Are D- and L-chiro-Phosphoinositides Substrates of Phosphatidylinositol-Specific Phospholipase C? 

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ABSTRACT: Derivatives of chiro-inositol have been recently shown to mediate many important biological processes. This work addresses the question of whether phosphatidylinositol-specific phospholipase C (PI-PLC) could be involved in the generation of these chiro-inositol derivatives. Two diastereomers of the analog of phosphatidylinositol containing 1D- and 1L-chiro-inositol have been synthesized. 1D-2-O-(1,2-O-Dipalmitoyl-sn-glycero-3-phospho)-chiro-inositol (1D-chiro-PI) was synthesized in 12 steps starting from 1D-2,3,4,5-O-tetrakis(methoxymethylene)-myo-inositol by the inversion of the hydroxyl group at the 1-position of inositol followed by several protection/deprotection and phosphorylation steps. 1L-2-O-(1,2-O-Dipalmitoyl-sn-glycero-3-phospho)-chiro-inositol (1L-chiro-PI) was synthesized in eight steps starting from 1L-chiro-inositol using regioselective silylation of the hydroxyl group at the 2-position of chiro-inositol in a key synthetic stage. Both diastereomers were subjected to cleavage by PI-PLC from Bacillus thuringiensis. The reaction of 1L-chiro-PI produced chiro-inositol 1,2-cyclic phosphate, however, at the rate of $10^3$ of that attained with the natural substrate, phosphatidylinositol. On the other hand, 1D-chiro-PI was found to be resistant to PI-PLC. These results suggest that the natural chiro-inositol derivatives should have the 1L-configuration if they are produced by PI-PLC, which is in contrast to the 1D-configuration reported by others. We therefore have isolated chiro-inositol from the total bovine liver lipid and determined its absolute configuration. The obtained chiro-inositol was found to be exclusively of the 1L-configuration, with the enantiomeric purity exceeding 99%.

Phosphatidylinositol-specific phospholipase C (PI-PLC) is an important enzyme in cleaving anchors of some membrane proteins and in generation of various inositol derivatives (Deckmyn et al., 1990; Rhee et al., 1989; Rhee & Choi, 1992; Meldrum et al., 1991; Bruzik & Tsai, 1994). The important classes of these inositol derivatives include the various isomers of inositol phosphates, cyclic inositol phosphates, and phosphoinositolglycan insulin mediators. It was a general understanding that all naturally occurring inositol phosphatases have the myo-configuration. However, several recent publications reported the presence of chiro-inositol (Futerman et al., 1985; Low et al., 1987; Larner et al., 1988a,b; Mato et al., 1987; Pak & Larner, 1992; Ostlund et al., 1993) in animal tissues (Figure 1). The phosphoinositolglycan generated in rat liver in response to insulin stimulation (Larner et al., 1988a,b) and by treatment of hepatoma cells with Staphylococcus aureus PI-PLC (Mato et al., 1987) were reported to contain chiro-inositol exclusively or predominantly. The identity of the cyclitol in those chiro-inositol derivatives was determined as 1D-chiro-inositol (Larner et al., 1988a,b). Recently, Larner's group reported the presence of a family of

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5 Abbreviations: GPI, glycosylphosphatidylinositol; IDDM, insulin-dependent diabetes mellitus; MOPS, 3-(morpholino)propanesulfonic acid; NIDDM, non-insulin-dependent diabetes mellitus; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; tR, retention time; SDC, sodium deoxycholate; TBDPS, tert-butyldiphenylsilyl; TFA, trifluoroacetic acid.
and 1L-chiro-inositols were found recently in urine and plasma of healthy human subjects (Ostlund et al., 1993), with the 1D-enantiomer predominating. There is, however, a disagreement over the levels of 1D-chiro-inositol in diabetes. According to the most recent work, the 1p-enantiomer was significantly elevated in NIDDM and IDDM patients (Ostlund et al., 1993). In contrast, an earlier study reported a deficiency of this enantiomer in NIDDM patients (Kennington et al. 1990) and in diabetic animals (Huang et al., 1993; Ortmeier et al., 1993a,b). The concentration of 1D-chiro-inositol in urine and plasma has been proposed as a diagnostic parameter for NIDDM (Kennington & Larner, 1991), and dietary 1D-chiro-inositol has been used to lower blood sugar levels in human and animal subjects (Larner & Kennington, 1991; Larner et al., 1992). A synthetic method for 1D-chiro-inositol has been developed (Kennington et al., 1992). A hypothesis has been put forward that the insulin signal is transduced by the receptor-mediated hydrolysis of GPI by an insulin-dependent GPI-specific PLC (Romero & Larner, 1993; Pak et al., 1992; Romero, 1991; Low & Saltiel, 1987). GPI-specific PLCs have been found in several organisms (Foucquier et al., 1990; Fox et al., 1986, 1987; Stieger et al., 1991; Buelow & Overath, 1986), with GPI-PLC from Trypanosoma brucei being homologous (Hereld et al., 1988; Carrington et al., 1989) to PI-PLCs from bacteria (Kuppe et al. 1989).

Despite the proposed biological importance of the chiro-inositol derivatives and the implicit assumption that they are also generated by PI-PLC or GPI-PLC cleavage of the corresponding phospholipids (Romero & Larner, 1993; Low & Saltiel, 1987), it has never been demonstrated that any of the PI-PLC or GPI-PLC enzymes can accept chiro-phosphatidylinositolides or chiro-glycosylphosphatidylinositolides as substrates. Two main reasons for the paucity in such studies are the difficulty in synthesizing the chiro-inositol substrates and in obtaining some of the enzymes in pure forms. Furthermore, the natural chiro-inositol derivatives were reported to exist in the 1D-configuration (Larner et al., 1988a,b), but our stereochemical analysis predicts that PI-PLC is more likely to accept 1L-chiro-PI for the following reasons: Recent studies on the mechanism of bacterial and mammalian PI-PLC (Volwerk et al., 1990; Leigh et al., 1992; Bruzik et al., 1992; Bruzik & Tsai, 1994) indicated that both types of enzymes have an absolute requirement for the 1D-configuration of myo-PI (D-1, Figure 1) and for the presence of an axially oriented 2-hydroxyl group adjacent to the equatorially oriented phosphatidyl moiety. Such an arrangement enables the nucleophilic attack of the 2-hydroxyl group at the phosphorus atom in D-1, the first step in PI cleavage by both mammalian and bacterial PI-PLC (Bruzik et al., 1992; Volwerk et al., 1990). Analogs of PI with the 1L-configuration of myo-inositol such as L-1 are not substrates for bacterial PI-PLC (Volwerk et al., 1990; Leigh et al., 1992). These results predict that, of the two diastereomers of chiro-PI (L-2 and D-2 in Figure 1), the 1L-isomer is more likely to be a substrate for PI-PLC since the orientation of the phosphatidyl moiety and adjacent hydroxyl groups in L-2 are the same as those of D-1. Notice that the numbering system and D/L designation differ between myo- and chiro-inositols as explained in the legend of Figure 1.

This report describes the first synthesis of both 1D- and 1L-chiro-phosphatidylinositolides in diastereomERICALLY pure forms and their substrate properties toward bacterial PI-PLC as studied by 31P NMR. In the absence of any information regarding the structure of natural chiro-inositol-containing phospholipids, the structure of analogs to be synthesized was selected based on the premise of the highest stereochemical similarity of these analogs to myo-inositol phospholipids. With these considerations the structure of 2-phosphatidy1-chiro-inositol was chosen (L-2 and D-2, Figure 1). The only difference between 2-phosphatidyl-chiro-inositol and 1-phosphatidyl-my0-inositol is the inversion of the hydroxyl group at the 3-position (in D-1). For comparison, in the alternative 3-phosphatidyl-chiro-inositol the phosphatidate and the adjacent hydroxyl groups would be in the unfavorable disquatorial arrangement, while in 1-phosphatidyl-chiro-inositol the required equatorial/axial arrangement of phosphatidate and 2-hydroxyl group, respectively, would require a flip of the inositol ring, placing four hydroxyl groups in an unfavorable axial orientation.

The results indicate that 1L-chiro-PI was accepted by PI-PLC at a reduced rate whereas the 1D-diastereomer was not a substrate. Such a result implied that the naturally occurring chiro-inositol derivatives should have the 1L-configuration if they are derived from phosphatidylinositolides by PI-PLC. This prompted us to isolate and characterize chiro-inositol from bovine liver lipid. The results indicated that the isolated natural chiro-inositol is indeed the 1L-enantiomer.

**MATERIALS AND METHODS**

**Materials.** 1D-2,3,4,5-O-Tetrakis(methoxymethylene)-myo-inositol (3a, [α]D + 3.6°) was obtained as described recently (Bruzik et al., 1992). 1L-Chiro-Insitol was obtained from 1L-(-)-quebrachitol (Aldrich) by its demethylation with 50% hydroiodic acid at 70 °C during 48 h (Tegge & Ballou, 1989). The product was essentially free of other inositol isomers, but contained ca. 10% of unhydrolyzed quebrachitol. It was used for further syntheses without additional purification. 1D-chiro-Insitol [α]pD + 62.4° (c 1.2, H2O) was obtained by acid hydrolysis of kasugamycin (Fluka) as reported (Kennington et al., 1992). Inositol l-phosphate was synthesized as described earlier (Pietrusiewicz et al., 1992). PI-PLC from Bacillus thuringiensis was a generous gift from Dr. T. L. Rosenberry (Case Western University, Cleveland, OH).

**Methods.** NMR spectra were obtained with Bruker 250-, 300-, and 500-MHz AM spectrometers. 1H and 31P NMR chemical shifts were indirectly referenced to tetramethylsilane and those of 31P to 85% H3PO4. The purity of products was assayed by spectroscopic methods (1H, 31C, and 31P NMR) and TLC technique using aluminum-foil-based silica gel plates visualized with phosphomolybic acid solution. Optical rotations were measured using Perkin-Elmer 241 MC spectropolarimeter. HPLC separations were performed using a Hitachi L-6200A system employing D-4500 photo-diode-array detector and Dionex PAD-2 electrochemical detector.

**Synthesis of 1D- and 1L-2-O-(1,2-O-Dipalmitoyl-sn-glycero-3-phospho)-chiro-inositol (D-2 and L-2, Respectively)**

1D-1-O-Benzoyl-2,3,4,5-O-tetakis(methoxymethylene)-myo-inositol (4a). 1.6-Diol 3a (520 mg, 1.46 mmol) in pyridine (4 mL) was treated with benzoic chloride (186 μL, 10% excess) at ~30 °C, and the progress of the reaction was monitored by TLC (hexane-acetone, 3:1). The mixture was kept at 4 °C for 12 h, and then water (10 mL) and ethyl acetate (20 mL) were added. The organic phase was separated, washed twice with water (10 mL) and concentrated. Crude product was chromatographed on silica gel (hexane-acetone, 10:1) to give pure 4a (540 mg, 80%). A small amount of the 1,6-dibenzoyl derivative (4%) was also recovered. 4a: TLC Rf 0.18 (hexane-
acetonitrile (5% each). The mixture was slowly warmed up to room temperature. The progress of the reaction was monitored by TLC (hexane-acetone, 3:1, product Rf 0.22). The ratio of the isomers was ca. 1:1 as determined by 1H NMR. The mixture was concentrated and chromatographed on silica gel (Sigma, 10-40 μm), giving 4a (240 mg, eluted off earlier) and 5a (248 mg). The same 1:1 mixture of 4a and 5a was also formed when pure 5a underwent isomerization under analogous conditions. The recovered isomer 4a was recycled to give a total yield of 66% after two cycles. 5a: 1H NMR (CDCl3) δ 8.1 (2H), 7.5 (1H), 7.4 (2H), (each m, Ph), 5.41 (tr, H-6, J 9.9 Hz, 1H), 4.82-4.63 (m, OCH2O, 8H), 4.02 (tr, H-2, J 2.3 Hz, 1H), 4.00 (tr, H-4, J 9.7 Hz, 1H), 3.87 (d, 1H), 3.63 (tr, J 9.5 Hz, 1H), 3.52 (m, 2H), 3.52, 3.38, 3.35, 3.05 (each s, Me, 3H); 13C NMR (CDCl3) δ 166.7 (C=O), 132.8, 130.0, 129.6, 128.1 (Ph), 98.5, 98.3, 98.0, 96.6 (OCH2O), 81.4, 78.5, 78.0, 76.9, 74.3, 70.2 (CHO), 56.3, 56.2, 55.9, 55.5 (OMe). 1d-6-O-Benzoyl-2,3,4,5-O-tetrakis(methoxymethylene)-myo-inositol (5a). The foregoing 1-benzoate 4a was isomerized to 6-benzoate 5a as follows: 4a (540 mg) was dissolved in THF (3 mL) and 0.1 mL of 1.0 M tetra-n-butylammonium fluoride was added. The mixture was left at room temperature for 12 h. TLC (hexane-acetone, 3:1) indicated the presence of another product (Rf 0.20) in addition to the substrate (Rf 0.22). Cleavage of chiro-Phosphoinositides by PI-PLC 4a was recycled to give a total yield of 66% after two cycles. 3a: 1H NMR (CDCl3) δ 5.90 (2H), 5.83 (1H), 5.74 (2H), (each m, Ph), 5.87 (tr, H-6, J 9.9 Hz, 1H), 4.9-4.6 (m, OCH2O, 8H), 4.15 (dd, H-1, J 9.6 Hz, 1H), 4.09 (brt, H-6, J 9.9 Hz, 1H), 4.00 (tr, H-4, J 9.5 Hz, 1H), 3.66 (dd, H-3, J 2.3, 10 Hz, 1H), 3.38, 3.41, 3.38 (each s, Me, 3H); 13C NMR (CDCl3) δ 165.3 (C=O), 133.5, 129.7, 129.2, 128.5 (Ph), 98.4, 98.2, 98.0, 97.4 (OCH2O), 77.6, 77.4, 77.1, 76.7, 76.6, 73.9 (CHO), 56.39, 56.35, 56.2, 55.8 (OMe). 1d-2-O-Benzoyl-1,3,4,5,6-O-pentakis(methoxymethylene)-chiro-inositol (9a). The foregoing nitrate 7a was subjected to hydrolysis in THF over 10% Pd/C at ambient hydrogen pressure and room temperature during 12 h. The progress of the reaction was monitored by TLC (hexane-acetone, 3:1, product Rf 0.2). The catalyst was filtered off and the filtrate was concentrated to give crude alcohol 8a. This product was treated with MOM-Cl (0.1 mL) and diisopropylethylamine (0.3 mL) in DMF (1 mL) at room temperature during 12 h followed by heating at 70 °C during 5 h (TLC, hexane-acetone, 3:1, product Rf 0.31). Silica gel chromatography (hexane-acetone, 10:1) afforded pure 9a (78 mg, 90% in two steps). 9a: 1H NMR (CDCl3) δ 8.27 (m, Ph), 7.03 (m, Ph), 5.87 (dd, H-2, 5.3, 9.6 Hz, 1H), 4.91 (m, 3H), 4.78 (d, J 6.6 Hz, 1H), 4.71 (d, J 6.6 Hz, 2H), 4.58 (d, J 6.7 Hz, 1H), 4.39 (m, 5H), 3.31 (s, Me, 6H), 3.17, 3.10, 3.06 (each s, Me, 3H). 1d-1,3,4,5,6-O-Pentakis(methoxymethylene)-chiro-inositol (10a). The foregoing benzoate 9a was cleaved by sodium hydroxide in MeOH (3%, 1 mL). Silica gel chromatography (hexane-acetone, 3:1, Rf 0.22) afforded pure alcohol 10a (47 mg, 75%). 10a: 1H NMR (CDCl3) δ 7.84-7.40 (m, OCH2O, 10H), 3.97 (dd, H-6, J 3.0, 4.1 Hz, 1H), 3.90 (dd, H-1, J 3.4, 4.2, 1.3H), 3.82 (dd, H-5, J 2.8, 10.0 Hz, 1H), 3.74 (tr, H-4, J 10.5 Hz, 1H), 3.77 (dd, H-2, 1H), 3.49 (tr, H-3, J 9.5 Hz, 1H), 3.38, 3.35, 3.33, 3.32, 3.30 (each s, Me, 3H), 2.93 (d, J 4.9 Hz, 1H); 13C NMR (CDCl3) δ 98.56, 98.23, 98.20, 97.30, 96.76 (OCH2O), 83.67, 78.0, 77.3, 76.2, 75.8, 69.9 (CHO), 56.0, 55.9, 55.8, 55.7, 55.5 (OMe). 1d-2-O-[(1,2-O-Dipalmitoyl-sn-glycero-3-phospho)-chiro-inositol (D-2). The alcohol 10a (45 mg, 112 μmol) and diisopropylethylamine (39 μL, 2-fold excess) in chloroform (0.5 mL) were treated with methyl N,N-diisopropylphosphoramidochloridite (26.5 mg, 20% excess) at room temperature. The progress of the reaction was followed by TLC (hexane-acetone, 3:1, substrate Rf 0.22, product Rf 0.41). After the phosphitylation had been completed, the mixture was evaporated to dryness and added with tetrazole (32 mg, 4-fold excess) and 1,2-dipalmitoyl-sn-glycerol (92 mg, 20% excess). The whole mixture was dissolved in THF–acetonitrile (1:1, 0.5 mL). The new product with an Rf of 0.5 (same solvent system as above) was formed within 0.5 h. The reaction mixture was treated with m-chloroperoxybenzoic acid (42 mg, 60%, 10% excess) at −30 °C and warmed up to room temperature within 0.5 h. The crude product (Rf 0.15) was purified by chromatography (hexane–acetone, 10:1) to give the pure phosphate triester 11a (55 mg, 46%, δF=−1.4 ppm, CDCl3). The above product 11a was dissolved in neat trimethylamine at −10 °C in a heavy-walled tightly closed screw-cap vial and heated at 50 °C during 21 h. TLC after this time showed complete demethylation. Trimethylamine was evaporated and the residue was treated with neat ethanol (1 mL) and boron trifluoride–ether (30 μL) during 30 min at room temperature. After this time TLC (chloroform–methanol–ammonia, 19:3:0.1) product Rf 0.26 showed a complete deprotection. The mixture was concentrated and the product was purified by chromatography (chloroform–methanol–ammonia, 70:30:0.6) to give pure D-2 (30 mg, 71%). D-2: [α]D+0.6 ± 0.2° (c 0.7, methanol–chloroform, 1:1); 1H NMR (CD3OD–CDCl3, 5:1) δ 5.23 (m, H-2′, 1H), 4.44 (dd, H-1′, 5.3, 12.0 Hz, 1H), 4.24 (dtr, H-2′, J 3.1, 9.1, 9.1 Hz, 1H), 4.18 (dd, H-1′B, J 6.8, 12.0 Hz, 1H), 4.13 (tr, H-1′, J 3.5 Hz, 1H), 4.04 (m, H-3′-AB, 2H), 3.93 (tr, H-6′ (1), J 3.3 Hz, 1H), 3.72 (tr, H-3, J 9.4 Hz, 1H), 3.68 (dd, H-5, J 3.1, 9.4 Hz, 1H), 3.59 (tr, H-4, J 9.6 Hz, 1H), 2.32, 2.29
(each tr, CH₃C=O, J 7.4 Hz, each 2H), 1.59 (m, 4H), 1.26 (brs, 48H), 0.87 (tr, 6H); 31P NMR (CD₂OD) δ 1.08 ppm.

1L-2-O-(tert-Butyldiphenylsilyl)-chiro-inositol (12b). 1L-chiro-Inositol (50 mg) containing ca. 10% (−)-quebrachitol and imidazole (30 mg) in DMF (1 mL) was treated with tert-butyldiphenylsilyl chloride at −10 °C and kept at room temperature during 2 h. DMF was evaporated under vacuum and the residue was chromatographed (chloroform–methanol, 4:1). The fraction containing silylated quebrachitol (Rₛ 0.58) was eluted off first and was followed by the main fraction of 2-TBDPS-chiro-inositol (12b, Rₛ 0.5, 30 mg, 26%).

The extension of the reaction time to 24 h resulted in the formation of a significant amount of another product with a slightly lower mobility (most likely 3-TBDPS-chiro-inositol). 12b: 1H NMR (DMSO-d₆) δ 7.75 (4H, Ph), 7.35 (6H, Ph), 3.87 (dd, H-2 (5), J 2.1, 9.6 Hz, 1H), 3.58 (m, 3H), 3.49 (dd, H-5(2), J 2.1, 9.6 Hz, 1H), 3.2 (tr, H-3 (4), J 9.2 Hz, 1H), 1.00 (s, Me, 9H); 13C NMR (DMSO-d₆) δ 136.0, 135.7, 135.0, 134.2, 129.47, 129.43, 127.5, 127.4 (Ph), 73.6, 73.1, 72.34, 72.26, 70.6 (CHO), 27.1 (C-Me), 19.4 (C₆).

1L-1,3,4,5,6-O-Pentakis(methoxymethylene)-chiro-inositol (10b). 2-TBDPS-chiro-inositol (12b, 140 mg, 0.33 mmol) and ethyldisopropylamine (0.5 mL) in DMF (1 mL) were treated with methoxymethylene chloride (0.25 mL) at room temperature for 12 h and for 3 h at 60 °C (product Rₛ 0.4, hexane-acetone, 3:1). The mixture was diluted with ethyl acetate and extracted three times with water and the organic phase was concentrated. The residue (204 mg) was rendered anhydrous by evaporation with dry dioxane. The above product was solubilized in a 1.0 M solution of tetra-n-butylammonium fluoride in THF (0.5 mL) and the mixture was heated at 55 °C during 3 h. The progress of desilylation was checked by TLC (product Rₛ 0.15). Chromatography on silica gel (hexane-acetone, 3:1) afforded pure 10b (78 mg, 59% in two steps). This product gave essentially the same proton spectrum as that of 10a, except that it contained 15% of the unidentified impurity. This product was used for further synthetic steps without additional purification.

1L-2-O(1,2-O-Dipalmitoyl-sn-glycero-3-phospho)-chiro-inositol (L-2). Phosphitylation of alcohol 10b was carried out essentially identically as described above for 10a. Starting from 75 mg of 10b 57 mg of 1L-chiro-PI (L-2) was obtained (38% in five steps). L-2. 31P NMR (CD₂OD-DCCl₃, 5:1) δ 11.11 ppm; [α]D = −12.7° (c 0.6, methanol–chloroform, 1:1). Despite the fact that L-2 and D-2 are not enantiomers (they both contain L-α-glycerol residue) the 1H NMR spectrum of L-2 was essentially identical to that of D-2.

Reactions of chiro-Phosphatidylinositols with PI-PLC from B. thuringiensis

(A). 1D-chiro-PI (D-2, 2.4 mg, 3 μmol) was dispersed in MOPS–Na buffer (0.4 mL, 50 mM, pH 7.0) containing 10 mM SDC and 25% D₂O. This sample was treated with 3.5 μg of PI-PLC and the reaction was monitored by 31P NMR. Only a single signal at −0.2 ppm due to the substrate was observed after 3 weeks at room temperature. To verify that PI-PLC was still active after such a long incubation time, synthetic dipalmitoylphosphatidylymyo-inositol (1 mg) was added. A rapid conversion of myo-PI to IpC (δₑ₃P 16 ppm) was observed within the time necessary to acquire the first 31P NMR spectrum (5 min.).

(B). 1L-chiro-PI (L-2, 2.7 mg, 3.3 μmol) was treated with PI-PLC as above. The conversion of the substrate (δ₀₃P −0.2 ppm) into the product (δₐ₃P 15.6 ppm) was observed and was complete within 10 days.

1L-chiro-Inositol 1,2-Cyclic Phosphate (L-14). 1L-chiro-PI (L-2, 4.2 mg, 5 μmol) and SDC (5 mg) were dispersed in water (0.5 mL containing 20% D₂O). The dispersion was treated with PI-PLC (10 μg) and the reaction was monitored by 31P NMR. After most PI had been used up (60 h) the mixture was passed through a C-18 cartridge and further chromatographed on Dowex 1X8-200 (ammonium form) using a step gradient of ammonium carbonate. The fraction eluted at 50 mM buffer was evaporated under vacuum to give pure L-14. 1H NMR (D₂O) δ 4.67 (dd, H-1, J 3.5, 5.2 Hz, 1H), 4.51 (ddd, H-2, J 5.3, 8.3, 13.6 Hz, 1H), 4.37 (brt, H-6, J 3.2 Hz, 1H), 1.89 (dd, H-3, J 8.4, 9.7 Hz, 1H), 3.86 (dd, H-5, J 3.1, 9.2 Hz, 1H), 3.74 (tr, H-4, J 9.5 Hz, 1H); 31P NMR (D₂O) δ 15.7 ppm.

Determination of Configuration of Naturally Occurring chiro-Inositol

Isolation and Determination of the Content of chiro-Inositol in the Total Lipid of Bovine Liver. Bovine liver purchased from a local store (330 g) was homogenized in ice-cold 5% TFA (1 L) using a Waring blender. The homogenate was centrifuged at 7000g, and the pellet was rehomogenized in chloroform–methanol (1:2, 600 mL). Supernatants were pooled and extracted three times with chloroform–0.5 M KCl (1:1, 200 mL). Organic phases were collected, washed twice with 0.5 M KCl (100 mL), and concentrated to give dark brown thick oil (9 g). The foregoing lipid extract (1 g) was solubilized in dioxane and 3% methanolic KOH (10 mL) and stored for 0.5 h. TLC (chloroform–methanol–water, 65:35:4) indicated that all phospholipids have been saponified. The mixture was adjusted to pH 1.5 with HCl and extracted five times with chloroform–methanol (2:1). The organic phase was discarded and the aqueous phase was concentrated to dryness. 31P NMR at this point indicated the presence of seven major signals in the range −1.2 to 1.0 ppm. The residue was redissolved in 6 N HCl (5 mL) and refluxed during 24 h. 31P NMR showed only one signal of inorganic phosphate, indicating the completion of hydrolysis (at 12 h 33% of monoesters was still present). The mixture was concentrated to dryness and the residue redissolved in water, and passed through the cation-exchange column (H⁺ form, Aldrich, Dowex 50X8, 1 cm × 12 cm) and the anion-exchange column (OH⁻ form, Bio-Rad, AG 1X8, 100–200 mesh, 1 cm × 12 cm). The final eluate was concentrated by evaporation. The oily residue contained >95% glycerol and the remainder was inositol. Glycerol was removed by distillation at 100°C under high vacuum to give the product which contained chiefly myo- and chiro-inositols. The content of chiro-inositol was determined by integration of its H-1/H-6 proton signals at 4.10 ppm and H-2 proton signals of myo-inositol at 4.13 ppm. These measurements gave the value of 8%, consistent with determination of the perbenzoylated derivatives (see below).

Hexa-O-benzoylinositols. General Procedure. The suspension of inositol in dry pyridine was treated with excess of benzoyl chloride at 75°C during 3 h. The product was purified by chromatography on silica gel (hexane–acetone, 3:1) and subsequent crystallization from acetone–hexane.

Hexa-O-benzoyl-myo-inositol: mp 265 °C (hexane–acetone, 3:1); 1H NMR (CDCl₃) δ 8.17 (m, 2H), 7.9 (m, 10 H), 7.7 (m, 1H), 7.6 (m, 2H), 7.45 (m, 5H), 7.3 (m, 10H), 6.38 (tr, H-4, H-6, J 10.2 Hz, 2H), 6.34 (tr, H-2, J 2.9 Hz, 1H), 6.06 (tr, H-5, J 10.2 Hz, 1H), 5.85 (dd, H-1, H-3, J 10.5 Hz, 3.0 Hz, 2H).

Hexa-O-benzoyl-1L-chiro-inositol was obtained starting from 1L-quebrachitol: mp 243–245 °C; [α]D = −68°, [α]D = 36
Cleavage of chiro-Phosphoinositides by PI-PLC

RESULTS

Synthesis of 1D-chiro-Phosphatidylinositol (D-2). Our recent synthetic study (Bruzik & Tsai, 1992) generated a number of intermediates useful toward synthesis of phosphoinositides including 1D-2,3,4,5-O-tetraakis(methoxymethylene)-myo-inositol (3a, Scheme 1). The conversion of this precursor into the starting material (10a) for the synthesis of chiro-PI necessitated only an inversion and a subsequent protection of the hydroxyl group at the 1-position of myo-inositol. Our synthetic procedure is shown in Scheme 1. The 1,6-diol 3a was benzoylated regioselectively at the 1-OH group and the resulting 6-alcohol 4a was subjected to base-catalyzed migration of the benzoyl group between 1- and 6-hydroxyl groups to give the derivative 5a. The 1-alcohol 5a was further treated with trifluoromethanesulfonic anhydride and the resulting sulfonylester 6a was reacted with tetra-n-butylammonium nitrate to afford the corresponding 1-nitro derivative of 1D-chiro-inositol (7a) (Tagliaferri et al., 1990). The subsequent hydrogenolysis of the nitro group and methoxymethylation of the resulting 1-hydroxyl group followed by saponification of the 2-benzoyl group afforded the corresponding 2-alcohol (10a, note the numbering of the chiro-inositol). The alcohol 10a was phosphorylated (Bruzik et al., 1992) to give a 1:1 mixture of the diastereomeric phosphotriesters (11a, Scheme 2). Demethylation of the phosphotriesters 11a with trimethylamine and the subsequent exhaustive deprotection of the inositol moiety with ethanethiol/BF3 completed the synthesis, affording 1D-chiro-PI (D-2). The 1L-isomer of chiro-PI (L-2) can be also obtained in an analogous way starting from 1D-1,2,5,6-tetrakis(methoxymethylene)-myo-inositol (enantomer of 3a). Since this derivative is less readily accessible than 3a, an alternative synthesis was devised as described in the next section.

Synthesis of 1L-chiro-Phosphatidylinositol (L-2). As shown in Scheme 3, 1L-chiro-inositol obtained from 1L-(-)-quebrachitol (Tegge & Ballou, 1989) was treated with tert-butylidiphenylsilyl (TBDPS) chloride/imidazole in DMF to afford regioselectively 1-TBDPS-chiro-inositol (12b) in 26% yield. This derivative was treated with methoxymethylene chloride (MOM-Cl) to give the fully protected derivative 13b. Further removal of the silyl group in 13b with tetra-n-butylammonium fluoride provided 2-alcohol 10b. Alcohol 10b was then converted into 1L-chiro-PI (L-2) analogously as described for the conversion of 10a into D-2. Despite the low yield obtained in the first silylation step, this scheme is superior to the synthesis used in the D-series due to the small number of steps needed to obtain PI-precursor 10b. The alcohol 10a could also be prepared in the analogous way to 10b starting from 1D-(-)-pinitol.
Activity of PI-PLC toward 1D- and 1L-chiro-Phosphatidylcholines. Both diastereomers of chiro-PI (D-2 and L-2) were subjected to the action of PI-PLC from B. thuringiensis. The 1L-isomer L-2 was cleaved by PI-PLC at a low rate to give 1L-chiro-inositol 1,2-cyclic phosphate (Figure 2). The product of the cleavage has been identified as a cyclic phosphate by its $^{31}$P NMR spectrum showing a signal at 15.6 ppm characteristic of five-membered ring phosphates and $^1$H NMR spectrum showing a large vicinal coupling constant of phosphorus to H-2 (19 Hz). The above values are very close to those of myo-inositol 1,2-cyclic phosphate (Cerdán et al. 1986). The rate of the cleavage of L-2 is 3 orders of magnitude slower than that of 1D-myo-PI (D-1). The 1D-isomer D-2 was found resistant to PI-PLC, even when treated with a large amount of enzyme and employing long incubation times. We estimate that the activity of PI-PLC toward 1D-chiro-PI under our assay conditions is <1 nmol/mg-min, or <10$^{-4}$ of that with 1D-myo-PI.

Although our result concerns only one type of bacterial PI-PLC and one type of chiro-phosphatidylcholine substrate, the stereochemical properties of enzymes are usually conserved among the same class of enzymes. For example, all known types of PI-PLC cleave substrate with the 1D-configuration of myo-inositol. Our results therefore imply that if the natural chiro-inositol-containing insulin mediators are derived from PI-PLC-catalyzed reactions, they should have the 1L-instead of the 1D-configuration. Such a possibility prompted us to isolate and characterize naturally occurring chiro-inositol as described in the following section.

Isolation of chiro-Inositol from Bovine Liver Lipid. The total liver lipid was hydrolyzed with sodium hydroxide in methanol and the resulting water-soluble phosphodiester compounds were further hydrolyzed in refluxing 6 N HCl. The hydrolysate was deionized to remove inorganic phosphate and then was evaporated under high vacuum to remove glycerol. The resulting mixture of myo- and chiro-inositol was subjected to exhaustive benzoylation to give a mixture of hexabenzoyl-myo- and chiro-inositols. The composition of this mixture was determined by: (i) the $^1$H NMR spectra of crude inositols and of the mixture of hexabenzoylated derivatives and (ii) HPLC of the mixture of hexabenzoyl derivatives using silica gel column with hexane–isopropyl alcohol (98:2) elution and UV detection at 254 nm. The elution profiles are shown in Figure 3, parts A–C. The composition of the inositol mixture was found to be 91.5 ± 0.5% myo-inositol and 8.5 ± 0.5% chiro-inositol by both NMR and HPLC methods.

**Figure 2**: Time courses of PI-PLC-catalyzed reaction with 1L- and 1D-2-phosphatidyl-chiro-inositol (L-2 and D-2, respectively) as monitored by $^{31}$P NMR. Dispersion of L-2 (7.5 mM) in SDC (10 mM, pH 7.0) was treated with PI-PLC (3.5 mg) and $^{31}$P NMR spectra were acquired after 1 h (A), 18 h (B), 100 h (C), 10 days (D). Spectrum E was obtained with analogously treated sample of D-2 after 3 weeks. Identities of peaks at ~0.2 and 15.6 ppm are chiro-PI and chiro-1cP, respectively.
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FIGURE 3: Determination of the content and configuration of chiro-inositol from bovine liver lipid by HPLC. Conditions: (A-C) HPLC on Microisorb-MV, 4.6 x 250 mm, detection at 254 nm, isocratic elution with hexane-isopropyl alcohol (98:2, v/v), flow rate 1 mL/min; (D-G) HPLC on the polymethacrylate CHIRALPAK OT (+) column, 4.6 x 250 mm, detection at 230 nm, flow rate 0.6 mL/min, isocratic elution with methanol-hexane-isopropyl alcohol (50:25:25, v/v) at 10 °C. Samples: (A) 1D-hexabenzyol-chiro-inositol, retention time (tR) 31.4 min.; (B) hexabenzyol-myo-inositol, tR 12.5 min.; (C) hexabenzyolinositols from bovine liver lipid, tR 11.4 and 12.7 min.; (D) 1L-hexabenzyol-chiro-inositol, tR 31.8 min.; (E) 1D-hexabenzyol-chiro-inositol, tR 25.4 min.; (F) the mixture of 1D (60%) and 1L (40%) enantiomers, tR 25.3 and 32.0 min.; (G) Hexabenzyol-chiro-inositol from bovine liver, tR 32.8 min.

Since hexabenzyol-myo-inositol is optically inactive, [α]D20 should be -106° after correcting for 91.5% myo-derivative. However, the quantitative polarimetric determination of the enantiomeric purity of chiro-inositol was difficult due to a large error of such measurement at a low concentration. We therefore developed an HPLC method for quantitative analysis of the chiro-inositol derivatives (for an alternative method see Ostlund et al., 1993). The benzoylated chiro- and myo-derivatives were preparatively separated by HPLC on silica gel (Figure 3C). The isolated hexabenzyol-chiro-inositol along with the synthetic standards were subjected to analysis on the chiral HPLC column [CHIRALPAK OT (+), Chiral Technologies, Inc.] with a helical polymethacrylate phase. This column was found to distinguish between individual enantiomers of hexabenzyol-chiro-inositol as shown in Figure 3 (traces D-F). The elution profile G shows very clearly that the chiro-inositol from bovine liver is entirely of 1L-configuration.

The possibility of epimerization of myo-inositol into chiro-inositol during the acidic hydrolysis of inositol phosphates was excluded by a control experiment in which samples of myo-inositol and synthetic myo-inositol 1-phosphate were separately refluxed in 6 N HCl and 50% TFA over 48 h. The final hydrolysates contained only myo-inositol (>99%) as indicated by their 1H NMR spectra.

DISCUSSION

The results presented in this paper are significant in several aspects: (a) 1D- and 1L-chiro-PI have been synthesized in the diastereomerically pure forms (D and L represent the configurations of the inositol moiety: the diacylglycerol moiety is 1-α in both cases). The configuration of D-2 is assigned as 1D based on the reaction sequence starting with a known precursor (Bruzik & Tsai, 1992), and that of L-2 is assigned as 1L based on the configuration of the starting material 1L-(−)-quebrachitol assigned by comparison with 1D- (+)-chiro-inositol (Posternak, 1936). These compounds will be useful for substrate-specificity studies of other PI-related enzymes. (b) The PI-PLC from B. thuringiensis has been shown to cleave 1L-chiro-PI at a substantially reduced rate, 10−3 relative to the natural substrate, 1D-myo-PI. 1L-chiro-PI and 1D-myo-PI differ only in the configuration at carbon 3 (in the numbering system of 1D-myo-PI, see Figure 1) of the inositol ring. This result indicates that the 3-OH group of 1D-myo-PI is important in PI-PLC-substrate interactions. (c) In spite of the low activity of 1L-chiro-PI, the result suggests that it is possible for PI-PLC to produce 1L-chiro-inositol derivatives. It further implies that the insulin signal may be mediated by GPI-PLC homologous to known PI-PLC. On the other hand, PI-PLC showed no activity at all toward the 1D-diastereomer (<10−6 relative to 1D-myo-PI). In terms of stereochemistry, this result is consistent with the earlier finding that PI-PLC is specific to 1D-myo-PI and does not hydrolyze 1L-myo-PI (Volwerk et al., 1990; Leigh et al., 1992). Although our results on chiro-PI are limited to one specific type of PI-PLC, they imply that, if PI-PLC or GPI-PLC is indeed responsible for the generation of chiro-inositol-containing insulin mediators, the chiro-inositol should have the 1L- instead of the 1D-configuration. (d) We then proceeded to isolate inositols from bovine liver according to published procedures and showed that the chiro-inositol, which constitutes 8.5% of total inositol, indeed has the 1L-configuration, with an enantiomeric purity of >99%. Again, this experiment was performed only for the lipid from a single source and is insufficient to conclude whether earlier reports are in error. However, our results suggest that the authors of earlier reports should re-evaluate their findings.

The finding of the 1L-configuration of naturally occurring chiro-inositol phospholipids is significant in the context of the latest finding of the occurrence of both enantiomers of chiro-inositol in human biological fluids (Ostlund et al., 1993). Our result suggests that only 1L-chiro-inositol is incorporated into a phospholipid pool of bovine liver. This result is also consistent with stereochemical similarity between 1D-myo- and 1L-chiro-phosphatidylinositols as explained in Figure 1. As shown in this work, PI-PLC, which requires the 1D-configuration of myo-phosphatidylinositols, can also cleave 1L-chiro-phosphatidylinositol. This result implies that 1L-chiro-inositol can be metabolized by enzymes known to accept myo-inositol. For example, phosphatidylinositol synthase from mouse brain, which selects the hydroxyl group at the 1D-position of myo-inositol to produce phosphatidylinositols, accepts small structural variations of myo-inositol at the 3-position (Johnson et al., 1993). It remains to be seen whether it would accept inositol analogs with an inverted 3-hydroxyl group such as in 1L-chiro-inositol. Incorporation of 1D-chiro-inositol into phospholipids and their further degradation would most likely require existence of an entirely different enzymatic system with a reversed stereospecificity.

REFERENCES


