Bis(monoacylglycerol) phosphate in rat uterine stromal cells: structural characterization and specific esterification of docosahexaenoic acid

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In rat uterine stromal cells (U_{III} cells), docosahexaenoic acid (DHA) was esterified extensively in alkenylacyl-glycerophosphoethanolamine and in an unknown phospholipid accounting for only 0.7 % of the total phospholipid. The latter was identified as a bis(monoacylglycerol) phosphate (BMP) using MS. Incorporation studies using $C_{18:3}n-3$ and $C_{20:5}n-3$ demonstrated that BMP had a high specificity to incorporate DHA and C_{22} polyunsaturated fatty acids of the $(n-3)$ series. By contrast, polyunsaturated fatty acids of the $(n-6)$ series were never incorporated into BMP. Incubation of U_{III} cells with 5 μM DHA for 24 h increased the DHA content of BMP from 36 to 71 % of the total acyl chains. [^{3}H]DHA-labelled BMP purified as a single TLC spot was resolved into three peaks using HPLC. These peaks were also observed when cells were labelled with [^{3}H]-phosphatidylglycerol, an exogenous BMP precursor, and with [^{33}P]P_i. Electrospray MS of BMP from control cells showed that the first two peaks contained the same molecular species (mainly $C_{22:6}n-3/C_{22:6}n-3$ and $C_{18:1}n-9/C_{22:6}n-3$) while the third peak mainly contained the $C_{18:1}n-9/C_{18:1}n-9$ species. The stereoconfiguration analysis of the compounds revealed an *sn*-glycero-3-phospho-1′-*sn*-glycerol configuration for the first peak and *sn*-glycero-1-phospho-1′-*sn*-glycerol configurations for the other two. BMP from rat testis was used to establish the positions of the acyl groups. More than 70 % of its acyl chains were $C_{22:5}$ $n-6$. It was separated on HPLC into three peaks that co-migrated with the three peaks of BMP from U_{III} cells. Lipase activity and NMR analysis of the second peak showed that fatty acids esterified the primary alcohol group on each glycerol moiety. We conclude that the three peaks are stereoisomeric compounds with different acyl-chain locations and may be the result of different metabolic fates depending on subcellular localization.

Key words: lysobisphosphatidic acid (LBPA), mass spectrometry, molecular species, NMR.

INTRODUCTION

We previously showed that the growth of rat uterine stromal cells (U_{III} cells) is somewhat increased by arachidonic acid, whereas docosahexaenoic acid (DHA) induced a strong dose-dependent inhibition of growth ($IC_{50} = 2$ μM) [1]. Studies with radiolabelled fatty acids of the $(n-3)$ series revealed that U_{III} cells are able to synthesize DHA from various precursors and that two distinct phospholipid (PL) pools accumulate this fatty acid [2]. DHA was found to be esterified in alkenylacyl-glycerophosphoethanolamine (plasmalogens-PE) and rapidly incorporated into another PL that was not identified.

In the first part of the present report, we identify this PL as bis(monoacylglycerol) phosphate (BMP). BMP, also designated as lysobisphosphatidic acid (LBPA), was first detected in pulmonary alveolar macrophages (PAMs) of lung from pig and rabbit by Body and Gray [3] and was shown to be synthesized from phosphatidylglycerol (PG) or lyso-PG [4–6]. Although BMP represents only 1 % of cellular PLs in most mammalian cells, it accumulates in rabbit alveolar macrophages [7–9], macrophage-like Raw 264.7 cells [10] and in several tissues in patients suffering from a number of natural and drug-induced lipid-storage diseases [11,12]. Renkonen's group determined that the stereoconfiguration of BMP from several sources is unique, having an *sn*-glycero-1-phospho-1′-*sn*-glycerol (*sn*-1:*sn*-1′) backbone [13–15], although the *sn*-glycero-3-phospho-1′-*sn*-glycerol (*sn*-3:*sn*-1′) isomer of BMP has also been observed in BHK cells [16]. The *sn*-3:*sn*-1′ isomer was postulated to be an obligatory intermediate in the synthesis of *sn*-1:*sn*-1′ BMP [6,17].

BMP was found to be preferentially located in lysosomal and endosomal membranes [7,10,18–20], where its biosynthesis is thought to occur. Despite numerous studies, the complete biosynthesis pathway from PG to BMP is not fully understood, particularly the step involved in the intramolecular reorientation of the glycerol moiety [6,17,21].

Moreover, the biochemical steps implicated in the fatty acid part of the BMP structure are not known. Conflicting results have been reported on the acyl-chain positions on the glycerol moieties of BMP [7,18], suggesting either multiple BMP isomers or cell-specific pathways for acylation. Different fatty acid compositions have been described for BMP, depending on the source. Oleic acid was found to represent 50–80 % of the acyl chains in BHK cells [13], PAMs [7,9] and rat liver [22]. In contrast, polyunsaturated fatty acids (PUFAs), mainly arachidonic acid and DHA, were found in high proportion in BMP from liver of rats treated with Triton or lysosomic drugs [22,23] and in tissues from patients having a lipidosis; in these latter observations, the high PUFA proportion was associated with a dramatic increase in BMP levels.

Abbreviations used: BMP, bis(monoacylglycerol) phosphate; DHA, docosahexaenoic acid; DPA, docosapentaenoyl chain; FAB, fast-atom bombardment; FAME, fatty acid methyl ester; FCS, foetal calf serum; LBPA, lysobisphosphatidic acid; PA, phosphatidic acid; PAM, pulmonary alveolar macrophage; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PUFA, polyunsaturated fatty acid; *sn*-1:*sn*-1′, *sn*-glycero-1-phospho-1′-*sn*-glycerol; *sn*-3:*sn*-1′, *sn*-glycero-3-phospho-1′-*sn*-glycerol; plasmalogens-PE, alkenylacyl-glycerophosphoethanolamine.

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Incorporation experiments using PAMs demonstrated that exogenous oleic acid, arachidonic acid and DHA are rapidly esterified into BMP [24,25], suggesting a rapid turnover of the fatty acid moiety of the PL. In PAMs and human fibroblasts, labelled DHA was more efficiently esterified into BMP than arachidonic acid [24] and incorporation was assumed to occur via a transacylation process. Various groups have studied the properties of candidate transacylases with the required acyl donor and acceptor specificity [6,21,26] in order to understand the formation of BMP from lyso-PG and/or PG and to explain why BMP is usually rich in PUFA species. Considering the differences in these reports, we felt it necessary (i) to more clearly define the PUFA specificity of BMP in U_{III} cells and (ii) to define its structure in terms of stereoconfiguration of the glycerol moieties and location of the acyl chains. These two points may be of importance with regards to BMP properties and biosynthesis. Moreover, two stereoisomers are found in cells and tissues, the *sn*-1:*sn*-1′ BMP and *sn*-3:*sn*1′ BMP, and earlier studies have reported different fatty acid locations on the glycerol moieties. During the present work, we found that TLC-purified BMP from U_{III} cells was resolved by HPLC into three distinct compounds that were characterized by MS, fatty acid composition and configuration of the glycerol moiety.

EXPERIMENTAL

Chemicals

M199 medium with Phenol Red, PBS and foetal calf serum (FCS) was obtained from TechGen International (Les Ulis, France). [1-^{14}C]Linoleic acid (52 mCi/mmol), [1-^{14}C]linolenic acid (50 mCi/mmol), [1-^{14}C]arachidonic acid (52 mCi/mmol), [4,5-^{3}H]DHA (58 Ci/mmol) and [^{33}P]P_i (100 Ci/mg) were obtained from New England Nuclear (Boston, MA, U.S.A.), [5,6,8,9,11,12,14,15,17,18-^{3}H]eicosapentaenoic acid (152 Ci/mmol) was obtained from Isotopchim (Ganagobie, France). [1,2,3-^{3}H]*sn*-3:rac-PG (herein referred to as [1,2,3-^{3}H]PG, 30 mCi/mmol), [3-^{3}H]*sn*-3:rac-PG (referred to as [3-^{3}H]PG, 15 mCi/mmol) and [1-^{3}H]*sn*-3-phosphatidylethanolamine ([1-^{3}H]PE, 15 mCi/mmol) were generous gifts from Dr M. Waite (Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC, U.S.A.); their synthesis and characterization were reported previously [6,17]. Phospholipase C (PLC, *Bacillus cereus*), phospholipase D (PLD, cabbage), phospholipase A_2 (PLA_2, porcine pancreas and *Crotalus adamanteus*), lipase (*Rhizopus arrhizus*), standard free fatty acids, fatty acid methyl esters (FAMEs) and PLs were obtained from Sigma (St Louis, MO, U.S.A.). Silica gel 60 TLC plates (20 × 20 cm, 0.25 mm) were from Merck AG, Darmstadt, Germany. The solvents and reagents used were of HPLC grade from Fluka (Basel, Switzerland).

Cell cultures and incorporation of radioactive fatty acids and PLs

U_{III} cells were grown in Falcon plastic flasks (75 cm²) in a 95% air/5% CO_2 humidified atmosphere at 37 °C. For stock cultures, the medium M199 contained 5% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells between passages 51 and 60, subcultured weekly [27], were seeded in 75-cm² Petri dishes and allowed to attach. The medium was replaced 24 h later with M199 containing 5% FCS. Confluent cultures were obtained 4–5 days later and were used as control cells and for labelling studies with fatty acids, PL and P_i. The medium was removed and cells [(0.5–2.0) × 10^7] were incubated for 0.5–72 h with fresh medium, with or without FCS, containing the labelled precursors. Fatty acids (5 nM–5 μM) and PL (1 μM) were added to the medium which was then sonicated for 10 min before use; [^{33}P]P_i (100 μCi/ml) was added directly to the corresponding medium without phosphate. At the end of incubation, the medium was removed and counted for radioactivity to estimate the amount of material taken up by the cells. The cells were washed twice with 10 ml of PBS, 5 ml of methanol was added, and the cells were scraped and used for lipid analysis and BMP purification. For labelling studies using cell suspensions, trypsinized cells were washed with PBS, resuspended in M199 (30 × 10^7 cells/ml), then 1 ml of medium containing the radioactive fatty acid was added. The suspension was incubated continuously at 37 °C and, at indicated times, 0.5 ml of the suspension was transferred into 5 ml of methanol/chloroform (1:1, v/v).

The cell number was determined on corresponding control cultures by measuring the total DNA [28]. For each experiment three to five independent assays were performed.

Lipid extractions

Total cell lipids were extracted using the Bligh and Dyer procedure [29] in the presence of 0.01% butylated hydroxytoluene, dried under vacuum and dissolved into chloroform/methanol (2:1, v/v). The radioactivity in aliquots of the total lipid extracts was determined. Total lipids from 10–120 g of rat testis were extracted using the Bligh and Dyer procedure and neutral lipids were removed using Sep-Pack silica chromatography (Waters, Milford, MA, U.S.A.). Total PLs were then submitted to PLC hydrolysis [30] using 50 units of PLC for 2 mg of PL and, after extraction, the diradyl glycerols were removed by Sep-Pack chromatography. The Sep-Pack was then eluted with methanol, and BMP and polar lipids not degraded by PLC were purified by TLC and HPLC.

TLC separation of lipid and PL classes

The radiolabelled lipid classes from U_{III} cells were separated by TLC using hexane/diethylether/acetic acid (40:10:1, by vol.) as the developing solvent. The radioactive bands were detected and integrated using a Berthold LB 511 TLC analyser (Berthold, Wildbad, Germany). The silica gel containing total PL was scraped and the fatty acid esters were converted directly to FAMEs as described previously [31]. In order to determine the distribution of radiolabel within individual PL, total PL were first purified using Sep-Pack silica chromatography. The individual PL classes were then separated by TLC using either chloroform/methanol/acetic acid/water (50:25:8:2, by vol.) or chloroform/methanol/40% methylamine (65:22:4, by vol.) as acidic and basic systems, respectively. The radioactive bands were detected and integrated using the TLC analyser, visualized under UV light after spraying with 0.05% 2′-7′-dichlorofluorescein in methanol and compared with known standards. The PL bands corresponding to BMP and other glycerophospholipids were scraped and the gel was either extracted using 3 × 4 ml of chloroform/methanol/water (5:5:1, by vol.) to isolate PLs for further analysis or was treated directly to obtain FAMEs. BMP was purified from total cellular PLs and from methanolic samples obtained from rat testis by TLC using the basic system (R_F values: BMP = 0.75; PE = 0.66; PG = 0.63) and, after extraction, checked for purity using the acidic system for TLC (R_F values: BMP = 0.73; PE = 0.48; PG = 0.64).

MS

Liquid secondary-ion MS (fast-atom bombardment, FAB) mass spectra and B/E-linked scans were obtained using a Micromass

Autospec tandem mass spectrometer using a 20-kV caesium beam (2 μA) under negative- or positive-ion conditions. Samples were desorbed from a glycerol matrix and ion species were accelerated to 8 kV. Daughter-ion-linked scans (B/E) were used to identify the fatty acids and the structure of glycerol linkages. The collision-activated dissociation (CAD) spectra were obtained by B/E-linked scanning using helium as a collision gas in the first field-free region of the mass spectrometer. All mass spectra were recorded using OPUS software. Electrospray MS analysis was conducted by the method of Lehmann et al. [32]. Samples, dissolved in methanol/dichloromethane (1:1, v/v), were injected into a VG quattro II triple quadrupole mass spectrometer and data analysed using Masslynx software. Parent ions were separated in the first quadrupole, collisioned in the second quadrupole and daughter ions corresponding to acyl chains esterified in BMP were detected in the third quadrupole.

NMR spectroscopy

BMP samples (170–750 μg) were dissolved in 100 μl of either [^{2}H]chloroform or [^{2}H]chloroform/[^{2}H]methanol (8:2, v/v) in a 5- or 2.5-mm sample tube. NMR spectra were recorded at 300 K with a Bruker DRX500 spectrometer. Unambiguous ^{1}H and ^{13}C assignments of BMP were established by heteronuclear multiple-bond correlation (HMBC) experiments [33].

HPLC analysis

Radioactive FAMEs were separated by reversed-phase HPLC using a 4.6 × 250-mm Ultrabase C_{18} column (SFCC, Shandon, Eragny, France) with 5-μm spherical particles and a Hewlett Packard 1190 system fitted with a diode array detector. The mobile phase was acetonitrile/water (80:20, v/v) with a flow rate of 2.0 ml/min. The absorbance was monitored at 205 nm and eluting lipids were identified by comparison with authentic standards. The radioactivity was determined by continuous-flow liquid scintillation counting using a Radiomatic Flow One β detector. The detector was operated with Ultima-Flow AP (Packard) at a flow rate of 3 ml/min in a 0.85-ml cell and the UV absorbance and radioactivity of the lipids was determined as described previously [2]. Total PLs from labelled cells were separated by HPLC on silica-NH_2 columns as described by Bernhard et al. [34] using UV detection at 205 nm, 0.9 ml/min flow rate and continuous radioactive detection.

HPLC analysis of purified BMP from U_{III} cells and rat testis was performed on silica-NH_2 (two tandem 200 × 4.6-mm columns were used to increase separation) using a flow rate of 1.2 ml/min for the solvent (acetonitrile/methanol/water/50% methyl phosphonic acid, 2920:1000:60:1, by vol.; pH adjusted to 6.2 with 28% NH_4OH). Peak detection was monitored at 205 nm and the radioactivity measured by continuous flow analysis. In some experiments, separate BMP peaks were collected, the solvent was partially removed under N_2 and PLs were extracted using the Bligh and Dyer procedure [29] with an additional washing step to remove methyl phosphonic acid. Samples were then analysed for fatty acid composition, MS and NMR.

GC

Fatty acid compositions were determined by GC after transmethylation. FAME and dimethylacetal derivatives were analysed with a Hewlett Packard 5590 gas chromatograph. The capillary column was a SP2380 (0.32 mm, 30 m; Supelco, Bellefonte, PA, U.S.A.). The temperature was programmed from 145 to 225 °C at 1.2 °C/min; helium was used as a carrier gas. FAME and dimethylacetal derivatives were identified by comparison with the relative retention times of known standards. The percentage and mass of each fatty acid were computed using an internal standard (pentadecanoic acid methyl ester) as described previously [31].

Biochemical and chemical analysis, and radioactivity measurements

Purified BMP (after TLC purification or HPLC separation) from cells and rat testis were submitted to various enzymic degradation procedures according to published protocols for PLC [30], PLD [35], PLA_2 [36] and *R. arrhizus* lipase [37]. Degradation products as well as non-reactive material were then measured by either GC and HPLC analysis (study of fatty acid release) or by TLC and HPLC (phosphate ester bond hydrolysis). Control experiments using equivalent amounts of labelled PE, phosphatidylcholine (PC) and PG were performed in order to standardize the extent of hydrolysis. The phosphorus content of total lipid extracts, PL classes and BMP samples was measured using the Bartlett procedure [38]. Radioactivity was measured using a Tricarb 460 spectrometer (Packard) and Ultimagold as scintillation fluid; lipid extracts were counted after drying under N_2.

Analysis of stereoconfiguration

Cells were labelled for 24 h with 1 μM [3-^{3}H]PG and total lipids were extracted. Labelled BMP was purified by TLC and then by HPLC in order to eliminate possible residual contamination by [3-^{3}H]PG and to separate the individual peaks of BMP. Control PL ([3-^{3}H]PG and [1-^{3}H]PE), total and BMP peaks were then analysed according to the procedure of Waite's group [6,39].

Statistical analysis

Data are expressed as means ± S.E.M. Statistical significance ($\alpha = 0.05$) was determined using the Mann–Whitney or Kruskal–Wallis non-parametric rank tests followed, when necessary, by a multiple comparison procedure.

RESULTS AND DISCUSSION

Characterization of U_{III} cell BMP

In the non-transformed rat uterine stromal cell line (U_{III} cells), exogenous DHA has been found to specifically exert a dose-dependent inhibition of cell proliferation [1] and to accumulate into PE-plasmalogens as well as into a minor unidentified PL [2]. This PL had an R_F value in different TLC systems between those of PE and tetraoleyl-bis phosphatidic acid (PA), and did not correspond to any of the standard PLs tested, including PG, PA and cardiolipin. To elucidate its structure, U_{III} cells were incubated with 5 μM [^{3}H]DHA (5 μCi) for 24 h, the total lipids were extracted and separated by TLC with the basic TLC system. The unknown compound was eluted, purified further by TLC using the acidic system, extracted and submitted to MS. The overall recovery of the ^{3}H in this PL was 66 ± 4% (mean ± S.E.M.; $n = 4$); the phosphorus assay on the purified PL represented only 0.7 ± 0.1% of the total cell PLs (62 ± 5 nmol of total PL/10^6 cells). Negative FAB-MS of this PL (Figure 1) provided a spectrum mainly composed of molecular ions (865.6 and 819.6) corresponding to PG masses and intense fragments at m/z 171, 153, 97 and 79 characteristic of the glycerophosphate structure (results not shown). When linked scan analysis was performed for molecular ions 865 and 819 and compared with linked scan analysis of dioleyl-PG and 1-palmitoyl,2-oleyl-PG, differences were noted (results not shown). Since a daughter ion of mass (M-H)-74 produced by the loss of a glycerol moiety was

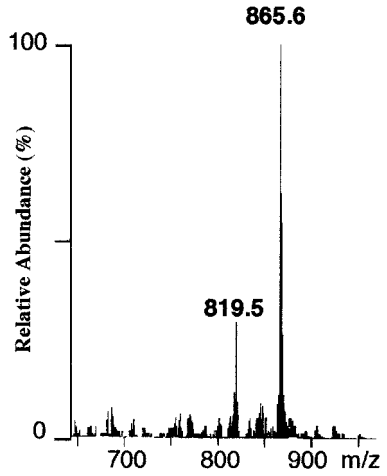

Figure 1 Typical mass spectrum of the minor PL isolated from U_{III} cells and purified by TLC

Confluent U_{III} cells were incubated for 24 h with 5 μM [^{3}H]DHA, then PLs were extracted and separated by TLC using the basic eluent. The labelled unknown PL was eluted from the gel and checked for purity by TLC with the acidic eluent. The purified sample was then submitted to negative FAB mass analysis. Further details can be found in the Experimental section. Extension of the 600–1000 *m/z* scale shows major molecular ions at *m/z* 865 and 819 corresponding to BMP esterified with two DHA and DHA/oleic acid, respectively.

Table 1 Fatty acid composition of BMP isolated from control U_{III} cells and cells incubated for 24 h with 5 μM DHA

Duplicate dishes of cells were cultured with standard medium up to confluency. Then, cells were cultured for 1 day with standard medium (control cells) and with standard medium supplemented with 5 μM DHA (supplemented cells). At the end of incubation, cells were washed twice, lipids extracted, and BMP isolated by TLC as described in the Experimental section. FAMEs from BMP were analysed by GC. Results are expressed as mol% ± S.E.M. for three independent experiments; tr, trace amount; nd, not detected. For simplification, mono-unsaturated fatty acid isomers have been pooled.

Fatty acid	Control cells	Supplemented cells
16:0	5.6 ± 1.1	3.7 ± 0.8
16:1	0.2 ± 0.2	tr
18:0	3.3 ± 0.7	2.5 ± 0.4
18:1	29.6 ± 1.4	12.3 ± 0.9
18:2 (*n*−6)	2.2 ± 0.5	0.4 ± 0.3
18:3 (*n*−3)	nd	nd
20:0	nd	nd
20:1	0.1 ± 0.1	tr
20:2 (*n*−6)	nd	nd
20:3 (*n*−6)	tr	tr
20:4 (*n*−6)	3.7 ± 0.5	1.2 ± 0.4
20:5 (*n*−3)	tr	tr
22:0	nd	nd
22:1	nd	nd
22:4 (*n*−6)	6.0 ± 0.9	1.1 ± 0.3
22:5 (*n*−6)	3.1 ± 0.7	2.4 ± 0.4
22:5 (*n*−3)	7.6 ± 1.1	5.5 ± 0.7
22:6 (*n*−3)	36.0 ± 3.1	70.8 ± 2.3

always observed for PG but not for the unknown compound, we concluded that the unknown was BMP for which the two glycerol moieties were esterified by one fatty acid, regardless the acyl position. Daughter ions of 865.6 were identified at *m/z* 327.2 [$RCOO^-$], 481.2 [M-$RCOOCH_2$-CHOH-CH_3] and 555.3 [M-RCO], indicative of the presence of $C_{22:6}n-3$. Daughter ions of 819.6 were for oleic acid as a second acyl chain in BMP. Thus U_{III} cells incubated for 24 h with 5 μM DHA contain BMP in which the major molecular species are $C_{22:6}n-3/C_{22:6}n-3$ and $C_{18:1}n-9/C_{22:6}n-3$. Using negative FAB, similar spectra and data were reported by Holbrook et al. [40] who also identified BMP from PC12 cells, but with different molecular species.

Fatty acid esterification into BMP

GC analysis of FAMEs from BMP extracted from cells cultured with or without 5 μM DHA for 24 h was performed. In control cells (Table 1), BMP contained over 50 % of C_{22} fatty acids, of which $C_{22:6}n-3$ was predominant, about 30 % of oleic acid and 9 % of saturated $C_{16:0}$ and $C_{18:0}$ fatty acids. Arachidonic acid and other C_{20} PUFAs were only present in trace amounts. The fatty acid composition of BMP was very different from those observed for the major PL classes of control U_{III} cells, in which the proportions of DHA and total PUFAs were significantly lower [2]. Taking into account the proportions of cellular PLs and their fatty acid compositions in confluent U_{III} cells, we calculated that about 10 % of the cellular DHA was esterified in BMP. The fatty acid composition of U_{III} cell BMP was also quite different from those reported for this lipid in other cell types studied [7,9,13,40]. For example, in BHK cells, oleic acid represents 80 % of the total fatty acids esterified in BMP and only a small proportion of PUFAs was observed in this model. As expected from MS spectra (Figure 1), cell incubation with DHA resulted in a dramatic increase of this fatty acid in BMP, which was compensated for by a significant decrease in the proportion of unsaturated and saturated fatty acids. Except for $C_{22:4}n-6$, the proportions of C_{22} PUFAs were weakly affected by the incubation with DHA, suggesting that these fatty acids might also be incorporated easily into BMP, as observed for DHA. Determination of fatty acid contents and phosphorus assays on purified BMP samples revealed that the cellular BMP content (0.54 ± 0.08 nmol/10^6 cells) was not affected by incubation with DHA. These results suggest that $C_{22:6}n-3$ is able to replace other fatty acids in existing pools of BMP.

To better understand the origin of the C_{22} fatty acids esterified in BMP, confluent cells were incubated for up to 72 h with 5 μM radioactive $C_{18:2}n-6$, $C_{20:4}n-6$, $C_{18:3}n-3$ and $C_{20:5}n-3$. These fatty acids have been found to be converted rapidly by U_{III} cells into longer homologues [2]. Table 2(a) shows that when cells were incubated with the precursors of DHA there was a time-dependent increased esterification into BMP. More radioactivity was incorporated into this PL when $C_{20:5}n-3$ was used as compared with $C_{18:3}n-3$, but, in both cases, the esterification was significantly lower than that of DHA. HPLC analysis of FAMEs obtained for all PL classes after 8-, 24- and 72-h cell incubations with 5 μM [^{3}H]DHA was then performed to determine the nature of the labelled fatty acid in each class. At all times and for all classes, more than 95 % of the radioactivity was recovered as [^{3}H]DHA and only trace amounts of radioactivity were recovered as $C_{22:5}n-3$ and $C_{20:5}n-3$. When the distribution of radiolabel among fatty acids was determined in PLs after labelled PUFA incubations, a specificity towards fatty acid esterification appeared for BMP (Table 2b). No detectable radioactivity could be observed in BMP when cells were incubated with radioactive linoleic and arachidonic acids, despite the fact that substantial amounts of labelled $C_{22:4}n-6$ and $C_{22:5}n-6$ were synthesized by the cells (results not shown). After 48 h of incubation with [^{14}C]$C_{18:3}n-3$, about 8 % of the radioactivity incorporated by the cells was recovered as labelled DHA whereas 60 and 30 % was recovered as $C_{22:5}n-3$ and $C_{20:5}n-3$, respectively (Table 2b). PC, PE and BMP exhibited very different distributions of the label. About 70 % of the total radioactivity

Table 2 Specificity of PUFA incorporation into BMP by U_{III} cells

(a) Confluent U_{III} cells were incubated with the labelled fatty acid stated (5 μM) for the indicated times. Cellular PLs were then separated by TLC and their radioactivity measured. The results represent the total radioactivity recovered in BMP and are expressed as the percentage radioactivity in the total PLs; mean ± S.E.M. for three or four independent determinations. nd, not detected; tr, trace amount. (b) Cells were incubated for 48 h with labelled linolenic acid or labelled eicosapentaenoic acid, the total PL, PC, PE and BMP classes were converted to their respective FAMEs, which were then analysed by reversed-phase HPLC as described in the Experimental section. Radioactivity associated with each radiolabelled fatty acid was measured and expressed as the percentage total radioactivity in the PL class. Results are means of three determinations; coefficients of variation were all under 10%, thus for simplification S.E.M. values were not reported.

(a)

Time (h)	[^{14}C]18:3 ($n-3$)	[^{3}H]20:5 ($n-3$)	[^{3}H]22:6 ($n-3$)
1	nd	nd	7.1 ± 0.2
2	nd	0.2 ± 0.1	9.3 ± 0.3
8	0.2 ± 0.1	1.9 ± 0.4	11.2 ± 0.3
24	1.1 ± 0.2	4.4 ± 0.4	14.5 ± 0.5
48	4.5 ± 0.4	6.9 ± 0.5	17.5 ± 0.4
72	6.7 ± 0.5	10.4 ± 0.8	18.1 ± 0.7

(b)

	Precursor ...	[^{14}C]18:3 ($n-3$)				[^{3}H]20:5 ($n-3$)		
PL class	Product ...	18:3	20:5	22:5	22:6	20:5	22:5	22:6
Total PLs		0.3	29.6	60.8	8.1	16.4	72.1	11.4
PC		tr	37.6	60.4	2.6	16.0	74.3	3.1
PE		0.5	16.1	63.3	18.2	12.7	69.3	23.4
BMP		nd	7.4	20.9	69.4	8.3	21.1	71.3

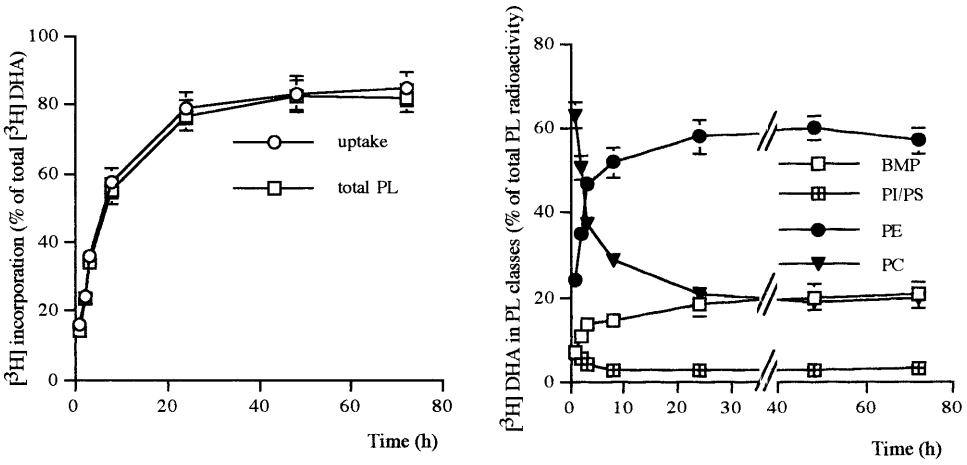

Figure 2 Radioactivity distribution within PL classes of U_{III} cells after incubation with radioactive DHA

Confluent U_{III} cells (5×10^6 cells/dish) were incubated with 5 μM [^{3}H]DHA (2 μCi) for the indicated times. At the end of incubation, cells were collected, their lipids extracted, PL classes separated by TLC and their radioactivity measured as described in the Experimental section. Uptake and total PL correspond to the percentage of initial [^{3}H]DHA incorporated into the cells (○) and incorporated into total PLs (□), respectively (left-hand panel). ^{3}H Radioactivity incorporated into each PL class was expressed as the percentage of activity incorporated into total PLs (right-hand panel). Results are means ± S.E.M. for independent triplicates. PI/PS, phosphatidylinositol/phosphatidylserine.

found in BMP was attached to DHA while this fatty acid represented only 18 and 3% of the label in PE and PC, respectively. The same patterns were observed when cells were incubated with [^{3}H]$C_{20:5}n-3$ (Table 2). These results, together with those presented in Figure 1 and Table 1, show that, regardless of the source of DHA (formed by elongation/desaturation or by direct addition), DHA was esterified selectively into BMP.

Then, a kinetic study of DHA uptake by U_{III} cells was undertaken. Uptake was nearly completed by 24 h and about 80% of the incubated DHA was found in cells. More than 90% of the incorporated radioactivity was in PL (Figure 2, left-hand panel). [^{3}H]DHA incorporation initially occurred into PC (Figure 2, right-hand panel). The percentage of [^{3}H]DHA recovered in PC and phosphatidylserine/phosphatidylinositol rapidly decreased with a concomitant increase in BMP and PE, which was nearly complete by 24 h. Free [^{3}H]DHA and [^{3}H]DHA in neutral lipids were maximal after 1 h of incubation, and represented 5 and 7% of the total cell radioactivity, respectively (results not shown). After 24 h of incubation, the ^{3}H distribution within PL classes remained stable and PC, PE, phosphatidylserine/phosphatidylinositol and BMP contained 19 ± 2, 58 ± 2, 3 ± 1 and 18 ± 1% of the total radioactivity incorporated in cells, respectively. At this time, the ^{3}H-specific radioactivity in BMP

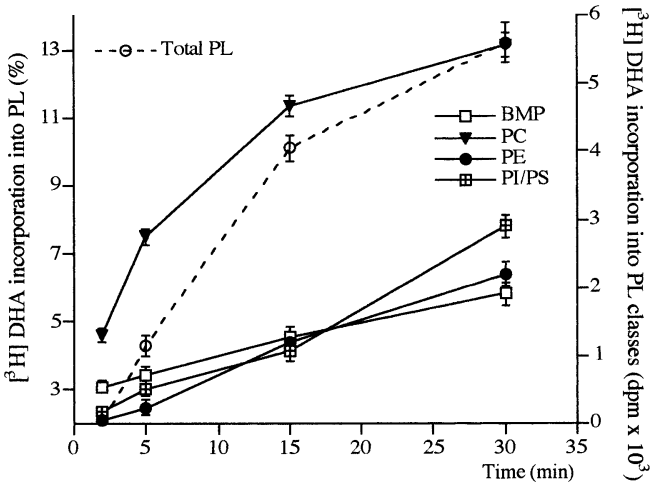

Figure 3 Radioactivity in PL classes of U_{III} cells incubated with radioactive DHA

Confluent U_{III} cells (55×10^6 cells) were suspended in 5 ml of culture medium supplemented with 0.1 μM [^{3}H]DHA (15 μCi) for the indicated times. At the end of incubation, aliquots of cell suspension were mixed immediately with 4 vols of methanol, lipids were extracted, PL classes separated by TLC and their radioactivity measured as described in the Experimental section. Total PL corresponds to the percentage of initial radioactivity incorporated into total PLs. ^{3}H Radioactivity incorporated into each PL class was expressed as d.p.m. Results are means $\pm$ S.E.M. for independent triplicates. PI/PS, phosphatidylinositol/phosphatidylserine.

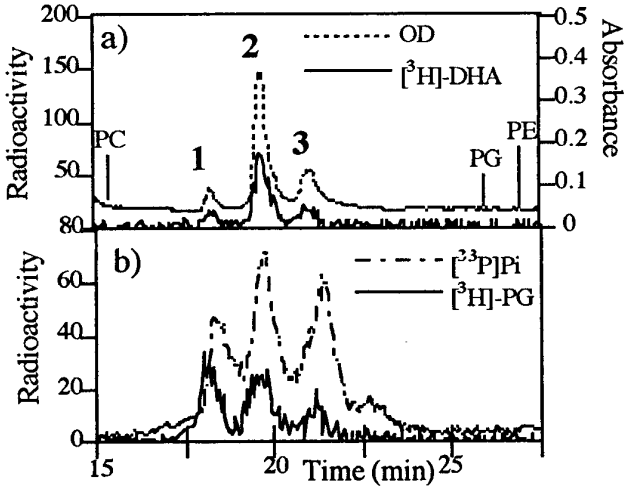

Figure 4 HPLC analysis of BMP

(**a**) Confluent control cells were incubated for 24 h with [^{3}H]DHA. Labelled BMP was purified by TLC and then analysed by HPLC using silica-NH_2 columns as described in the Experimental section. Absorbance (OD, arbitrary units) was recorded at 205 nm and radioactivity was measured online. BMP was eluted as three peaks (1, 2 and 3); PC, PG and PE correspond to the respective elution times for standard PC, PG and PE. (**b**) Control cells were incubated for 24 h with either [^{33}P]P_i or [^{3}H]PG. Labelled BMP was then analysed by HPLC. Chromatograms are from one experiment but are representative of more than 15 analyses.

(d.p.m./nmol of PL phosphorus) was 13 ± 2- and 47 ± 4-fold higher than observed for PE and PC, respectively. Since after 2 h of incubation the radioactivity associated with DHA and neutral lipids was low, modifications of the [^{3}H]DHA distribution within PL classes reflected mainly a transacylation process rather than direct acylation. Our results suggest that either PC is an acyl donor to PE and BMP or the two latter, being richest in DHA, are connected together for transacylation.

Short incubations of cells in suspension were done to examine these possibilities. As shown in Figure 3, [^{3}H]DHA was rapidly esterified into BMP (accounting for its low amount) and PC. After 2 min of incubation, 25 % of the radioactivity incorporated in PL was in BMP, 60 % in PC and only a trace amount was in PE. For incubation times less than 15 min, the radioactivity incorporated in BMP was greater than in PE, suggesting that PE was not an acyl donor to BMP. Taking into account the respective pool size of BMP and PC, our results suggest that DHA incorporation into BMP occurred first by direct acylation rather than transacylation from PC. For longer incubation times, we could not exclude the possibility of a transacylase process. BMP was shown to be synthesized from PG or lyso-PG [4–6] but the pathways are not fully known [21]. This PL is exclusively located in the endosomal/lysosomal compartment of the cell [18–20] and was found in high proportions in late endosomes [20]. Acylation of the BMP glycerophosphate backbone by fatty acids is thought to involve mainly transacylases [17,24,41] and to occur either during the conversion of PG to *sn*-1:*sn*-1′ BMP [6,21] or after its complete synthesis by a remodelling process, as suggested by Huterer and Wherrett [8]. Both lysosomal PLA_1 [41] and transacylase [21] activities have been suggested in the putative biosynthetic pathway of BMP, neither of which would explain the esterification of PUFAs into BMP. If a transacylase is involved in the BMP esterification, our data suggest that it should be rather specific for C_{22} PUFAs of the $(n-3)$ series or should use a donor containing a high proportion of PUFAs. A better knowledge of the PL and fatty acid compositions of the different endocytic vesicles should be of interest to understand the mechanism(s) involved in the acylation of BMP. Previous results have shown that arachidonic acid as well as DHA is rapidly incorporated into BMP of fibroblasts [24]. A high proportion of DHA has been also reported in BMP of rat liver after Triton and chloroquine treatment [11,12,18] and in PC12 cells [40]. Arachidonic acid was shown to incorporate rapidly in PAMs in which it represented about 20 % of total fatty acids, whereas oleic acid represented about one-half [25]. In this model, other PUFAs of the $(n-6)$ series were also present in substantial amounts, by contrast to what was observed for U_{III} cells. As indicated below, BMP isolated from rat testis contains a very high proportion of PUFAs of the $(n-6)$ series, especially 22:5 $(n-6)$, and low proportions of mono-unsaturated and saturated fatty acids. Taken together, these data suggest that the fatty acid composition of BMP is intimately dependent on the model studied and could correspond either to the cellular function of BMP, as described for PAMs [9,25], or to the capacity of the cell organelles to synthesize and/or accumulate PUFAs.

Structural analysis of BMP

Since BMP is found in cells and tissues as both *sn*-3:*sn*-1′ and *sn*-1:*sn*-1′ isomers [6,13,16,39] and possibly with different acyl positions [7,9,18], assays using phospholipase and lipase hydrolysis were conducted with the [^{3}H]DHA-labelled BMP isolated from U_{III} cells. Whatever the assay conditions used, no significant hydrolysis of BMP was obtained with PLC from *B. cereus*, PLD from cabbage and PLA_2 from porcine pancreas or from *C. adamanteus*, despite a near complete degradation of standard PLs and [^{3}H]DHA-labelled PC and PE under the same conditions. When BMP was incubated with lipase from *R. arrhizus*, 43–64 % of [^{3}H]DHA was released from BMP, while total release of the *sn*-1 fatty acid from PC, PE and PG was obtained. These results suggest several possibilities: (i) BMP from U_{III} cells has an abnormal stereoconfiguration (*sn*-1) at the first glycerol moiety; (ii) a significant proportion of the fatty acid is esterified on

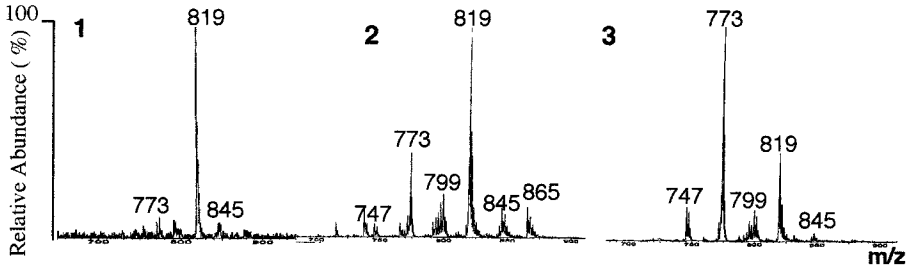

Figure 5 Parent ions from control-cell BMP peaks separated by HPLC

TLC-purified BMP from control cells was analysed by HPLC as described in the Experimental section and resolved into three peaks as indicated in Figure 4. Individual peaks (1, 2 and 3 corresponding to increasing retention times) were collected, extracted and analysed by tandem ESI MS. Major molecular ions are indicated for each compound. The data shown are from one experiment representative of the three experiments performed.

Table 3 Major molecular ions and molecular species identified in the three BMP peaks resolved by HPLC

TLC-purified BMP from control cells (−DHA) and from cells incubated for 24 h with 5 μM DHA (+DHA) was resolved into three peaks (1–3) by HPLC as indicated in Figure 4. Peaks were collected, extracted and analysed by tandem MS as described in the Experimental section. Major molecular ions detected for each sample are reported as well as their direct relative abundances (not corrected for isotopic masses). Daughter-ion analysis was performed to establish the molecular species. The species in greatest abundance was set at 100 and others were calculated relative to that.

	Molecular ion ...	747	773	819	821	845	865	867	869
Peak	Species ...	18:1/16:0	18:1/18:1	22:6/18:1 22:5/18:2	22:6/18:0 22:5/18:1	22:6/20:2 22:5/20:3	22:6/22:6	22:6/22:5	22:5/22:5 22:6/22:4
+DHA									
1		–	4	46	–	12	100	54	48
2		–	6	56	7	16	100	43	36
3		7	26	100	45	7	3	–	–
−DHA									
1		–	12	100	28	5	–	–	–
2		–	37	100	13	14	–	–	–
3		10	100	36	–	–	14	–	–

secondary alcohols of glycerol(s); or (iii) U_{III} cell BMP is a mixture of isomers with different stereoconfigurations and fatty acyl locations.

Using HPLC with silica-NH_2 columns, we were able to resolve BMP from control or [^{3}H]DHA-supplemented cells into three peaks (peaks 1–3; Figure 4). These peaks had retention times very different from that of PG and of other cellular PLs. Since in several models BMP biosynthesis is reported to occur from labelled P_i [8,20] or from exogenously added PG [4,5], similar studies were conducted with U_{III} cells. The three separated peaks were always labelled, but to different extents, when [^{33}P]P_i or [^{3}H]PG were used as precursors (Figure 4). The same relative distribution of the three compounds, with each labelled precursor, was obtained when paired samples consisting of total PL freshly extracted from the cells and TLC-purified BMP were compared by HPLC. Therefore, we conclude that the three BMP peaks were not formed during purification steps by acyl migration as reported for lyso-PL [42], but more probably corresponded to related compounds produced by the cell.

For control cells, the proportions of each compound, expressed as absorbance units at 205 nm, were 14 ± 2, 42 ± 4 and $41\pm3\%$ ($n=4$) for peaks 1–3 respectively. These proportions were altered markedly after 24 h of incubation of cells with 5 μM [^{3}H]DHA, to 16 ± 1, 61 ± 3 and $24\pm2\%$ of the total, respectively. The [^{3}H]DHA distribution (13 ± 1, 65 ± 4 and $22\pm3\%$) was very close to the optical trace, as indicated in Figure 4. The individual peaks collected after HPLC were estimated to be 12, 38 and 45 mol% in control cells, when quantified using phosphorus assay. These proportions were not significantly modified after incubation of the cells with DHA.

Electrospray tandem MS was then used to compare and identify the molecular species of these three BMP peaks. Typical spectra obtained for individual peaks from control cells showed differences between them in terms of relative abundance and m/z of parent ions (Figure 5): m/z at 773 was the predominant ion in peak 3 while peaks 1 and 2 contained mostly BMP with m/z at 819. Since all the parent ions detected could be a mixture of different species, daughter ions were determined. Peak 819 yielded fatty acid daughter ions at m/z 281 ($C_{18:1}n-9$), 327 ($C_{22:6}n-3$), 279 ($C_{18:2}n-6$) and 329 ($C_{22:5}n-6$) (results not shown), which permitted identification of $C_{22:6}n-3/C_{18:1}n-9$ and $C_{22:5}n-6/C_{18:2}n-6$ as the molecular species. Table 3 reports the relative abundance of the molecular species for the three BMP peaks from control and DHA-incubated cells. These results show that in both sets of experimental conditions used, BMP peak 3 contained less PUFAs than the two other peaks and a higher proportion of oleic acid. Addition of DHA in the culture medium resulted in an increased DHA proportion in the three BMP peaks, with a high level of $C_{22:6}n-3/C_{22:6}n-3$ species in peaks 1 and 2. Since the three BMP peaks were clearly resolved by HPLC but contained common major molecular species (see peaks 1 and 2 in Figure 5), their resolution cannot be

Table 4 Stereoconfiguration analysis of U_{III} cell BMP

U_{III} cells were incubated for 24 h with 1 μM [3-^{3}H]PG (0.2 μCi/dish), cells were scraped and total lipids extracted. BMP was purified by TLC and HPLC as described in the Experimental section. Total BMP or individual peaks were collected and then submitted to stereoconfiguration analysis to determine the relative positions of the radiolabel and phosphodiester bond. [1-^{3}H]*sn*-3-PE was derived from *Escherichia coli* incubated with [1-^{3}H] glycerol. [3-^{3}H]PG was obtained by a transphosphatidylation reaction using [3-^{3}H]PE, glycerol and savoy cabbage PLD. Between 10000 and 50000 d.p.m. of each sample and control were analysed. The results are the means of three determinations with a deviation of less than 4%.

Substrate	Filtrate (%)	Precipitate (%)
[1-^{3}H]PE	9	91
[3-^{3}H]PG	87	13
^{3}H-Labelled BMP	28	72
Peak 1	59	41
Peak 2	30	70
Peak 3	25	75

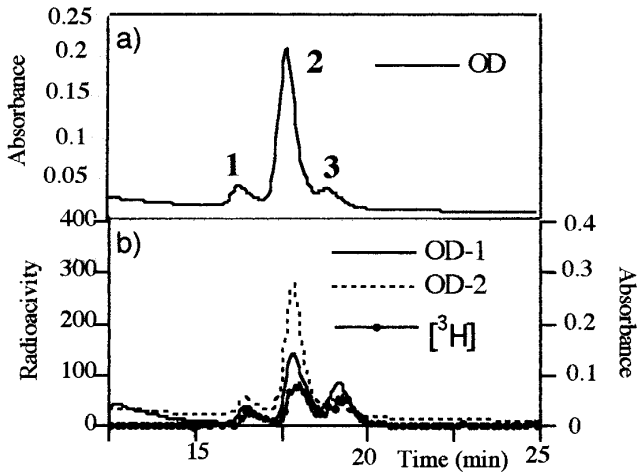

Figure 6 HPLC analysis of purified rat testis BMP

(**a**) Rat testis BMP was extracted and purified by TLC as mentioned in the Experimental section and then analysed by HPLC. Absorbance (OD) was recorded at 205 nm. BMP was eluted as three peaks (1, 2 and 3). (**b**) When TLC-purified rat testis BMP was co-injected with purified [^{3}H]DHA-labelled BMP from U_{III} cells (OD-2) the three BMP peaks of the two different sources co-eluted. OD-1 represents the absorbance obtained with cellular BMP alone and [^{3}H] indicates the radioactivity measured in this sample.

explained by differences in fatty acid length and degree of unsaturation but possibly by different acyl positions on the glycerol moieties and/or glycerol configurations. Moreover, these results demonstrated a clear difference between the BMP components in their ability to accumulate DHA, which suggests a difference in their biosynthetic pathways and/or in their cellular location.

When U_{III} cells were incubated for 24 h with 1 μM [3-^{3}H]PG, 27–32% of the ^{3}H label was taken up. HPLC analysis of the ^{3}H distribution in cellular lipids showed that 83 ± 5% of the radioactivity ($n = 4$) was recovered in BMP, about 10% in [3-^{3}H]PG and less than 5% in lysoPG. ^{3}H-Labelled BMP peaks were then recovered and submitted to the well-established stereoconfiguration assay described by Waite's group [6,39]. As indicated in Table 4, expected results were obtained with control experiments on [1-^{3}H]PE and [3-^{3}H]PG. When total ^{3}H-labelled BMP was analysed, 72% of the label was associated with the dimedone-formaldehyde precipitate and 28% was recovered in the filtrate. Taking into account the positional ^{3}H distribution in the BMP precursor [3-^{3}H]PG and the accuracy of the method, we estimate that 70–80% of the BMP molecules were reoriented and had an *sn*-1 configuration for the labelled glycerol instead of the usual *sn*-3 configuration found in the other PLs. Analysis of individual BMP peaks showed that more than 85% of peaks 2 and 3 had an *sn*-1 configuration for the labelled glycerol. By contrast, about 65% of BMP from peak 1 exhibited an *sn*-3:*sn*-1′ configuration, indicating that this compound was either a real mixture of the two stereoisomers or was contaminated by peak 2, which was about 3-fold more abundant.

Table 5 Fatty acid composition of rat testis BMP peaks resolved by HPLC

Rat testis BMP was extracted and purified by TLC as described in the Experimental section. Peaks 1, 2 and 3 were then separated by HPLC, recovered and extracted. FAMEs from BMP peaks were analysed by GC. Results are expressed as mol% and are the means of three independent experiments; S.E.M. values are not reported as all coefficients of variation were lower than 8%. tr, trace amount; nd, not detected. For simplification, mono-unsaturated fatty acid isomers have been pooled.

Fatty acid	Peak 1	Peak 2	Peak 3
16:0	4.9	1.8	3.1
16:1	1.1	0.8	1.0
18:0	3.0	1.5	2.2
18:1	4.9	2.1	8.8
18:2 ($n-6$)	1.1	1.5	0.6
20:3 ($n-6$)	tr	tr	tr
20:4 ($n-6$)	3.9	2.7	2.0
20:5 ($n-3$)	nd	nd	nd
22:4 ($n-6$)	5.1	2.5	1.9
22:5 ($n-6$)	69.9	81.5	74.7
22:5 ($n-3$)	0.1	0.2	tr
22:6 ($n-3$)	5.3	6.5	5.1

NMR analysis of such compounds is a method of choice for establishing the position of the acyl groups [43–45]. Because NMR analysis requires a large amount of BMP, rat testis were used as a source of BMP for these studies. Total BMP content of rat testis was 1.0 ± 0.05% ($n = 9$) of total PLs and was recovered, after TLC, with an overall yield of 47 ± 2%. This purified BMP was then separated by HPLC into three peaks (Figure 6), which co-migrated, when co-injected, with the three peaks of BMP from U_{III} cells. The relative proportions, in UV units, from the two sources were very different, but in each source the second peak was the most abundant. Table 5 shows the fatty acid composition of the three peaks of BMP from rat testis as determined by GC. These three isomers were very similar in composition and $C_{22:5}n-6$ represented more than 70% whereas DHA was about 6%. As a consequence, the majority of BMP molecules are expected to be acylated with two molecules of $C_{22:5}n-6$, which should facilitate further structural studies. HPLC purification of BMP yielded 120, 785 and 23 μg of peaks 1, 2 and 3, respectively, with purity greater than 94% as measured by HPLC. Tandem MS confirmed their BMP structure and the high predominance of the $C_{22:5}n-6/C_{22:5}n-6$ species (results not shown). As for U_{III} cells, BMP from rat testis showed a strong selectivity for C_{22} PUFA esterification but $C_{22:5}n-6$ was shown to replace $C_{22:6}n-3$ despite the fact that DHA synthesis in this tissue was found to be more efficient [46]. The fact that the distributions of the three peaks for U_{III} cells BMP and rat testis BMP were clearly different and did not vary significantly for each model, for several independent experiments, strongly suggests that these peaks were not produced during sample preparation, but corresponded to naturally occurring products with a tissue-dependent distribution.

NMR analysis required accurate identification of the docosapentaenoyl chain (DPA, $C_{22:5}n-6,\Delta4$) carbons. We determined the spectrum for the DPA methyl ester, since the signals for the acyl groups did not markedly differ from those in simple esters or acylglycerol. Since the chemical shift is affected by the nearest double bond, ^{13}C-NMR spectra of $(n-3)$ and $(n-6)$ unsaturated acyl chains are clearly distinguishable. A complete assignment of the ^{13}C resonances of DPA was obtained using comparison with the data sets of DHA ($C_{22:6}n-3,\Delta4$) and those of arachidonic acid and $C_{22:4}n-6,\Delta7$ to identify the head chain and the end chain, respectively [44].

The high selectivity and dispersion of ^{13}C-NMR spectra in glycerophospholipids allowed the identification and elucidation of the distribution of fatty acids in BMP between the α and β positions of the glycerol backbone [43–45,47] (Figure 7a) without the possibility of determining the configuration (*S* or *R*) of the two asymmetric carbon atoms (Gl_2 and Gl'_2) of the glycerol backbones, as in the case of rac-glycerol 3-phosphate [45]. The ^{13}C-NMR spectrum of peak 2 from rat testis BMP was relatively simple, the two acyl glycerol moieties were strictly identical and therefore the two acyl chains were attached to the glycerols at the same positions, α and α' (or β and β').

Assignments of ^{1}H and ^{13}C spectra were made using chemical shift, coupling constants, intensity data and heteronuclear shift correlation, which is a sensitive method for determining long-range (two- and three-bond) ^{1}H and ^{13}C connectivity [33]. The correlation (Figures 7b and 7c) of the single signal (173.273 ppm) of the C_1 carbonyl atom of the acyl chain with the signal (4.03–4.04 ppm) of the two equivalent protons itself correlated with the signal (64.435 ppm) of the Gl_3 carbon atom of the glycerol backbones, indicating unambiguously that the acyl chains are attached to the Gl_3 and Gl'_3 carbon atom glycerols (α and α' positions). NMR analysis shows also that the signal (3.89–3.91) of the methyne proton is correlated with the signal (68.053) of the Gl_2 asymmetric carbon atom; meanwhile, the two signals (3.89–3.91 and 3.83–3.84 ppm) of the two non-equivalent protons are correlated with the signal (64.146 ppm) of the Gl_1 carbon atom of the glycerol backbones. This result was confirmed further by experiments using the lipase from *R. arrhizus* which released more than 96% of the fatty acid chains.

Peak 1 did not yield clear results due to the low amounts available. However, its ^{1}H-NMR spectra shows one signal with a very low field ($\approx$ 5 ppm), characteristic of a methyne proton of the glycerol backbone which suggested acyl occupancy of the secondary alcohol.

Further NMR experiments requiring more material will be needed to determine the location and the nature of the fatty acyl chains for the two other BMP peaks, particularly peak 3, which corresponds to a BMP with a lower capacity to incorporate DHA. Since peak 3, isolated from DHA-supplemented cells, contained the same molecular species as peak 2 isolated from control cells, and because these two peaks were clearly resolved by HPLC but have common stereoconfiguration, we conclude that these compounds are differentiated by the acyl location: at least one chain should be located at the *sn*-2 position in peak 3.

In conclusion, U_{III} cell BMP is a mixture of three compounds that can be labelled by [^{33}P]P_i, [^{3}H]DHA and exogenous [^{3}H]PG. They correspond either to intermediates in a common biosynthetic pathway or to products formed in different cell compartments. Analysis of BMP isoforms in the different samples after cellular fractionation could help us to answer that question. Recent data have shown that the late endosomes from BHK cells contain a higher proportion of BMP than that observed in early endosomes and lysosomes [20,48], suggesting specialized functions or properties for late endosomes. Comparison of BMP structures and fatty acid composition with other PL classes of these organelles may also be of interest to better understand why fatty acid composition of BMP is so dependent on the cell studied and what the mechanisms involved in BMP acylation are.

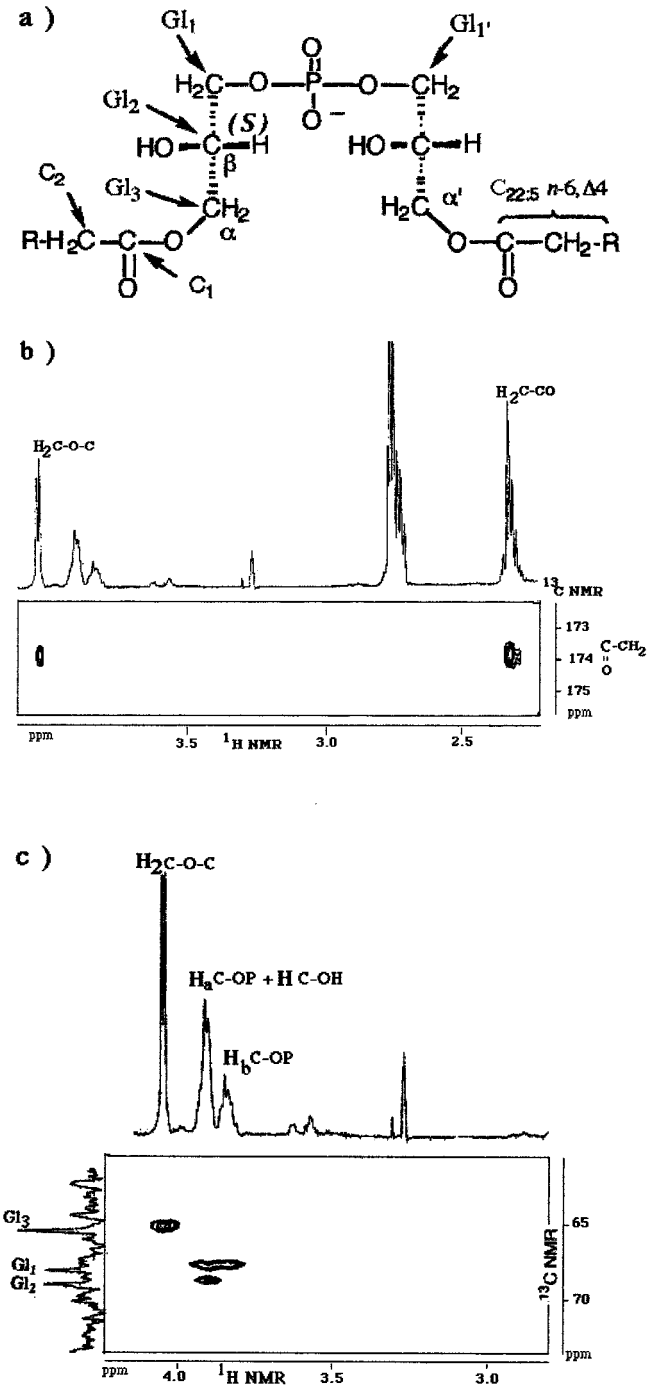

Figure 7 Relevant regions (b, c) of the ^{1}H–^{13}C correlation spectrum of peak 2 from rat testis BMP, and hypothetical structure of the compound (a)

See the text for details.

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