Biological variability in the structures of diphosphoinositol polyphosphates in *Dictyostelium discoideum* and mammalian cells

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Previous structural analyses of diphosphoinositol polyphosphates in biological systems have relied largely on NMR analysis. For example, in *Dictyostelium discoideum*, diphosphoinositol pentakisphosphate was determined by NMR to be 4- and/or 6-PPInsP_5, and the bisdiphosphoinositol tetrakisphosphate was found to be 4,5-bisPPInsP_4 and/or 5,6-bisPPInsP_4 [Laussmann, Eujen, Weisshuhn, Thiel and Vogel (1996) Biochem. J. **315**, 715–720]. We now describe three recent technical developments to aid the analysis of these compounds, not just in *Dictyostelium*, but also in a wider range of biological systems: (i) improved resolution and sensitivity of detection of PPInsP_5 isomers by microbore metal-dye-detection HPLC; (ii) the use of the enantiomerically specific properties of a rat hepatic diphosphatase; (iii) chemical synthesis of enantiomerically pure reference standards of all six possible PPInsP_5 isomers. Thus we now demonstrate that the major PPInsP_5 isomer in *Dictyostelium* is 6-PPInsP_5. Similar findings obtained using the same synthetic standards have been published [Laussmann, Reddy, Reddy, Falck and Vogel (1997) Biochem. J. **322**, 31–33]. In addition, we show that 10–25% of the *Dictyostelium* PPInsP_5 pool is comprised of 5-PPInsP_5. The biological significance of this new observation was reinforced by our demonstration that 5-PPInsP_5 is the predominant PPInsP_5 isomer in four different mammalian cell lines (FTC human thyroid cancer cells, Swiss 3T3 fibroblasts, Jurkat T-cells and Chinese hamster ovary cells). The fact that the cellular spectrum of diphosphoinositol polyphosphates varies across phylogenetic boundaries underscores the value of our technological developments for future determinations of the structures of this class of compounds in other systems.

INTRODUCTION

Among the considerable range of inositol polyphosphates now known to occur in cells, the discovery of diphosphate derivatives ('inositol pyrophosphates') [1–4] has presented us with a particularly difficult challenge to understand both their structures and their functions. Yet what little knowledge we do have about these compounds strongly suggests that they fulfil some important physiological purpose. For example, it has been established that diphosphoinositol pentakisphosphate (PPInsP_5 or 'InsP_7') is synthesized *in vivo* by ATP-dependent kinase-mediated phosphorylation of InsP_6 [2,3]. PPInsP_5 is itself then further phosphorylated to bisPPInsP_4 (or 'InsP_8') [2–4]. These compounds are predominantly dephosphorylated by fluoride-sensitive diphosphatases [3,4]; the fact that PPInsP_5 and bisPPInsP_4 are interconverted through these two closely coupled substrate cycles is generally believed to be of some significance, on the basis of precedents set by many other such cycles. What is of particular interest is the high rate of metabolic flux through these substrate cycles. It is sufficient to ensure the turnover of 30–50% of the cellular InsP_6 pool every hour [3]. Attention has also been drawn to the considerable free-energy change that is likely to result during the hydrolysis of these diphosphates, which has been speculated to drive transphosphorylation reactions [2]. It may also be significant that the levels of PPInsP_5 are sensitive to changes in cellular calcium homoeostasis [5]. Finally, these polyphosphates have also been shown to be high-affinity ligands for proteins that are involved in vesicle traffic, such as coatomer [6], AP-2 [7] and AP-3 [7].

While we continue with the difficult search for some satisfactory physiologically relevant explanation for these characteristics, even the structures of PPInsP_5 and bisPPInsP_4 have proved difficult to elucidate. In part, this is because the concentrations of PPInsP_5 and bisPPInsP_4 are normally 1 μM or less in mammalian cells [2–4]. Since these compounds are up to 300-fold more abundant in *Dictyostelium* [1,2], this is where most of the previous structural work has been performed. Originally, fast-atom-bombardment MS and ^{31}P-NMR led to the tentative assignment of the diphosphate of PPInsP_5 to either the 1- or 3- position [2]. The two diphosphate groups retained by bisPPInsP_4 were proposed to be at the 1/3- and 4/6- positions. However, Vogel and co-workers [8] subsequently applied the greater resolving power of two-dimensional ^{1}H/^{31}P-NMR to this problem and came to some different conclusions: the diphosphate of PPInsP_5 was now determined to reside at either the 4- or 6- position, and the bisPPInsP_4 was found to contain diphosphate groups at either

Abbreviations used: Micro-MDD-HPLC, microbore metal-dye-detection HPLC; RT, retention time; PPInsP_5, diphosphoinositol pentakisphosphate; bisPPInsP_4, bisdiphosphoinositol tetrakisphosphate (the location of the diphosphate groups, when known, are designated according to IUPAC [the 'D-1 nomenclature'; see Biochem. J. (1989) **258**, 1–2].

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the 4,5- and/or 5,6- positions. At that time, Vogel and colleagues [8] did not have a means of distinguishing between these enantiomeric alternatives. This is an important question. Knowledge of the absolute configurations of the diphosphoinositols is a prerequisite for a molecular understanding of the catalytic sites of the enzymes that synthesize and metabolize these compounds, since in general these will be chirally selective. The same argument is true of any 'receptors' that might mediate the physiological actions of these compounds. Moreover, the functional characterization of the diphosphoinositols will also benefit from the availability of a variety of defined isomers for specificity testing.

Thus complete knowledge of the structure, metabolism and functions of inositol diphosphate derivatives necessitates that we can differentiate between all possible isomers of PPInsP_5, including the enantiomerically related alternatives. A combination of several recent technical advances has enabled us to attain this goal. First there have been some improvements [9] in both the sensitivity and resolution of the metal-dye-detection HPLC procedures [10,11], and we focus here on the particular benefits of these with regards to the analysis of PPInsP_5 isomers. Second, we have previously chemically synthesized enantiomerically pure reference standards of 1-PPInsP_5 and 3-PPInsP_5 [12]. We have now completed the synthesis of all remaining possible PPInsP_5 isomers. Third, we have used a hepatic diphosphatase [12] as a chirally specific diagnostic tool to distinguish between 4- and 6-PPInsP_5.

While this manuscript was under review, Vogel and colleagues [13] also developed an enantiomerically selective assay using our 4- and 6-PPInsP_5 standards. They demonstrated that a PPInsP_5 kinase purified from *Dictyostelium* did not phosphorylate 4-PPInsP_5. Instead, both *Dictyostelium* PPInsP_5 and 6-PPInsP_5 were phosphorylated to a similar extent [13]. This group concluded that the naturally occurring diphosphoinositol polyphosphates in *Dictyostelium* were 6-PPInsP_5 and 5,6-bisPPInsP_4 [13]. Our data from a different enzyme assay are largely consistent with these conclusions, but in addition we have found that 10–25 % of the cells' complement of PPInsP_5 is comprised of 5-PPInsP_5. The biological significance of this observation is underscored by our demonstration that 5-PPInsP_5 is in fact the predominant PPInsP_5 isomer in four different mammalian cell lines.

MATERIALS AND METHODS

Materials

The 1- and 3-isomers of PPInsP_5 were synthesized as described elsewhere [12]; these procedures were modified slightly to prepare 4- and 6-PPInsP_5 by asymmetric total synthesis (see below and Scheme 1). The preparation of 2-PPInsP_5 and 5-PPInsP_5 involved a somewhat different approach which is described elsewhere [14]. For these syntheses, chromatography, compositional and optical analyses, and routine laboratory manipulations were as reported previously [15]. For TLC, ammonium molybdate reagent was used for visualization of phosphate-containing compounds [16]. Note that we (results not shown) and others [17] did not observe any degradation of the diphosphoinositols during these TLC procedures. Unless otherwise stated, $^1H/^{13}C$-NMR spectra were measured in [^2H]chloroform on a Bruker AC-250 MHz spectrometer and reported relative to tetramethylsilane as internal reference.

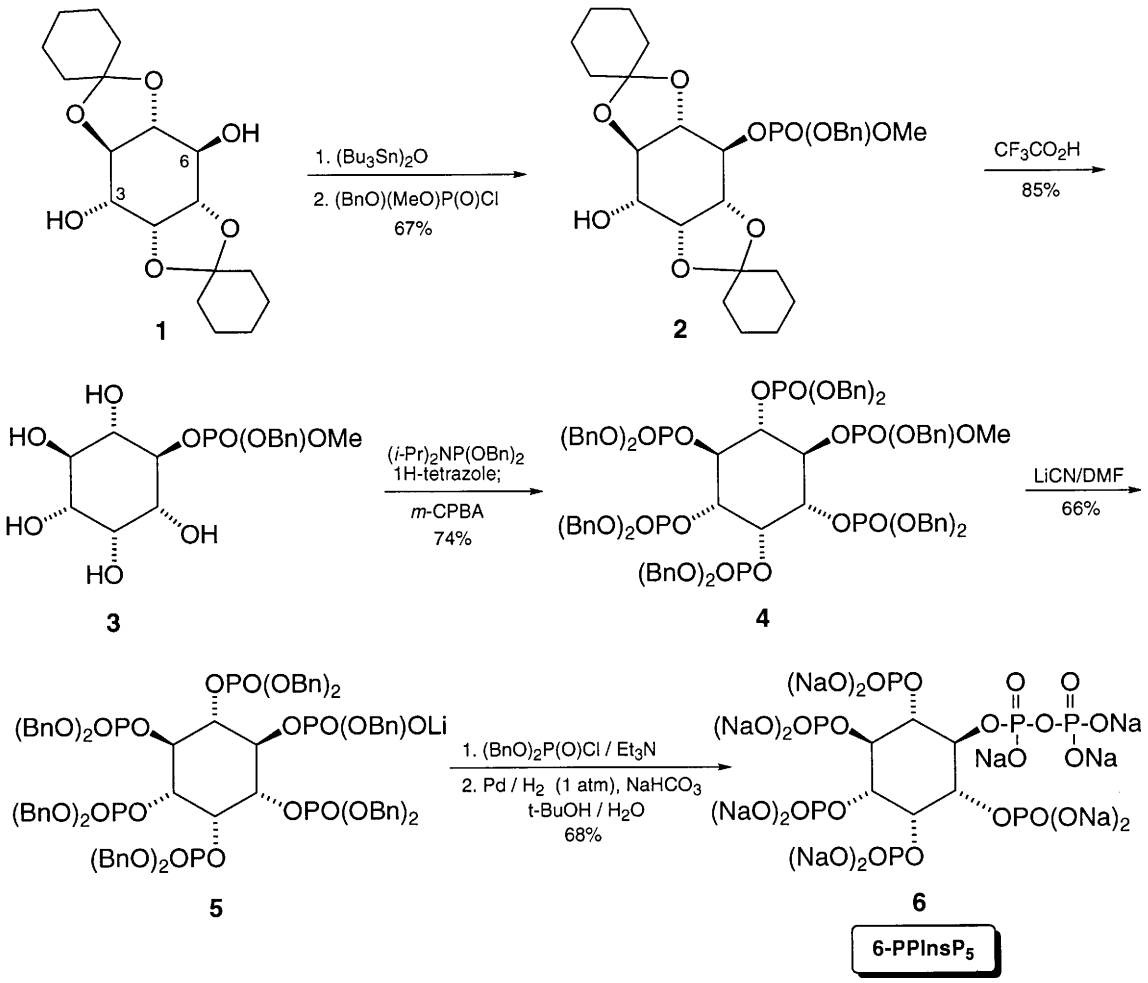
(−)-1,2:4,5-Dicyclohexylidene-D-*myo*-inositol (compound **1** in Scheme 1) [12] (1 g; 2.94 mmol) and bis(tributyl)tin oxide (1.06 ml; 2.08 mmol) in toluene (30 ml) were heated under reflux with water removal by activated 4Å molecular sieves. After 3 h, the reaction mixture was cooled to 0 °C and neat benzyl methylchlorophosphate [18] (1.29 g; 4.38 mmol) was added. The mixture was warmed to room temperature and after 2 h was concentrated under reduced pressure. Flash chromatography (SiO_2) using diethyl ether gave 1,2:4,5-*O*-dicyclohexylidene-6-*O*-benzyl(methyl)phosphoryl-D-*myo*-inositol (compound **2** in Scheme 1) (1.18 g; 67 %), m.p. 51 °C; TLC (SiO_2) ether, $R_F \sim 0.34$; ^1H NMR δ1.21–1.80 (m, 20H), 2.49 (d, *J* 8.6 Hz, 1H ^2H_2O exchangeable), 3.42–3.44 (m, 1H), 3.80 (d, *J* 11.2 Hz, 1.5 H), 3.83 (d, *J* 11.2 Hz, 1.5 H), 3.84–3.92 (m, 1H), 3.96–4.06 (m, 1H), 4.19–4.25 (m, 1H), 4.46 (t, *J* 4.8 Hz, 1H), 4.62–4.72 (m, 1H), 5.18 (dd, *J* 2.9, 7.15 Hz, 2H), 7.25–7.44 (m, 5H); ^{13}C NMR δ 23.2, 24.9, 35.1, 36.2, 37.3, 54.0, 54.9, 67.2, 69.8, 76.6, 77.0, 77.2, 77.9, 80.2, 111.0, 113.8, 127.0, 128.5, 136.3.

Phosphate triester (compound **2** in Scheme 1) (90 mg; 0.14 mmol) was dissolved in CF_3COOH/CH_2Cl_2/MeOH (1.5:3:0.5, by vol.; 5 ml) at 0 °C. After 4 h, the solvents were evaporated and the residue was triturated with ether (2 × 10 ml), ethyl acetate (2 × 10 ml), methylene chloride (2 × 10 ml) to give 6-*O*-benzyl(methyl)phosphoryl-D-*myo*-inositol (compound **3** in Scheme 1) (56 mg; 85 %), m.p. 144 °C, sufficiently pure to be used without further purification: TLC (SiO_2) 20 % MeOH/CH_2Cl_2, $R_F \sim 0.24$; ^1H NMR ($C^2H_3O^2H$) δ 3.35–3.41 (m, 2H), 3.45–3.62 (m, 2H), 3.68–3.82 (m, 3H), 3.95–4.01 (m, 1H), 4.41–4.56 (m, 1H), 5.20 (dd, *J* 0.95, 7.1 Hz, 2H), 7.25–7.41 (m, 5H); ^{13}C NMR δ 56.0, 70.62, 72.31, 73.18, 74.39, 74.45, 75.10, 83.67,129.17, 129.69, 137.20.

To a suspension of the above pentaol (compound **3** in Scheme 1) (50 mg; 0.11 mmol) and 1*H*-tetrazole (119 mg; 1.7 mmol), in CH_2Cl_2 (10 ml) at 0 °C was added dibenzyl *N*,*N*-di-isopropylphosphoramidite (379 mg; 1.1 mmol). After 2 h at room temperature, the reaction mixture was cooled to −78 °C and a solution of *m*-chloroperbenzoic acid (85 %; 303.8 mg; 1.37 mmol) in CH_2Cl_2 (5 ml) was added slowly. The reaction mixture was stirred at −20 °C for 4 h, diluted with CH_2Cl_2 (50 ml), washed with aq. 5 % $Na_2S_2O_2$ (2 × 25 ml) and satd. $NaHCO_3$ (2 × 25 ml). The aqueous wash solution was back-extracted with CH_2Cl_2 (25 ml). The combined organic extracts were washed with satd. NaCl (30 ml) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by chromatography (SiO_2) to give compound **4** (Scheme 1) (141 mg; 74 %) as a colourless oil: TLC (SiO_2) CH_2Cl_2/EtOAc (3:2), $R_F \sim 0.21$; ^1H NMR δ 3.61 (dd, *J* 3.7, 11 Hz, 3H), 4.35–4.42 (m, 3H), 4.85–5.29 (m, 24H), 5.59–5.62 (m, 1H), 7.05–7.39 (m, 55H); ^{13}C NMR δ 55.0, 69.7, 69.9, 73.2, 74.9, 76.0, 127.8, 127.9, 128.1, 128.3, 128.4, 136.2.

To a solution of compound **4** (Scheme 1) (310 mg; 0.18 mmol) in anhydrous dimethylformamide (4 ml) was added LiCN (0.5 M solution in dimethylformamide; 400 μl). After 12 h at ambient temperature, the solvent was removed *in vacuo* at ambient temperature. The residue was chromatographed (SiO_2) to yield the lithium salt (compound **5** in Scheme 1) (202 mg; 66 %) as a colourless syrup: TLC (SiO_2) 10 % MeOH/CH_2Cl_2, $R_F \sim 0.4$; ^1H NMR δ 4.65–5.30 (m, 28H), 6.90–7.21 (m, 55H); ^{13}C NMR δ 69.2, 69.7, 73.0, 75.0, 76.1, 126.5, 127.9, 128.0, 128.3, 136.2.

To a 0 °C solution of compound **5** (Scheme 1) (100 mg; 0.06 mmol) and triethylamine (9.8 μl, 0.071 mmol) in anhydrous CH_2Cl_2 (1.5 ml) was added chlorodibenzylphosphate [18] (26.5 mg; 0.071 mmol) in CH_2Cl_2 (0.5 ml). After 2 h at ambient temperature, the volatiles were removed *in vacuo*. The residue was dissolved in *t*-BuOH/water (6:1, v/v; 20 ml) and shaken under H_2 (345 kPa) in a Parr apparatus for 8 h in the presence of $NaHCO_3$ (70.5 mg; 0.84 mmol) and Pd black (100 mg). The catalyst was removed by filtration over a pad of Celite 577® and the filter cake was washed with water (10 ml), EtOH (10 ml) and EtOAc (5 ml). The combined filtrates were evaporated *in vacuo*



Scheme 1 Pathway for the chemical synthesis of 6-PPInsP_5

For details see the Materials and methods section. DMF, dimethylformamide; *m*-CPBA, *m*-chloroperbenzoic acid.

at room temperature. The crude material was purified by chromatography (Q Sepharose fast flow) eluted with a gradient from 0.5 to 2.0 M ammonium acetate (pH 5.0). The fractions that contained 6-PPInsP_5 were converted into its sodium salt by pooling and evaporation *in vacuo*. The residue was dissolved in water, combined with 13 equivalents of $NaHCO_3$, and then lyophilized to give compound **6** (42.5 mg; 68% overall from compound **5**) as a white amorphous solid: TLC [poly(ethyleneimine) cellulose] 1.5 M HCl, $R_F \sim 0.42$; ^{31}P NMR (202 MHz, ^2H_2O, 85% H_3PO_4 external reference) $\delta -7.80$ to -6.95 (m, 1P), -3.50 (br s, 1P), 2.40–5.80 (m, 5P); $[\alpha]$ 20/D $-2.4°$ (c 0.2, H_2O). The enantiomer of compound **6**, i.e. 4-PPInsP_5, was identical in all respects except for an opposite sign of optical rotation. The 4-PPInsP_5 was obtained analogously as described above using the enantiomer of compound **1** [12]. The bisphosphatase was purified approximately 130000-fold from the soluble fraction of a rat liver homogenate using the following procedures: polyethylene glycol precipitation, monoQ anion-exchange chromatography, heparin–agarose affinity chromatography, Green A dye-ligand chromatography, size-exclusion chromatography using Cellufine GCL-90 and Blue A dye-ligand chromatography (Safrany, S. T., Bembenek, M. E. and Shears, S. B., unpublished work).

Cell culture

The Jurkat human T-lymphocyte cell line was cultured as described [19]. NIH-Swiss 3T3 cells were grown in 90% Dulbecco's modified Eagle's medium (Gibco–Life Technologies) supplemented with 580 mg/l glutamine, 4.5 g/l D-glucose, 10% fetal calf serum (inactive), 100 units/ml penicillin and 100 μg/ml streptomycin in monolayers on 15 cm Petri dishes to about 70% confluence. FTC cells (follicular thyroid carcinoma cells, strain 133 [20]) were grown in the same dishes to similar density in 88% Coon's modified Ham F12 medium supplemented with 10% fetal calf serum (inactive), 1% non-essential amino acids (Gibco–Life Technologies), a five-hormone mixture (final concentrations: 10 ng/ml Gly-His-Lys; 10 μg/ml insulin; 10 ng/ml somatostatin; 5 μg/ml transferrin; 3.2 ng/ml hydrocortisone (all from Sigma Chemical Co., St. Louis, MO, U.S.A.), 100 units/ml penicillin and 100 μg/ml streptomycin. CHO-TSHr cells (clone c7-6) were grown on 15 cm Petri dishes in 90% Coon's modified Ham F12 medium supplemented with 10% fetal calf serum.

Cell incubation conditions and extraction of inositol phosphates

Cells were incubated at 37 °C in minimal medium (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4,

5.5 mM glucose and 20 mM Hepes/NaOH, pH 7.4) either in adherent layers or (in the case of Jurkat cells) in suspension at a density of 2×10^6 cells/ml. Cells were treated with final concentrations of either 10 mM NaCl or 10 mM NaF for up to 1 h. Jurkat cells were quenched by centrifugation (600 ***g***; 5 min; room temperature) followed by lysis of the cell pellet (see below). Adherently growing cells were treated with trypsin for 3 min by adding 10 ml of minimal medium and 5 ml of a 0.05 % (w/v) solution of trypsin in PBSE buffer [10 mM NaH_2PO_4, 154 mM NaCl, pH 7.4, supplemented with 0.02 % (w/v) titriplex III]. Trypsinized cells from three dishes were combined, washed once in 10 ml of minimal medium (as above) and pelleted by centrifugation (600 ***g***; 5 min; room temperature). Pelleted cells were lysed by adding 1 ml of 8 % (w/v) ice-cold trichloroacetic acid plus 50 μl of 0.2 M EDTA, followed by twice freezing and thawing in liquid nitrogen. After no more than 30 min, the precipitate was removed by centrifugation (3500 ***g***; 10 min; 4 °C) and the supernatants were extracted three times with 3 ml of water-saturated diethyl ether. After the pH had been adjusted to about 6 with Tris (0.5 M), the extract was treated with charcoal three times with 25 μl of a 20 % (w/v) suspension of acid-washed Norit A suspended in 0.1 M NaCl/50 mM sodium acetate, pH 4.0. After centrifugation (3500 ***g***; 5 min; 4 °C) the supernatant was saved and combined with the additional supernatants resulting from washing each of the three charcoal pellets with 100 μl of 0.1 M NaCl/25 mM NaF/0.5 mM EDTA, pH 6. The final extract was analysed by HPLC (see below).

HPLC analysis

Microbore metal-dye-detection HPLC (Micro-MDD-HPLC) was performed on MiniQ columns using a SMART system (Pharmacia) as described previously [8,9] with the following modifications. The flow rate of the eluates was 400 μl/min instead of 500 μl/min, and the MDD reagent containing 2-(4-pyridylazo)resorcinol was pumped at 300 μl/min. In order to optimize the separation of individual isomers of PPInsP_5 and of bisPPInsP_4 without significant loss of sensitivity, a modified gradient protocol was employed. The column was prewashed for 4.6 min with 4 % solvent B. Samples were injected in 0.5–1 ml of application buffer [11], and the column was washed for 1 min with 4 % solvent B. Then the following gradient was applied: 0–2 min, solvent B increased linearly from 4 to 5 %; 2–7.4 min, solvent B increased linearly from 5 to 55 %; from 7.4 to 22.4 min, solvent B increased linearly from 55 to 70 %; from 22.4 to 24.4 min, solvent B increased linearly from 70 to 100 %, where it remained for a further 2 min; 26.4–28.4 min, solvent B decreased linearly to 4 %. With this gradient very stable retention times (RTs) were observed (maximal run-to-run variation range $\pm$ 0.02 min). Chromatograms shown here were obtained using two different MiniQ columns; the column-to-column variation in elution time for PPInsP_5 isomers was maximally 0.3 min (results not shown).

Absorbance data, read continuously at 546 nm by a UV-M 2 recorder, were stored in an inverted form and further processed as ASCII files with the SMART software and SIGMAPLOT®. In composite Figures, the original baseline-subtracted chromatograms have been placed on a single Figure by adding fixed absorbance terms to each individual sampling point by means of SIGMAPLOT. Subtracted baselines were the averages from at least two individual blank chromatograms. Averaging was performed using SIGMAPLOT transforms. Relative peak sizes were integrated using either the SMART software or an off-line integration program.

Diphosphatase assays

Purified rat hepatic diphosphatase [4,12] was incubated at 37 °C in 50 μl of medium comprised of 40 mM KCl, 50 mM NaCl, 40 mM Hepes (pH 7.2 with KOH), 3.2 mM CHAPS, 0.04 mg/ml BSA, 0.8 mM disodium EDTA, 2 mM $MgSO_4$, 0.2 μM (i.e. the K_m concentration) [5β-^{32}P]PPInsP_5 (approx. 2000 d.p.m., prepared by M.E.B.), plus 0.001–3 μM either 4-PPInsP_5 or 6-PPInsP_5 (synthesized as described above) or 5-PPInsP_5 (M.E.B.), or PPInsP_5 purified from *Dictyostelium* [1]. Both the incubation time and the enzyme concentration were adjusted so that less than 30 % of the ^{32}P-labelled substrate was consumed. The reactions were quenched with 250 μl of ice-cold 8 % (v/v) perchloric acid containing 1 mg/ml InsP_6. Samples were then neutralized by the addition of an appropriate volume of medium containing 1.7 M KOH, 75 mM Hepes and 60 mM EDTA. The release of [^{32}P]P_i was assayed by loading acid-quenched KOH-neutralized samples (diluted to 10 ml with water) on to 0.6 ml of gravity-fed anion-exchange columns (Bio-Rad; AG1-X8 200–400 mesh; formate form). [^{32}P]P_i was eluted with 10 ml of 0.4 M ammonium formate/0.1 M formic acid. [β-^{32}P]PPInsP_5 was eluted with 10 ml of 2 M ammonium formate/0.1 M formic acid. Radioactivity was assessed from the Čerenkov radiation.

RESULTS

Micro-MDD-HPLC chromatography of PPInsP_5 isomers

The optimized micro-MDD-HPLC technique [9] employs a small-volume high-resolution MiniQ column that improves sensitivity 5–10-fold compared with the original technique [10,11], such that picomol of inositol polyphosphates can be detected. However, the absence of structurally defined standards of PPInsP_5 has hitherto prevented a detailed characterization of the elution properties of this group of compounds. Thus the chemical synthesis of all possible PPInsP_5 isomers ([12,14], and see the Materials and methods section) represents a substantial development. Figure 1 shows that 5-PPInsP_5 (RT = 10.13 min) was the first to be eluted from the HPLC column, followed closely by 4- and 6-PPInsP_5 (RT = 10.40 min). Although there was not a baseline separation, the reproducibility of these chromatograms (see Figure 1 legend) enabled us to discriminate routinely the 4/6 enantiomeric pair from 5-PPInsP_5. The next isomers eluted were 1- and 3-PPInsP_5 (RT = 11.57 min); the latter were eluted fractionally (but reproducibly, see Figure 1 legend) before 2-PPInsP_5 (RT = 11.66 min). Thus the micro-MDD-HPLC technique can resolve all but enantiomeric pairs of PPInsP_5 isomers.

Micro-MDD-HPLC and partial hydrolysis of bisPPInsP_4 isolated from *Dictyostelium*

Our analysis of the diphosphoinositol polyphosphates in *Dictyostelium* began with bisPPInsP_4 fraction (RT = 12.72 min; profile b in Figure 2). This compound has previously been rigorously characterized to be either 4,5-bisPPInsP_4 or 5,6-bisPPInsP_4 [8]. Minor contaminants were also present, one of which was not identified (RT = 5.11 min), and another (RT = 6.88 min, profile b in Figure 2) that was co-eluted with InsP_6 (see Figure 1). The peak from a further contaminant (RT = 10.41 min, profile b in Figure 2) was co-eluted with 4/6-PPInsP_5 (see Figure 1 and h in Figure 2); this particular peak was always eluted asymmetrically with a preceding shoulder. These contaminants are likely to be breakdown products of the bisPPInsP_4.

It has previously been shown that the β-phosphates can be

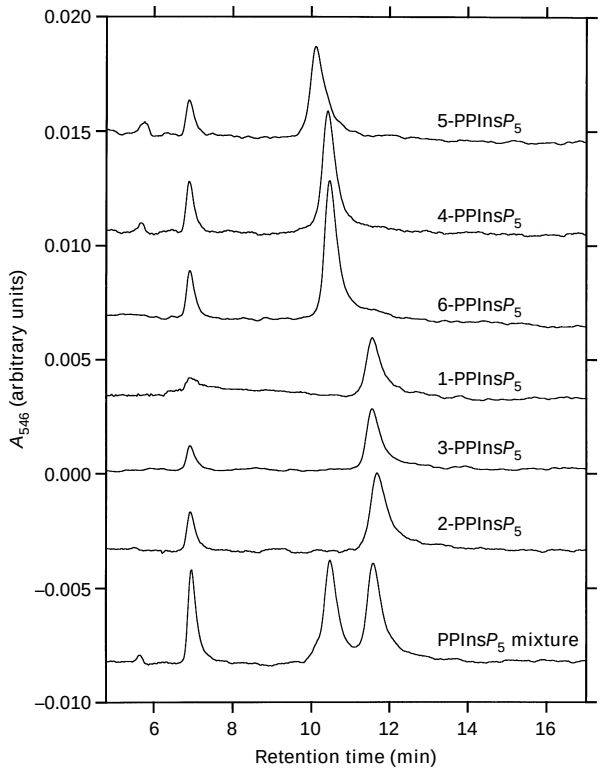


Figure 1 Micro-MDD-HPLC analysis of isomers of PPInsP_5

For details of the MDD-HPLC, see the Materials and methods section. The chromatograms of synthetic standards are shown, followed in parentheses by their retention times (RTs) (mean ± standard errors): 250 pmol of 5-PPInsP_5 (10.13 ± 0.003 min, $n = 16$); 450 pmol of 4-PPInsP_5 or 550 pmol 6-PPInsP_5 (10.40 ± 0.011 min, $n = 11$); 260 pmol of 1-PPInsP_5 or 240 pmol of 3-PPInsP_5 (11.57 ± 0.007 min, $n = 7$); 300 pmol of 2-PPInsP_5 (11.66 ± 0.003 min, $n = 6$). The retention time of 2-PPInsP_5 was significantly different from that of 1- or 3-PPInsP_5 (*t* test: $P < 0.005$). The bottom trace contains 900 pmol of a mixture of synthetic PPInsP_5 isomers generated as described [1]. The mass amounts of PPInsP_5 isomers were quantified after correction for minor contaminants (mainly InsP_6, RT = 6.88 ± 0.004). Note some slight variation in detection sensitivity for each isomer.

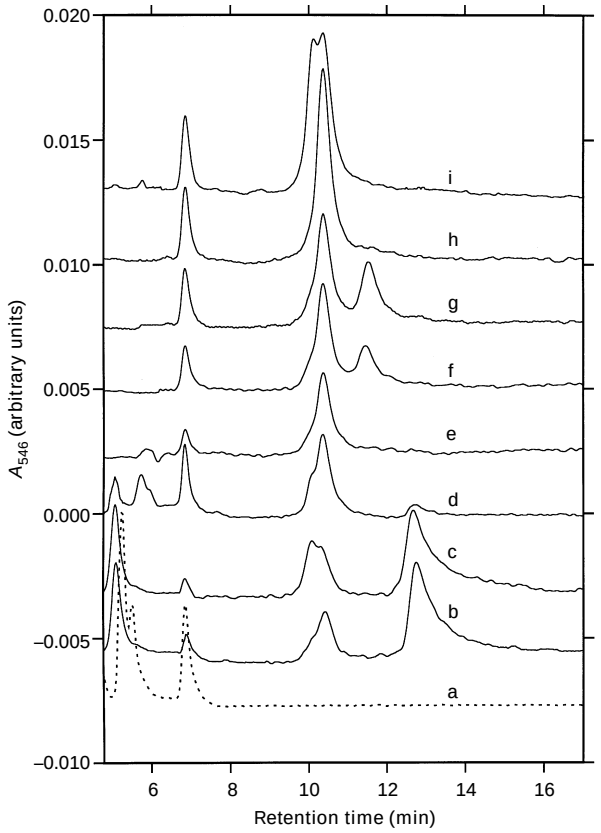


Figure 2 Micro-MDD-HPLC analysis of PPInsP_5 and bisPPInsP_4 isolated from *Dictyostelium*

The individual chromatograms are: (a) partial InsP_6 hydrolysate containing 280 pmol of D/L-Ins(1,2,4,5,6)P_5 (RT = 5.25 ± 0.003 min, $n = 3$) plus 29.4 pmol of Ins(1,3,4,5,6)P_5 (RT = 5.57 ± 0.003 min, $n = 15$), and 226 pmol of InsP_6; (b) 1.5 nmol of *Dictyostelium* bisPPInsP_4 (RT = 12.72 ± 0.021 min, $n = 4$); (c) 1.5 nmol of *Dictyostelium* bisPPInsP_4 plus 100 pmol of 5-PPInsP_5 standard; (d) 1.35 nmol of *Dictyostelium* bisPPInsP_4 hydrolysed for 90 s in 1 M trichloroacetic acid at 100 °C as described [1,4]; (e) 460 pmol of *Dictyostelium* PPInsP_5 isolated as described [1]; (f) 460 pmol of *Dictyostelium* PPInsP_5 plus 180 pmol of 1-PPInsP_5 standard; (g) 460 pmol of *Dictyostelium* PPInsP_5 plus 220 pmol of 2-PPInsP_5 standard; (h) 460 pmol of *Dictyostelium* PPInsP_5 plus 400 pmol of 6-PPInsP_5 standard; (i) 460 pmol of *Dictyostelium* PPInsP_5 plus 300 pmol of 5-PPInsP_5 standard. For further details of the micro-MDD-HPLC, see the Materials and methods section and legend to Figure 1.

cleaved from the diphosphate groups of bisPPInsP_4 on boiling in trichloroacetic acid [1]. We therefore considered that such a procedure could be structurally diagnostic, provided we could identify the resulting PPInsP_5 isomers. Treatment of the bisPPInsP_4 from *Dictyostelium* for 1.5–2 min gave the maximum recovery of PPInsP_5 isomers, before they were themselves further hydrolysed. The two incompletely resolved PPInsP_5 peaks (profile c, in Figure 2) were identified, by their co-elution with internal standards, as 4- and/or 6-PPInsP_5 (profile h in Figure 2) and 5-PPInsP_5 (profile i in Figure 2). The relative contributions of these two isomers to the overall peak (Figure 2, profile c and Figure 3, profile c) was estimated by subtracting a height-equalized chromatogram for a genuine 6-PPInsP_5 standard (Figure 3, profile a); the resultant peak was attributed to 5-PPInsP_5 (Figure 3, profile c), which we estimated to comprise 19 ± 3 % of the total ($n = 3$). It has previously been shown that alkaline hydrolysis of inositol phosphates also results in unequal rates of removal of different phosphate groups [21].

Acid-catalysed hydrolysis of *Dictyostelium* bisPPInsP_4 (profile d in Figure 2) did not lead to the accumulation of any material that was co-eluted with 1-, 2- or 3-PPInsP_5 (profiles f and g in Figure 2). This result consolidates the previous determination [8] that the diphosphate groups in bisPPInsP_4 are present on the 4/6- and 5- positions.

Micro-MDD-HPLC of PPInsP_5 isolated from *Dictyostelium*

When purified *Dictyostelium* PPInsP_5 was analysed by micro-MDD-HPLC, the material predominantly consisted of a peak (RT = 10.4 min, e in Figure 2) with the same retention time as the 6-PPInsP_5 standard (Figure 1 and h in Figure 2). However, the *Dictyostelium* PPInsP_5 peak (e in Figure 2) was reproducibly asymmetrical in comparison with the 6-PPInsP_5 standard (Figure 1), because of a leading shoulder. This minor contaminant (estimated to be 10 ± 3 % of the total, see the legend to Figure 3) has the exact elution properties of 5-PPInsP_5 (see above).

The PPInsP_5 isolated from *Dictyostelium* (e in Figure 2) did not contain any 1-PPInsP_5, 2-PPInsP_5 or 3-PPInsP_5 (f and g in Figure 2). This is an important point because earlier tentative

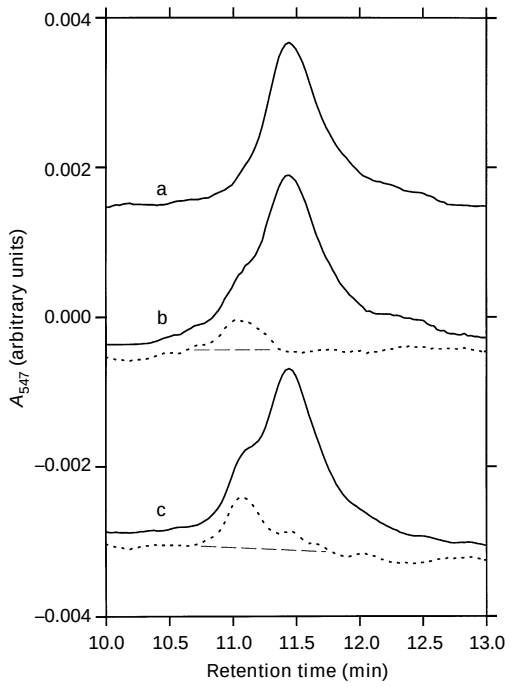


Figure 3 Micro-MDD-HPLC quantification of PPInsP_5 isomers obtained from *Dictyostelium* and from a partial hydrolysate of bisPPInsP_4

Micro-MDD-HPLC was performed as described in the Materials and methods section, except that the ramp from 55 to 70% solvent B was extended by 2 min (i.e. 7.4 to 24.4 min), to opitmize the resolution between 5-PPInsP_5 (RT = 11.05 ± 0.01 min) and 4- or 6-PPInsP_5 (RT = 11.43 ± 0.006 min); correspondingly, the retention times are later than in the other experiments (cf. Figures 1, 2 and 5). (a) An average micro-MDD-HPLC profile derived from three HPLC runs for 0.4 nmol of a 6-PPInsP_5 standard. (b) ——: an average chromatogram derived from three HPLC runs of about 0.4 nmol of PPInsP_5 purified from *Dictyostelium*. The peak was transformed so that its height became identical with that of the standard shown in (a), without modification to the proportionate shape of the peak. (b) · · · · · : a difference chromatogram obtained by subtracting the solid lines in (a) and (b). The residual peak (5-PPInsP_5, see the text) was thereby estimated to contribute 10 ± 3% ($n = 3$) to the total in the original peak. (c) ——: an average chromatogram derived from three HPLC runs for the PPInsP_5 that accumulated after acid-catalysed hydrolysis of bisPPInsP_4 purified from *Dictyostelium*. The peak was transformed so that its height became identical with that of the standard shown in (a), without modification to the proportionate shape of the peak. (c) · · · · · : a difference chromatogram obtained by subtracting the solid lines in (a) and (c). The residual peak (5-PPInsP_5, see the text) was thereby estimated to contribute 19 ± 3% ($n = 3$) to the total in the original peak. The dashed lines indicate the baseline that has been estimated to correspond to the peak in each of the difference chromatograms.

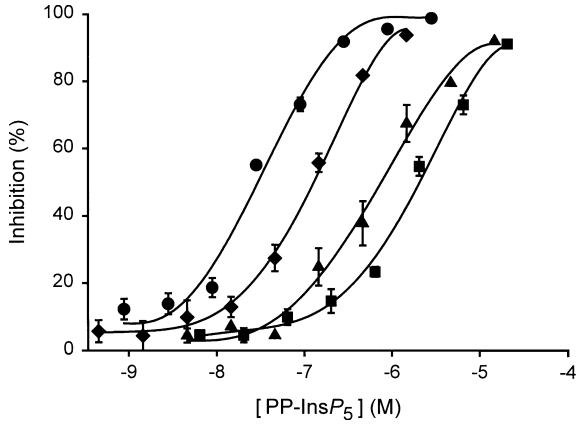


Figure 4 Rat hepatic diphosphatase activity in the presence of various isomers of PPInsP_5

Diphosphatase-catalysed [^{32}P]P_i release from [5-β-^{32}P]PPInsP_5 was assayed as described in the Materials and methods section in the presence of the indicated amount of the following PPInsP_5 isomer (apparent IC_{50} values, estimated by Graphpad Prism, follow each isomer in parentheses): ●, 4-PPInsP_5 (0.03 μM); ■, 6-PPInsP_5 (2.1 μM); ▲, *Dictyostelium* PPInsP_5 (0.92 μM); ◆, 5-PPInsP_5 (0.14 μM). Data are means ± S.E.M. from three experiments (error bars are not shown when they are smaller than the symbol). For comparative purposes, an IC_{50} of 3.5 μM was obtained for InsP_6 (not shown).

structural assignments by one of us (G.W.M.) with others [2], which relied on one-dimensional NMR, led to the proposal that *Dictyostelium* PPInsP_5 contained a 1- or 3-diphosphosphate group. At the time of these proposals, we lacked the definite ^{31}P resonance assignments which were recently made possible by the two-dimensional experiments of Vogel and colleagues [8]. In any case, the complete absence of 1-, 2- and 3-PPInsP_5 in *Dictyostelium* is now confirmed.

Enantiomeric resolution of the PPInsP_5 in *Dictyostelium*

Earlier data led to the proposal that the PPInsP_5 in *Dictyostelium* has its diphosphate group at either the 4- or 6- position [8], but in that particular study it had not been possible to distinguish between these two enantiomeric alternatives. It has been our goal to resolve this issue. We have therefore incubated a rat hepatic diphosphatase [12] with chemically synthesized standards of 4- and 6-PPInsP_5. These compounds differentially inhibited diphosphatase activity (Figure 4), with 4-PPInsP_5 being a 70-fold more potent inhibitor (Figure 4). The diphosphatase was also incubated with PPInsP_5 isolated from *Dictyostelium*. The resultant inhibition curve indicated that this material was approximately 2-fold more potent at inhibiting the diphosphatase than was 6-PPInsP_5 (Figure 4). Thus the development of an assay that is very enantiomerically selective (Figure 4) was pivotal in our reaching the conclusion that the predominant PPInsP_5 isomer in *Dictyostelium* is 6-PPInsP_5.

The fact that *Dictyostelium* PP-InsP_5 was slightly more potent at inhibiting the diphosphatase than was the 6-PPInsP_5 standard is consistent with *Dictyostelium* also containing some 5-PPInsP_5 (see above). By inserting the IC_{50} data (Figure 4 legend) into equations describing inhibition by two competitive and exclusive inhibitors [22], we estimate that *Dictyostelium* PPInsP_5 contains the 5-isomer and the 6-isomer in the ratio 25:75. This calculation agrees reasonably well with the independent chromatographic estimate of 10:90 (see above). Vogel and colleagues [13] have recently also identified 6-PPInsP_5 as being present in *Dictyostelium*, following experiments with a purified PP-InsP_5 kinase and the 4- and 6-PP-InsP_5 standards that we describe here. However, this group did not detect any 5-PPInsP_5 in *Dictyostelium*.

Micro-MDD-HPLC of PPInsP_5 isolated from four mammalian cell lines and the effect of fluoride

Although 6-PPInsP_5 is the major constituent of the PPInsP_5 fraction in *Dictyostelium*, we have now identified 5-PPInsP_5 as a minor isomer (see above). This new observation naturally leads to the queston as to what is the wider biological distribution of isomers of PPInsP_5. In fact, the 5-isomer is the major PPInsP_5 in both *Phreatamoeba balamuthi* [23] and *Entamoeba histolytica* [24]. We therefore investigated which isomers were present in four different mammalian cell types: FTC human thyroid cancer cells, Swiss 3T3 fibroblasts, Jurkat T-cells and Chinese hamster ovary (CHO) cells (Figure 5). Under control conditions, the

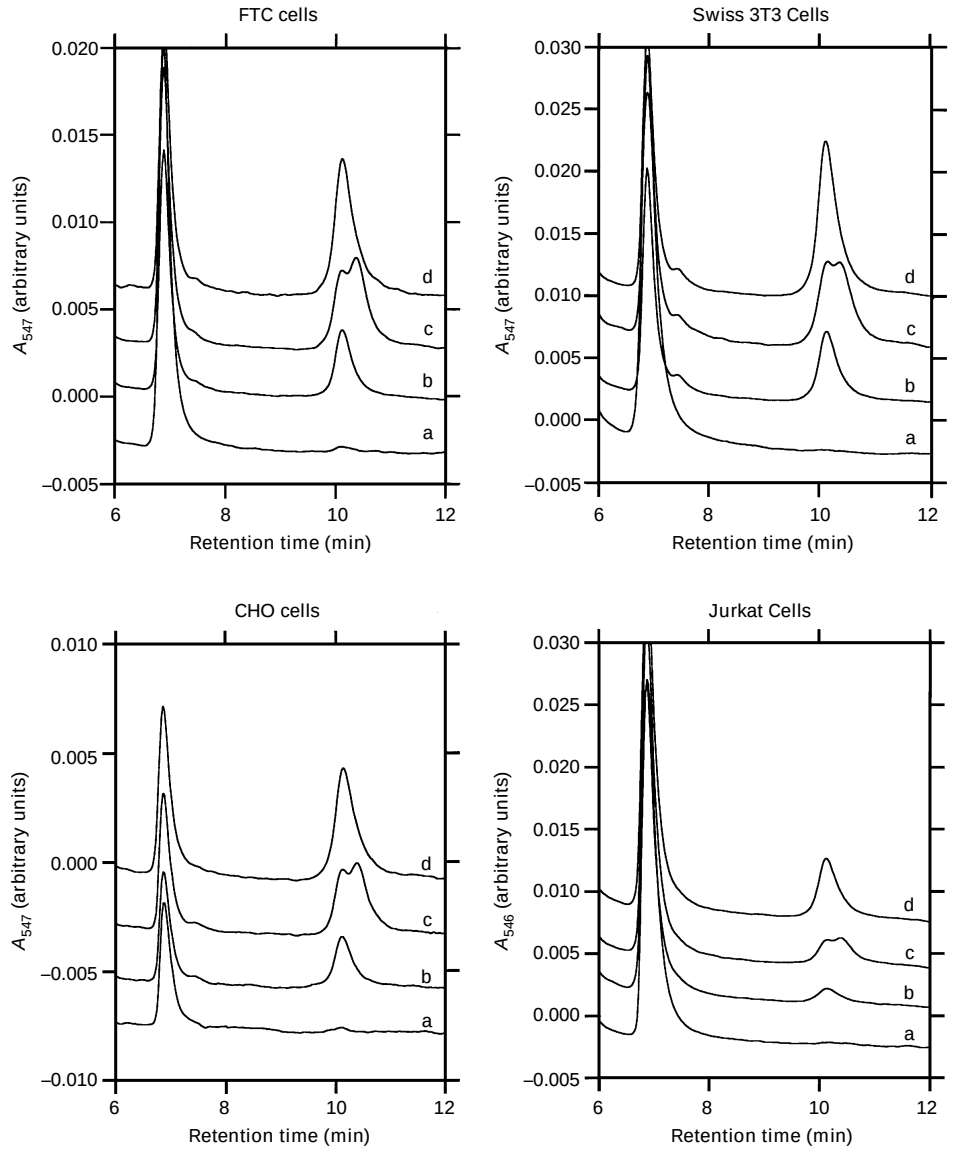


Figure 5 Micro-MDD-HPLC analysis of PPInsP_5 isomers in mammalian cell lines

Micro-MDD-HPLC was performed on extracts obtained (as described in the Materials and methods section) from 50 mg of wet packed FTC cells (top left panel), 1.5×10^7 Swiss 3T3 cells (top right panel), 20 mg of wet packed CHO cells (bottom left panel) and 1.5×10^7 Jurkat cells (bottom right panel). In each panel the extracts are prepared from: profile a, control cells; profile b, cells treated with 10 mM fluoride for 1 h; profile c, fluoride-treated cells chromatographed with an internal standard of 4-PPInsP_5 (225, 225, 150 and 90 pmol for FTC, 3T3, CHO and Jurkat cells respectively); profile d, fluoride-treated cells chromatographed with an internal standard of 5-PPInsP_5 (250, 250, 150 or 125 pmol synthetic 5-PPInsP_5 for FTC, 3T3, CHO and Jurkat cells respectively). Shown is only the range of each chromatogram where InsP_6 and PPInsP_5 isomers were eluted. See the text for details of mass levels of InsP_6 and 5-PPInsP_5 with and without fluoride treatment from these and additional experiments ($n = 2$–4).

levels of PPInsP_5 were close to the limits of detection, although a small peak with the elution properties of 5-PPInsP_5 (Figure 5, a profiles) was evident in both FTC cells (0.27 ± 0.12 pmol/mg of packed cells, $n = 2$) and CHO cells (0.32 ± 0.16 pmol/mg of packed cells $n = 2$). No bisPPInsP_4 was detected in these experiments, even when the size of the cell extract was increased up to 3-fold above those shown in Figure 5 (results not shown).

The low levels of PP-InsP_5 detected by these mass assays confirm data obtained from previous [^{3}H]inositol radiolabelling experiments with several other mammalian cell types [2–5]. However, as there is a very active kinase/bisphosphatase substrate cycle interconverting InsP_6 with PPInsP_5, inhibition of bisphosphatase with fluoride causes PPInsP_5 levels to increase dramatically [3–5]. A similar result was obtained in our present experiments (Figure 5). In each of the four different cell lines, a 1 h treatment with 10 mM fluoride (Figure 5, b profiles) brought about a substantial increase in the levels of PPInsP_5 [to 2.76 ± 0.72 ($n = 2$) and 2.12 ± 0.29 ($n = 2$) pmol/mg of packed cells for FTC and CHO cells respectively, and to 24.5 ± 14.6 ($n = 4$) and 5.17 ± 1.98 ($n = 3$) pmol/10^6 cells for 3T3 cells and Jurkat cells respectively]. The increase in PPInsP_5 was accompanied by a decrease in InsP_6 levels (from 14.2 ± 2.1 to 9.8 ± 0.8 pmol/mg in FTC cells, from 6.0 ± 0.5 to 5.0 ± 0.3 pmol/mg in CHO cells, from 71.4 ± 12.1 to 43.5 ± 4.3 pmol/10^6 cells in 3T3 cells, and from 69.4 ± 16.4 to 61.8 ± 13.6 pmol/10^6 cells in Jurkat cells). As previously indicated for other mammalian cells [4], this fluoride

treatment did not elevate levels of bisPPInsP_4 substantially (results not shown).

After fluoride treatment, the biological PPInsP_5 was co-eluted precisely with an internal standard of 5-PPInsP_5 (Figure 5, d profiles); internal standards of 6-PPInsP_5 (c profiles) were eluted fractionally later. We therefore conclude that 5-PPInsP_5 is the predominant isomer in all four of these mammalian cell lines.

General conclusions

The steady-state intracellular concentrations of the diphosphoinositol polyphosphates are considerably higher in *Dictyostelium* than in mammalian cells [1–4]. Thus the slime mould has been a particular focus of attention with regards to ascertaining the structures of these compounds. Yet even in this organism it has been technically challenging to determine the structures of these compounds. Considerable progress was recently made by the application of two-dimensional NMR to this problem, which revealed that the diphosphoinositol pentakisphosphate was either 4- and/or 6-PPInsP_5 [8]. We have now developed enantiomerically specific techniques that enabled us to determine that 6-PPInsP_5 is the major naturally occurring isomer in this organism. The defined chemical synthesis of relatively large quantities of the 6-PPInsP_5 and its enantiomer should facilitate our goal of understanding the specific functions of these polyphosphates in subsequent bioassays.

Yet it turns out to be of additional significance that *Dictyostelium* also contains 5-PPInsP_5 (Figure 2). The latter was not detected in two earlier studies of the diphosphoinositol polyphosphates of *Dictyostelium*, using two-dimensional NMR [8] and more recently when the diphosphoinositol pentakisphosphate kinase was used to discriminate between 4- and 6-isomers of PPInsP_5 [13]. The fact that we were able to detect an additional isomer (approx. 10–25 % of total PPInsP_5) illustrates the value of having a sensitive mass-detection technique with the ability to resolve individual isomers. Both yeast [25] and mammalian systems [4] possess the necessary enzymology for removing either of the β-phosphates from the two diphosphate groups of bisPPInsP_4; perhaps this is how both 5-PPInsP_5 and 6-PPInsP_5 are formed in *Dictyostelium*. Another possibility is that *Dictyostelium* contains InsP_6 kinases that phosphorylate both the 5- and 6-positions.

In fact, 5-PPInsP_5 was the only isomer of PPInsP_5 that others could detect in both *Phreatamoeba balamuthi* [23] and *Entamoeba histolytica* [24], and that we could detect in four different mammalian cell types (Figure 5). Even after 1 h treatment of mammalian cells with fluoride, when up to 40 % of the InsP_6 pool was driven into PPInsP_5, only the 5-isomer was detected (Figure 5). Thus 5-PPInsP_5 may turn out to be the most widely distributed PPInsP_5 isomer in nature.

With regards to the structure of *Dictyostelium* bisPPInsP_4, our data are consistent with the bisphosphate groups being in the 5- and 6- positions, as proposed recently [13]. The next challenge is to ascertain the structure of mammalian bisPPInsP_4. The techniques we describe in this paper are pertinent to this goal. However, the best approach to this problem may be to analyse the structures of the greater quantities of material that can be obtained from the purified kinases that synthesize the diphosphoinositol polyphosphates [17].

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