Abstract  Phosphatidylinositol (PI) is the precursor of many important signaling molecules in eukaryotic cells and, most probably, PI also has important functions in cellular membranes. However, these functions are poorly understood, which is largely due to that i) only few PI species with specific acyl chains are available commercially and ii) there are no simple methods to synthesize such species. Here, we present a simple biochemical protocol to synthesize a variety of labeled or unlabeled PI species from commercially available phosphatidylcholines. The protocol can be carried out in a single vial in a two-step process which employs three enzymatic reactions mediated by i) commercial phospholipase D from Streptomyces chromofuscus, ii) CDP-diacylglycerol synthase overexpressed in E. coli and iii) PI synthase of Arabidopsis thaliana ectopically expressed in E. coli. The PI product is readily purified from the reaction mixture by liquid chromatography since E. coli does not contain endogenous PI or other coeluting lipids. The method allows one to synthesize and purify labeled or unlabeled PI species in 1 or 2 days. Typically, 40–60% of (unsaturated) PC was converted to PI albeit the final yield of PI was less (25–35%) due to losses upon purification.

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Phosphatidylinositol (PI) is a crucial component of eukaryote membranes, because it is the precursor of many important signaling molecules, such as diacylglyceride activating protein kinase C, inositol-1,3,5-triphosphate releasing Ca$^{2+}$ from the endoplasmic reticulum lumen, and polyphosphoinositides regulating membrane trafficking and protein-membrane interactions (1). Yet, PI is a precursor of glycosylated PI derivatives anchoring certain proteins to cellular membranes and, most probably, has other important functions in eukaryote membranes as well. However, the relevant biochemical/biophysical studies addressing these functions have not been carried out yet, probably because only a few (and costly) PI species with definite acyl chains have been commercially available, as well as due to lack of simple methods for their synthesis. This is in notable contrast to other glycerophospholipids (GPLs), which can be easily prepared from commercially available phosphatidylcholines (PCs) by using phospholipase D (PLD)-mediated transphosphatidylation providing, e.g., phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol (PG), or hydrolysis yielding phosphatic acid (PA) (2). Commercially available PLDs cannot be used to produce PI because they do not use inositol as the acceptor of the phosphatidyl group of PC. Although mutant PLDs have been generated that do produce PI from PC, multiple isomers were generated because any of the six hydroxyl groups of inositol can accept the phosphatidyl moiety (3) and the natural isomer cannot be readily isolated from the mixture of isomers. Later studies have described more selective PLD mutants, but still a significant amount of an unnatural PI isomer was formed (4–6).

Several chemical methods to synthesize PI have also been published (7–13). However, those methods have been rarely employed, most probably because they require considerable expertise in organic chemistry (e.g., 10, 12), only saturated PI species can be prepared (13–15), or because the yield of PI is low and/or variable (see 14).

Here, we describe a simple biochemical method that allows preparation of unlabeled or labeled PI molecular species from commercial PCs by employing three sequentially acting enzymes. The whole synthesis can be carried out in a single vial and can be completed within a day.

Supplementary key words  phospholipids/biosynthesis • enzymology • molecular biology • methods/HPLC • mass spectrometry

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MATERIALS AND METHODS

Reagents and chemicals

Luria-Bertani medium, chloramphenicol, and kanamycin were obtained from HUSLAB Laboratory (Helsinki, Finland), DH1/pCD100 strain of Escherichia coli overexpressing CDP-diacylglycerol synthase (Cds) was from E. coli Genetic Stock Center (New Haven, CT), and C43/pET28-PI S1 expressing phosphatidylinositol synthase 1 (PI S1) was kindly provided by Dr. Ingo Heilmann. Butyryl hydroxyozone (BHT), isopropyl β-D-thiogalactoside, Triton X-100, Streptomyces chromofuscus PLD, myo-inositol, 2-mercaptoethanol, formic acid, ammonium formate, and ammonium hydroxide were purchased from Sigma-Aldrich, glycerol from VWR Chemicals (Helsinki, Finland), and DC™ protein assay reagents from Bio-Rad Laboratories (Vantaa, Finland). Unlabeled PC species were purchased from Avanti Polar Lipids (Alabaster, AL) and 16:0/Pytherol was synthesized as previously (16). D6-myoinositol was from CDN Isotopes (Quebec, Canada), silica gel 60 TLC plates from Merck-Millipore (Darmstadt, Germany), and LC-MS grade solvents from Fisher Scientific (Vantaa, Finland).

Preparation of E. coli membranes containing Cds or PIS1

E. coli strain DH1/pCD100 overexpressing Cds (17) was grown as described (18). Briefly, cells were grown in Luria-Bertani medium (3 l) containing 30 µg/ml kanamycin at 37°C as described (18). When OD600 reached 0.8, the expression of the enzyme was induced by isopropyl β-D-thiogalactoside (1 mM), and cells were grown for 4 h. The cells were collected by centrifugation for 10 min at 4,500 g at 4°C. The DH1/pCD100 cell pellets were resuspended in a 100 mM Tris-HCl buffer (pH 7.5) containing 8% glycerol, while the C43/pET28-PI S1 cell pellets were suspended in 50 mM Tris-HCl (pH 8.0) containing 8% glycerol and 8 mM 2-mercaptoethanol. The cells were then disrupted by a single 5 min sonication (70% duty cycle) on ice with a Branson 450 Sonifer equipped with a 3.2 mm tip. The remaining intact cells were collected and resonicated as above and the combined sonicates were centrifuged (100,000 × g) for 1 h at 4°C to pellet the membranes. The DH1/pCD100 membranes were suspended in 100 mM Tris-HCl, pH 7.5, and the C43/pET28-PI S1 membranes in 20 mM Tris-HCl, pH 8.0. Both buffers contained 8 mM 2-mercaptoethanol. After determination of the protein concentration (20), the membrane suspensions were diluted in aliquots and stored at −80°C. Both Cds and PIS1 maintained their activity for several months under these conditions. The yield of membrane protein was 104 ± 15 and 79 ± 20 mg for the PIS1 and Cds preparations (n = 5), respectively. In a typical preparation, the specific activity of Cds with di-18:1-PA as the substrate was 6.02 ± 1.12 nmol·min⁻¹·mg⁻¹ (n = 5) and that of PIS1 with di-18:1-CDP-DAG as the substrate was 3.47 ± 0.54 nmol·min⁻¹·mg⁻¹ (n = 4).

Synthesis of PI from PC

PC (1 µmol) and BHT (5 nmol) were mixed in chloroform/methanol (C/M) 9:1 in a Kimax screw-cap tube and the solvent was evaporated under an N2 stream. Then 2 ml ethanol-free chloroform, 100 µl buffer (1 M Tris-HCl, 1 M CaCl₂, pH 7.5), and S. chromofuscus PLD (up to 200 units) were added. The reaction mixture was shaken vigorously in an orbital shaker for 3 h at 37°C. Then 2 ml chloroform, 2 ml methanol, and 1.5 ml of 0.5 M HCl were added and the tube was vortexed vigorously. After centrifuging for 10 min at 3,000 g at room temperature, the upper phase was removed and the lower phase was washed once with 3.5 ml of the theoretical upper phase. Then 100 µl C/M (9:1) containing 10% Triton X-100 and 5 nmol of BHT were added and the solvent was removed as above. After addition of 1 ml of a 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, 0.5 mg BSA, and 0.125 mM DTT the mixture was sonicated in a water bath sonicator for 10 min at 4°C. Next, myo-inositol or D6-myoinositol (1.5 mM), CTP (5 mM), MgCl₂ (10 mM), and MnCl₂ (2.5 mM) were added followed by E. coli membranes containing Cds (1 mg protein) and PIS1 (0.5 mg protein). The tube was incubated in a 30°C water bath for 3–5 h after which 3 ml methanol, 6 ml chloroform, and 0.75 ml of 0.5 M HCl were added to stop the reaction. After extraction of the lipids (see above) and evaporation of the solvent from the lower phase, the residue was dissolved in C/M 1:2 and PI was purified by preparative HPLC as outlined below.

LC-MS analysis

An aliquot of the lipid extract of the reaction mixture was mixed with a D6-PI standard with the same acyl chains as the PI species synthesized. Conversely, the corresponding unlabeled PI species were used as standard when a D6-labeled PI species were synthesized. After evaporation of the solvent, the residue was dissolved in 5 µl chloroform and then 25 µl methanol was added, followed by vortexing (Note: PI is poorly soluble in neat methanol). A 5 µl sample was then analyzed on a Waters Acquity UPLC system equipped with an Acquity BEH-C18 column (1 × 100 mm) connected to a Waters Quattro Micro triple-quadrupole mass spectrometer operated in the positive ion mode (21). Multiple reaction monitoring was used to detect the PI analyte and the coeluting internal standard. The precursor ion was [M + H]+, while the product ion was 263 (unlabeled PI) or 269 (D6-PI) m/z units less than that of the precursor. Purity of the PI species synthesized was checked by directly infusing the lipid dissolved in C/M (1:2) containing 1% ammonium hydroxide to the mass spectrometer operated in the negative ion mode as described previously (22).

Purification of PI

Triton X-100 was first removed from the lipid extract of the reaction mixture using a Sep-Pak silica cartridge (500 mg. Waters). The lipid extract in C/M (9:1) was applied to the cartridge preequilibrated with C/M (9:1) and the cartridge was first eluted with 5 ml C/M (9:1), which elutes Triton X-100 and other nonpolar lipids and then with 10 ml C/M/H₂O (5:4:1), which elutes the phospholipids. The phospholipid fraction was taken to dryness under an N2 stream and the residue was dissolved in 1 ml chloroform. PI was purified from this solution using a HPLC system consisting of a LKB 2249 gradient pump equipped with a semi-preparative diol-modified silica column (Interchim Uptisphere Diol 6UM, 250 × 10 mm). The column was eluted with a gradient consisting of solvent mixture A [hexane/2-propanol (82:17) plus 1% glacial acetic acid and 0.08% of triethylamine] and solvent mixture B [2-propanol and distilled water (85:14) plus 1% glacial acetic acid and 0.08% of triethylamine] (23). At time zero, the proportion of solvent mixture B was 10% and increased to 20% at 40 min, then to 40% at 50 min, to 100% at 70 min and stayed at...
RESULTS

Three enzymes (PLD, Cds, and PIS1) were employed to convert various PC species to the corresponding PI via PA and CDP-DAG. We initially tested whether PC could be converted to PI in a single reaction mixture containing PLD. We also confirmed that PI conversion by PLD is not affected by triethylamine originating from the HPLC solvent. After removing the upper phase, the lower one was washed once with the theoretical upper phase (24) and taken to dryness under N\textsubscript{2} flow and then under a high vacuum for overnight. After addition of 1 ml C/M (9:1) and 10 µl NH\textsubscript{4}OH (to convert PI to ammonium salt), the solvent was evaporated and the residue was dissolved in C/M (9:1) and kept at ~80°C. The yield of PI was determined by a phosphate assay (25).

![Plate showing PI species with varying acyl chains](image)

**Fig. 1.** PI species with varying acyl chains were synthesized from the corresponding PC (1 µmol). After lipid extraction, 1/20 each extracts was run on TLC with standards and controls. Std 1: PC, 50 nmol; Std 2: PA, PI and CDP-DAG (50 nmol of each); E. coli lipid extract from 1.5 mg membranes. Notes: The mobility of PI species varies depending on their acyl chain hydrophobicity, and 160/Pyr10-PI is stained more weakly by iodine than the other PI species. The upper part of the plate showing only prominent Triton X-100 bands have been cut away from the image.

Removal of Triton by a silica cartridge before the HPLC. This protocol provided a polar lipid fraction free of any detectable Triton peaks as assessed by TLC (data not shown). The polar lipid fraction was then taken to dryness, dissolved in chloroform, and injected to the diol column. As shown in **Fig. 2**, no peaks due to Triton X-100 were detected, thus confirming that it had been removed by chromatography on the silica cartridge.

PI eluted from the HPLC column at ~60 min; i.e., well after PE (~28 min), cardiolipin (~50 min), and PG (~52 min) deriving from *E. coli* membranes. The remaining PA elutes well before PI at ~33 min, while the remaining CDP-DAG elutes later than PI at ~65 min. MS spectra obtained for the purified PIs showed only peaks with the expected masses (Fig. 3 and data not shown), thus showing that insignificant amounts of endogenous PA or CDP-DAG species were present in the *E. coli* membrane preparations used. Consistent with the MS data, TLC analysis of removal of Triton by a silica cartridge before the HPLC. This protocol provided a polar lipid fraction free of any detectable Triton peaks as assessed by TLC (data not shown). The polar lipid fraction was then taken to dryness, dissolved in chloroform, and injected to the diol column. As shown in **Fig. 2**, no peaks due to Triton X-100 were detected, thus confirming that it had been removed by chromatography on the silica cartridge.

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| Species (sn1/sn2 acyl) | PC Converted to PI (%) | Final Yield (%) |
|------------------------|------------------------|----------------|
| 14:1/14:1              | 22 ± 1.3               | 7              |
| 16:1/16:1              | 47 ± 4.7               | 29             |
| 18:1/18:1              | 61 ± 10.2              | 35             |
| 16:0/18:1              | 54 ± 2.6               | 36             |
| 18:0/18:2              | 49 ± 1.2               | 24             |
| 16:0/20:4              | 57 ± 9.4               | 32             |
| 18:0/22:6              | 49 ± 6.5               | 30             |
| 14:0/14:0              | 8 ± 0.8                | ND             |
| 16:0/16:0              | 0.2 ± 0.0              | ND             |
| 17:0/17:0              | 5 ± 0.3                | ND             |
| 16:0/Pyr10             | 37 ± 2.5               | 24             |

ND, not determined.

*Conversion was assessed by LC-MS from three independent reaction mixtures.*

*(Determined with a phosphate assay.)*
the isolated PIs showed only a single band coeluting with a PI marker (data not shown).

The yields of PIs after the HPLC purification were significantly lower than what was observed for reaction mixture (Table 1), thus showing that significant amounts of the produced PI was lost during the purification and/or the extraction protocols used to remove the polar modifiers (acetic acid and triethylamine) deriving from the eluent. Notably, control experiments showed that only 58 ± 8% (n = 4) of neat liver PI was recovered after HPLC purification. The highest final yields (35% and 36%) were observed for 16:0/18:1 and 18:1/18:1-PI, whereas somewhat less of the other unsaturated PI species were obtained (Table 1). The final yields of the saturated PI species were not determined because of the poor conversion of saturated PCs to PI.

**DISCUSSION**

This study describes a simple and rapid biochemical method to synthesize PI species with specific acyl chains from corresponding PCs. The method employs three enzymes: i) a commercially available PLD, ii) Cds of *E. coli* and iii) PIS1 from *A. thaliana* expressed in *E. coli*. The synthesis can be completed within one day in contrast to previous methods that require several days or even weeks. Purification of PI from the reaction mixture is straightforward, because the *E. coli* membranes used do not contain PI, while PE or PG deriving from those membranes elute well before PI from the LC column. Mass spectrometric analyses of the reaction mixtures showed that 45–60% of unsaturated PC was converted to PI, but after purification the final yield of PI was significantly less (25–35%). This rather low recovery was due to splitting of the effluent between the detector and the sample collection line (see Materials and Methods) as well as absorption to the column matrix as shown by only 58 ± 8% of a liver PI standard injected to the column being recovered in the PI fraction. Thus, the yield of PI could be increased significantly by bypassing the detector during the purification steps.

**Fig. 2.** Purification of PI from the lipid extract of the reaction mixture. The purification was accomplished as detailed in Materials and Methods. The elution positions of the different lipids originating from *E. coli* membranes [i.e., PE, PG, cardiolipin (CL), PA; Ref. 36], or included or formed in the reaction mixture (PC, PI, PA, CDP-DAG) are indicated by arrows. The elution times were confirmed by running the appropriate lipid standards. Triton X-100 (if present) elutes between 8 and 13 min.

**Fig. 3.** Mass spectra obtained for purified 16:0/20:4-D6-PI in the negative ion mode. A: MS/MS spectrum for the precursors of m/z 247. B: MS spectrum. C: Product ion spectrum. The purified lipid in C/M (1:2) containing 1% ammonium hydroxide was infused to the mass spectrometer and the spectrum was recorded either from m/z 600 to m/z 950 (A, B) or from m/z 200 to m/z 900 (C). The major peak at m/z of 863.6 corresponds to the predicted mass of 16:0/20:4-D6-PI -H+, whereas the minor peak at m/z 885.6 in the MS spectrum (B) has the predicted mass of Na+ adduct of PI minus H+. Product ion scan for 16:0/20:4-D6-PI precursor (C) yielded all expected fragments (37), including the 16:0 and 20:4 acyl anions (m/z 255.2 and 303.2, respectively) as well as a cyclic inositol phosphate anion (m/z 247.1) originating from the head group containing six deuteriums. Analogous data were obtained for all labeled unlabeled and PI species synthesized.
elution of the PI peak (not attempted here for convenience) and possibly by using another type of chromatographic column material. However, such modifications are of lesser importance because the prices of most PC species are extremely low (0.2–2.0 $/mg) as compared with those of the (very few) PI species (>500 $/mg) commercially available. Also, the cost of the enzymes used are negligible because E. coli expressing Cds and PIS1 can be produced in large quantities at a low cost and the price of the commercial S. chromofuscus PLD is low compared with the need.

In contrast to unsaturated PCs, the conversion of the saturated PCs to PIs was low (Table 1). The reason for this was not established, but saturated PAs may not properly disperse in Triton X-100 micelles, thus impairing the action of Cds (27). Although this problem might be circumvented by using lipid vesicle membranes instead of micelles as the macrosubstrate (27), this was not attempted here due to the additional work involved and possible problems when reconstituting PIS1 in an active form in vesicles. Notably, as far as we are aware, no biological membrane contains significant amounts of saturated PI species.

An advantage of the present method is that it allows one to readily prepare PI species labeled to the head group or an acyl chain. PI species with deuterium-labeled head group were obtained simply by replacing an unlabeled inositol with a deuterium-labeled one in the reaction mixture. We have synthesized many such head group-labeled PI species and, as expected, the yields were comparable to those of corresponding unlabeled ones (data not shown). Heavy isotope-labeled PI species are the optimal internal standards for LC-MS-based lipidomics (28) because they elute from the column simultaneously with the corresponding analyte PI, thus avoiding potential bias due to differential ion suppression when the internal standard and the corresponding analyte elute at different times (29). PIs with a heavy isotope-labeled head group are also expected to be highly useful when acyl chain remodeling is studied in cells (21) or when studying (the yet poorly understood) biophysical properties of PI species in membranes (12). On the other hand, PI species containing a fluorescently labeled acyl chain allow one to construct on-line assays to study, for example, transfer or binding of PI species by specific transfer proteins (30, 31) or the activity of phospholipases (32).

Recently, another biochemical method was described employing a genetically engineered PLD to prepare PI from PC (6). Although this method is even simpler than the present one, it seems to provide a lower yield of PI and also produces some unnatural PI isomers that can have harmful effects as indicated by the authors.

Very few CDP-DAG species are commercially available at present, which is a significant drawback when studying, for example, the substrate specificity of PI synthases, as has been stressed recently (33). The present method allows the preparation of various CDP-DAG species simply by omitting PIS from the reaction mixture. The CDP-DAG species produced are easily isolated by using the chromatographic system employed to purify PI (Fig. 2). If required, CDP-DAG could then be converted to cardiolipin using cardiolipin synthase. Notably, instead of PC, the present method can be used to prepare PI from the available PA or CDP-DAG species by omitting PLD or PLD and Cds, respectively (data not shown). Although we did not test it, it seems likely that also PCs or PAs with another type of fluorescent moiety, attached to an acyl chain, can be converted to corresponding PI. Finally, PI species synthesized with the present method can be converted to various labeled or unlabeled polyphosphoinositide species using appropriate kinases (34, 35) that are commercially available.

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