quantitative determination of the inhibition parameter in this assay system is possible. A Lineweaver−Burk plot of the effect of $cICH_2P$ on cIP hydrolysis is presented in Figure 4B. Clearly, $cICH_2P$ acted as a competitive inhibitor, and the K_i determined from the data was 12.3 mM. This K_i is significantly lower than the K_m for cIP (29 mM in the same assay system; Zhou et al., 1997). This means $cICH_2P$ binds to the PI-PLC active site significantly better than cIP. Two possible reasons can account for this observation: First, the stereochemistry of $cICH_2P$ and cIP is very similar, while $cICH_2P$ is slightly more hydrophobic because one oxygen has been replaced by a methylene group. This hydrophobicity may contribute to the better binding of $cICH_2P$ to the enzyme (it does not enhance partitioning of the compound to detergent micelles). Second, the bond length of P−C is slightly (about 0.1 Å) longer than P−O, making $cICH_2P$ a little more like a transition state analog.

Key information learned from these inhibition studies is that while PI-PLC needs to bind to a membranous interface for full activation toward cIP, the binding of the water-soluble inhibitor $cICH_2P$ to PI-PLC is also enhanced by the presence of an interface. This is the first observation of detergent enhancing the effectiveness of a water-soluble inhibitor competing with a water-soluble substrate. Clearly, when PI-PLC binds to the interface, a conformational change must be induced which allows both substrate and inhibitor to bind more tightly at the active site. The effect of $cICH_2P$ on cIP hydrolysis is summarized in Figure 5A.

$cICH_2P$ Inhibition of PI Hydrolysis. $cICH_2P$, with stereochemistry at C-2 of the inositol ring comparable to PI, was also moderately effective at inhibiting the enzymatic hydrolysis of PI in Triton X-100 mixed micelles (Figure 6). The PI/Triton X-100 assay system shows a maximum in activity at 1:2 substrate/detergent (Zhou et al., 1997), and this ratio was used for assessment of the inhibitory potency of $cICH_2P$. Like cIP, the inhibitor only has a strong affinity for the A·E complex and not E. The IC_{50} value for $cICH_2P$ inhibiting PI-PLC hydrolysis of PI determined by Figure 6B was about 8 mM for the D-isomer. The K_i for the inhibition of the phosphotransferase activity can be estimated from this IC_{50} value, if the real K_m is known, and assuming competitive inhibition. To a first approximation, the latter is a good assumption since $cICH_2P$ was shown to be a competitive inhibitor of cIP hydrolysis by PI-PLC (Figure 4B). In this particular case, sufficient detergent (Triton X-100) was added to maximize its activation of PI-PLC (by forming an

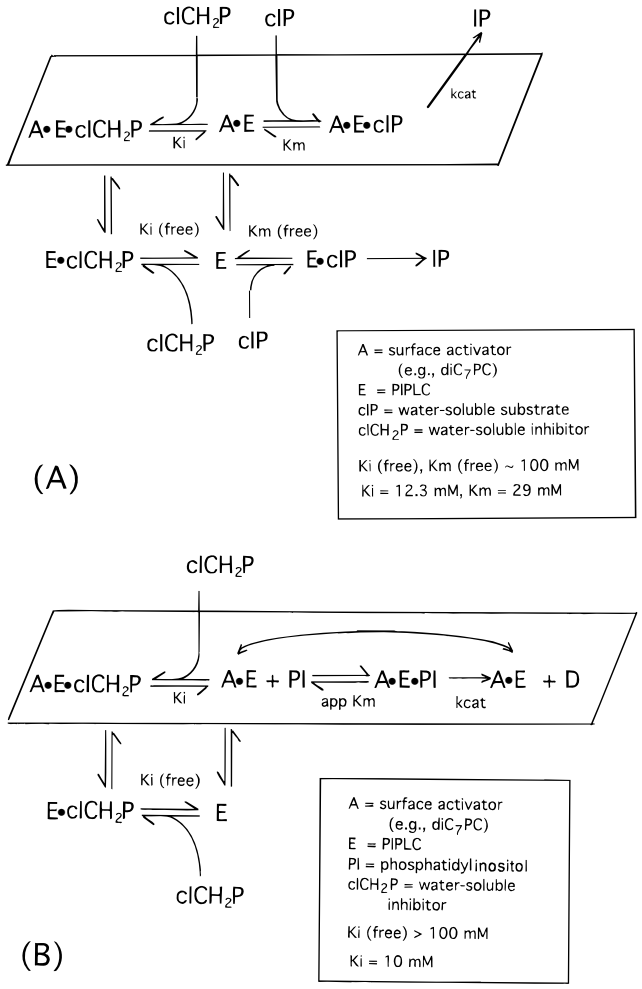

FIGURE 5: Kinetic scheme for how the water-soluble cIP analog, $cICH_2P$, inhibits PI-PLC hydolysis of (A) cIP or (B) PI. The inhibitor ($cICH_2P$ = I) does not partition to the interface except by binding to enzyme; experimentally, it was shown that the K_i for the inhibitor binding to "free" enzyme (enzyme that is not bound to an interface) is much greater than the K_i to the surface activated A·E complex.

activator−enzyme complex, A·E). It is to this A·E complex that the water-soluble inhibitor binds (Figure 5B). Binding of $cICH_2P$ to soluble E is much weaker (in Figure 5 this means that K_i(free) $\gg K_i$). While the calculation of a true K_i for the first step is problematic, an apparent $K_i' \sim 2$ mM for $cICH_2P$ could be obtained from the IC_{50} by using an apparent K_m of 1 mM for PI. This latter is the apparent K_m extracted from assays where the PI concentration was varied but the Triton X-100 used to solubilize the mixed micelles was fixed at a ratio of 1:2 PI:Triton X-100 (Zhou et al., 1997). Thus, in this assay system, the apparent K_i' for $cICH_2P$ inhibiting the PI phosphotransferase reaction was significantly lower than the K_i determined for the cIP hydrolysis reaction.

The difference in K_i and K_i' in the PI/Triton X-100 assay system could be explained by cIP hydrolysis (and hence $cICH_2P$ binding) occurring at a site different from the PI binding site on the PI-PLC enzyme. It has been noted that phospholipase D enzymes have both phospholipase and phosphomonoesterase activities and they appear to be functionally and spatially distinct (Friedman et al., 1996). Therefore, an alternate micellar assay system, PI/diC_7PC, was examined for the effect of $cICH_2P$ on PI-PLC cleavage of

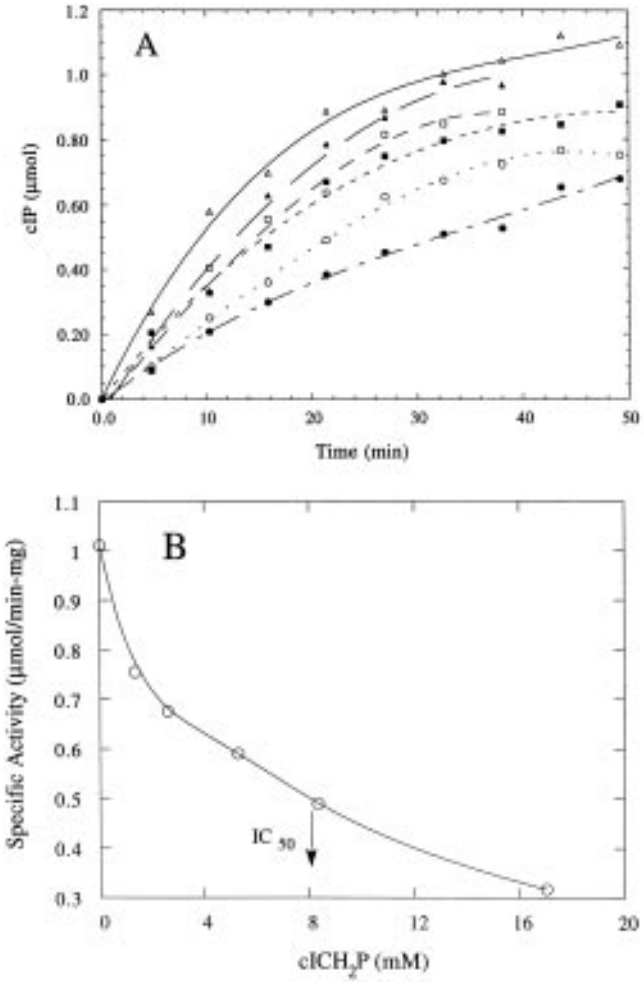

FIGURE 6: (A) Reaction progress curves for PI-PLC hydrolysis of PI in the absence of $cICH_2P$ (△), or the presence of 1.3 mM (▲), 2.6 mM (□), 5.3 mM (■), 8.3 mM (○), or 17.0 mM (●) $cICH_2P$. Reaction conditions included 4 mM PI dispersed in 8 mM Triton X-100 micelles, 50 mM HEPES buffer, pH 7.5, and 58 ng of PI-PLC. The concentration of $cICH_2P$ has been converted to reflect only the D-isomer. (B) Inhibition of PI-PLC phosphotransferase activity by $cICH_2P$. The initial rates for different concentrations of $cICH_2P$ were determined from (A); the IC_{50} value was estimated as 8 mM.

PI to cIP. DiC_7PC was the most "activating" detergent in cIP assays; its presence also diminished the cooperativity in the cIP kinetics. When PI was the substrate it exhibited unusual "surface dilution" behavior (Zhou et al., 1997); the optimum PI-PLC activity was observed at ~1:6 PI/diC_7PC, well above the amount of detergent needed to solubilize the PI. Furthermore, the apparent K_m for PI in that PC detergent mixed micelle system varied with the PI/PC ratio: the value was 6.5 mM at 1:2 and 1.2 mM at 1:8. While this complicates the analysis of PI hydrolysis, it has less effect on assessing the inhibitory potency of $cICH_2P$. As shown in Figure 7, the IC_{50} for $cICH_2P$ inhibition of the PI-PLC phosphotransferase activity was estimated as 15 mM. Assuming competitive inhibition (and an apparent K_m for PI in this micellar assay system of 2 mM) of the reaction, and given conditions that include 1 mM PI and 8 mM diC_7PC, the K_i is estimated as 10 mM. This value is now nearly the same as the K_i estimated for inhibition of cIP hydrolysis by $cICH_2P$ (12.3 mM). Thus, the phosphotransferase and cyclic phosphodiesterase activities do not occur at different sites, and $cICH_2P$ is a competitive inhibitor of PI as well as water-soluble cIP processing by PI-PLC. The kinetic scheme for

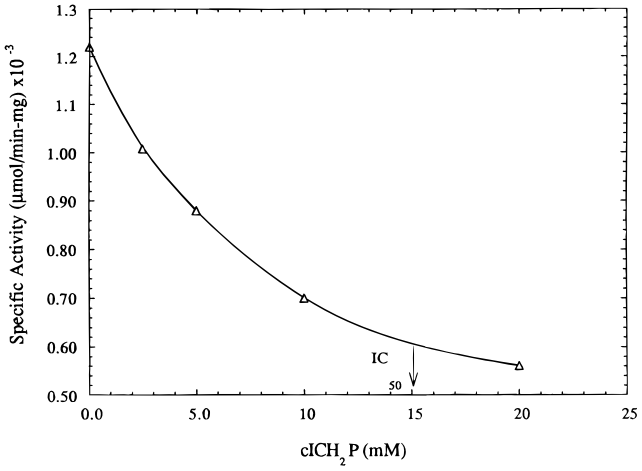

FIGURE 7: Inhibition of PI-PLC phosphotransfersae activity by $cICH_2P$. Reaction conditions include 1 mM PI, 8 mM diC_7PC, 50 mM HEPES, pH 7.5, 15 ng of PI-PLC, and 30 °C. The IC_{50} value was estimated as 15 mM (corresponding to an apparent K_i of 10 mM assuming competitive inhibition).

$cICH_2P$ inhibition of PI-PLC cleavage of PI is summarized in Figure 5B. When diC_7PC is used as the activating detergent, K_i (cyclic phosphodiesterase) = K_i' (phosphotransferase) and the system is amenable to treatment with a simple Michaelis−Menten analysis.

The two different micelle assay systems yield two different K_i values for the water-soluble compound $cICH_2P$ inhibiting PI-PLC conversion of PI to cIP. The PI/diC_7PC micelles exhibit simpler kinetics, with a K_i for $cICH_2P$ that is similar to what was observed with cIP kinetics. This is what would be expected for a water-soluble competitive inhibitor. However, with the PI/Triton X-100 micelles, the estimated K_i for $cICH_2P$ is much lower (~2 mM). Triton X-100 leads to only a 2-fold increase in the V_{max} of PI-PLC toward cIP with little effect on the K_m for cIP or the observed cooperativity. In this assay system, the enzyme is not poised in its most active state and the disparity in K_i values most likely reflects this. At this point, a detailed explanation is not possible. The lesson learned, however, is that assessing a water-soluble inhibitor's potency with an interfacial enzyme requires either a soluble assay system or an interfacial system with the enzyme in a maximally activated form.

An extension of these inhibitor studies to some of the mammalian PI-PLC isozymes may be particularly useful in understanding why those species produce cIP and I-1-P simultaneously from PI hydrolysis. The inhibition potency of $cICH_2P$ coupled with different substrate systems (cIP or PI) and activators (e.g., diC_7PC or Triton X-100) may provide mechanistic insights for these crucial enzymes and their interactions in signal transduction pathways.

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