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The molecular selectivity of phospholipase D in HL60 granulocytes

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Abstract The molecular selectivity of PLD in PMA-stimulated HL60 granulocytes was determined by HPLC analysis of [^{3}H]butanol incorporation into phosphatidyl[^{3}H]butanol (Ptd[^{3}H]But) molecular species. Comparison with phospholipid compositions confirmed that PLD acted primarily on phosphatidylcholine (PtdCho). Apparent enzyme selectivity was suggested by negligible formation of PB16:0/16:0 and preferential synthesis of Ptd[^{3}H]But species containing *sn*-1 18:0. Culture with exogenous 18:2n-6 or 20:4n-6 readily modified both PtdCho and Ptd[^{3}H]But compositions, and accentuated the apparent selectivity of stimulated PLD for *sn*-1 18:0 species of PtdCho. Such modifications to PLD-based signalling mechanisms may contribute to the modulatory effects of altered dietary lipid intakes on cellular functions.

Key words: Phospholipase D; HL60 granulocyte; Phospholipid molecular species; Phosphatidylbutanol; Transphosphatidylation

1. Introduction

Receptor-mediated activation of phospholipase D (PLD) is a widely distributed mechanism for the signal transduction of a broad range of agonists in many different cell types [1]. PLD is believed preferentially to hydrolyse phosphatidylcholine (PtdCho), the most abundant membrane phospholipid component [2]. The primary product of PLD hydrolysis of PtdCho, phosphatidic acid (PtdOH), has been identified as a significant transduction signal involved in protein phosphorylation [3]. PtdOH can also be converted by phosphatidate phosphohydrolase to diacylglycerol (DAG), and thus also contribute to the well-recognised activation of protein kinase C (PKC) isoforms by DAG [4].

DAG is produced directly from phosphatidylinositol 4,5-bisphosphate (PIP_2) by the action of phospholipase C (PLC), and can in turn be converted to PtdOH. The molecular species compositions of PtdCho and PIP_2 are very different, and it has been proposed that formation of different molecular species of PtdOH or DAG by hydrolysis of phospholipid substrate pools of different composition may regulate the activation of individual isoforms of PKC [5]. Fatty acid supplementation either in vitro or by diet can alter membrane phospholipid composition, and is also well-recognised to alter many cellular responses such as mitogen-induced proliferation [6]. It is possible that generation of PtdOH and/or DAG with altered compositions, and consequent altered characteristics of PKC activation, may be one mechanism underlying such dietary-induced changes.

Determination of the molecular composition of signalling molecules synthesised by phospholipase activation in intact cells by indirect estimation of differences in compositions after cell stimulation is dependent on relative rates of synthesis and hydrolysis. In this study, we have devised an HPLC technique for the efficient resolution of intact, underivatised acidic phospholipids. This has been applied to the analysis of phosphatidyl[^{3}H]butanol (Ptd[^{3}H]But) molecular species formed by the PLD transphosphatidylation reaction in PMA-stimulated HL60 granulocytes in the presence of [^{3}H]butanol [7]. This analysis has detailed both the inherent molecular substrate selectivity of PLD in control HL60 cells, and the modifications to this signalling mechanism caused by culture of cells in the presence of exogenous fatty acids.

2. Materials and methods

2.1. Materials

Cell culture supplies were obtained from Gibco (Paisley, Scotland) or Sigma (Poole, Dorset). Egg PtdBut was purchased from Lipid Products (Redhill, Surrey); other phospholipid standards, fatty acids and general chemicals were from Sigma. HPLC solvents were supplied by Rathburn (Walkerburn, Scotland), and bacterial PLD was a kind donation from Dr. H. Marriage (Genzyme Fine Chemicals, Maidstone, Kent). [^{3}H]Butanol (25 Ci/mmol) was purchased from Amersham.

2.2. Synthesis of rat liver Ptd[^{3}H]But and dimyristoyl Ptd[^{3}H]But

PtdCho isolated from rat liver or dimyristoyl PtdCho (PC14:0/14:0) (20 nmol each) was incubated was incubated with [^{3}H]butanol (1 mCi, 2.7 mmol), 300 μl *t*-butylmethyl ether, 25 U bacterial PLD and 450 μl 0.05M PIPES buffer, pH 7.0 (total volume 1.1 ml) at room temperature for 24 h. Synthesised Ptd[^{3}H]But was extracted using chloroform/methanol and purified by TLC.

2.3. Cell culture and fatty acid supplementation

HL60 cells were cultured in RPMI 1640 medium with 10% FCS, and were induced to differentiate to the granulocyte phenotype at a density of 2×10^5 cells/ml by addition of 1 μM retinoic acid for four days. Fatty acid/albumin complexes (1:1 molar ratio) were added at 30 μM on day 0, and retinoic acid and fatty acid supplements were renewed when the medium was changed on day 2.

2.4. PLD assay in HL60 cells

The PLD assay was performed essentially according to Randall [7] Briefly, HL60 granulocytes (2×10^7) in 540 μl of medium were preincubated with [^{3}H]butanol (167 μCi) for 5 min at 37°C before addition of 12-*O*-tetradecanoyl-phorbol 13-acetate (PMA) (100 nM) or an equivalent volume (10 μl) of DMSO vehicle. After incubation at 37°C for 30 min, reactions were terminated by pelleting cells and extracting total lipids using chloroform and methanol [8] after adding 10 μg egg PtdBut and 5 nCi dimyristoyl Ptd[^{3}H]But as carrier and internal standard, respectively. Ptd[^{3}H]But was isolated by thin layer chromatography (TLC) for HPLC analysis of molecular species composition. PtdCho and phosphatidylethanolamine (PtdEth) were isolated from chloro-

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; PIP_2, phosphatidylinositol bisphosphate; PtdOH, phosphatidic acid; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PMA, 12-*O*-tetradecanoyl-phorbol 13 acetate; PUFA, polyunsaturated fatty acid.

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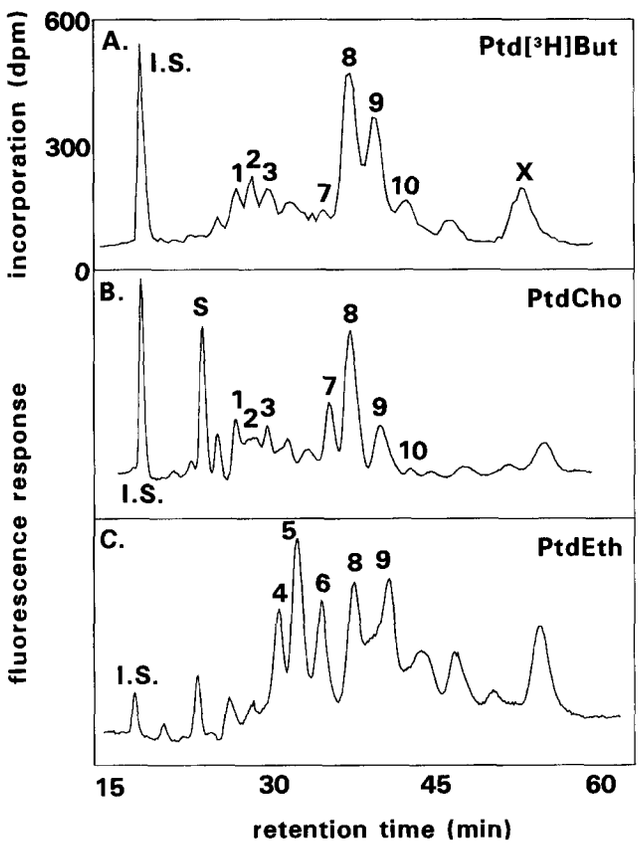

Fig. 1. Typical HPLC chromatograms of phospholipid molecular species from HL60 granulocytes cultured under control conditions in medium containing fetal calf serum (10%). (A) The molecular composition of Ptd[^{3}H]But species synthesised from [^{3}H]butanol in cells simulated with 100nM PMA. (B) PtdCho species. (C) PtdEth species. Equivalent peaks in each chromatogram are numbered sequentially, and correspond to the peaks identified in Table 1, except for peaks 4, 5 and 6 which were alkenyl species not significantly present in synthesised Ptd[^{3}H]But. I.S. = internal standard comprising the equivalent 14:0/14:0 species; S = sphingomyelin contaminant in PtdCho; X = component of unknown composition.

form/methanol extracts of parallel cell cultures using BondElut NH_2 cartridges [9].

2.5. HPLC analysis of phospholipid molecular species

Phospholipid molecular species were resolved by HPLC on a 250 × 4.6 mm 5 μ Apex II ODS column (Jones Chromatography, Glamorgan, Wales) maintained at 50°C. Mobile phase for PtdCho and PtdEth was methanol/water (37:3 v/v) containing 40 mM choline-Cl at 1 ml/min. PtdBut species were resolved using 40 mM choline-Cl in methanol/water/phosphoric acid (96:4:1 v/v). The UV absorbance response of eluted phospholipid species at 205 nm was proportional to degree of acyl unsaturation, while sequential fluorescence detection following post-column derivatisation with 1,6-diphenyl-1,3,5-hexatriene (DPH) provided a quantitative determination of phospholipid mass [10]. The identities of eluted PtdCho and PtdEth species were determined both by comparison between these absorbance and fluorescence responses [10] and by reference to authentic standards. Incorporated radioactivity was determined by collecting 250 μl HPLC fractions followed by liquid scintillation counting. Ptd[^{3}H]But species were routinely identified by retention time relative to that of the [^{3}H]PB14:0/14:0 internal standard, and by comparison of retention times with those of molecular species present both in carrier egg PtdBut, detected by UV absorbance at 205 nm, and in the Ptd[^{3}H]But standards synthesised from rat liver PC.

3. Results

3.1. Analysis of phosphatidylbutanol molecular species

Intact individual molecular species of PtdBut were resolved by reverse phase HPLC using a methanol-based mobile phase containing both phosphoric acid and choline-Cl. As PtdBut is an acidic phospholipid, it was ionised and did not elute from the HPLC at neutral pH. Phosphoric acid proved essential for resolution of PtdBut molecular species, presumeably by protonation of the phosphobutanol headgroup to uncharged species. The addition of an ion suppressant reagent such choline-Cl was also required to prevent gross tailing of PtdBut peaks, an effect routinely observed for analysis of neutral phospholipid molecular species [10]. The resolution obtained for Ptd[^{3}H]But synthesised from rat liver PtdCho in vitro by bacterial PLD was comparable to the original substrate composition (result not shown). The six major species of PtdBut corresponded to the major unsaturated species present in rat liver PtdCho, and were used to calibrate the resolution of Ptd[^{3}H]But species synthesised by HL60 cells. To our knowledge, this technique represents the first description of the HPLC analysis of PtdBut molecular species.

3.2. Synthesis of phosphatidyl[^{3}H]butanol by HL60 granulocytes

The resolution obtained for Ptd[^{3}H]But synthesised by PMA-stimulated HL60 granulocytes is shown in Fig. 1A. The major species synthesised were PB16:0/18:1, PB18:1/18:1 and a late-eluting component of unknown composition. The molecular species composition of PtdCho from HL60 granulocytes (Fig. 1B) was characterised by a predominance of three species, PC16:0/16:0, PC16:0/18:1 and PC18:1/18:1; species containing polyunsaturated fatty acids (PUFA) were present only as minor components. The composition of PtdEth molecular species from HL60 cells was very different (Fig. 1C), containing both more unsaturated and more alkenyl species. The composition of the principal Ptd[^{3}H]But molecular species synthesised in response to PMA is compared in Table 1 with the distributions of the same species in PtdCho and PtdEth. These species

Table 1
Synthesis of molecular species of phosphatidylbutanol by control HL60 granulocytes

Molecular species	Peak number	Phospholipid concentration/ [^{3}H]butanol incorporation (% total)		
		Ptd[^{3}H]But incorporation	PtdCho mass	PtdEth mass
16:0/16:1	1	5.47 ± 0.33	5.93 ± 0.64	2.96 ± 0.32
16:0/20:4	2	6.06 ± 0.27	5.93 ± 0.40	3.38 ± 1.09
16:0/18:2	3	6.10 ± 0.58	4.62 ± 1.38	0
16:0/16:0	7	3.60 ± 0.11	11.01 ± 0.21	0
16:0/18:1	8	24.15 ± 0.46	27.93 ± 3.10	7.07 ± 2.77
18:1/18:1 + 18:0/20:4	9	18.12 ± 0.75	13.62 ± 2.64	21.12 ± 5.25
18:0/18:2	10	7.58 ± 0.60	0.86 ± 0.62	0
Unknown	*x*	11.50 ± 0.80	2.65 ± 0.34	0
% total		83.83	76.17	34.53

Incorporation of [^{3}H]butanol into Ptd[^{3}H]But molecular species was determined in PMA-stimulated cells (mean ± S.D., n = 3). The distribution of the same molecular species is detailed in PtdCho and PtdEth from unstimulated cells (mean ± S.D., n = 3). Peak numbering refers to the resolutions shown in Fig. 1.

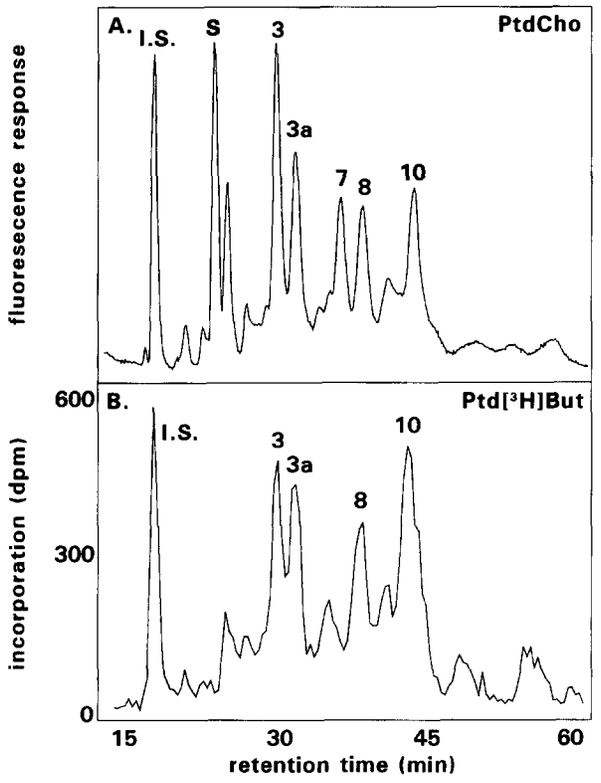

Fig. 2. The molecular species compositions of (A) cellular PtdCho and (B) synthesised Ptd[^{3}H]But from HL60 granulocytes supplemented in culture with 30 μM 18:2n-6. The peak numbering and identifications refer to Fig. 1 and Table 1, except for peak 3a which was the *sn*-1-*alkyl*16:0/*sn*-2 18:2 species.

were major components of PtdCho (76% total) but were present at considerably lower concentration in PtdEth (35% total), especially as peak 9 comprised at least two poorly resolved 18:1/18:1 and 18:0/20:4. The UV absorbance response of this peak at 205 nm (result not shown) suggested strongly that it was predominantly 18:1/18:1 for PtdCho but was 18:0/20:4 for PtdEth. The retention time of peak 9 in synthesised Ptd[^{3}H]But (Fig. 1A) compared to the rat liver PtdBut standards also suggested a predominant composition of 18:1/18:1 rather than 18:0/20:4. This comparison was consistent with previous reports that PtdCho is the preferred substrate for PLD in HL60 cells, a view supported by the low content of alkenyl species in Ptd[^{3}H]But. The Ptd[^{3}H]But composition, however, was not identical to that of PtdCho; there was negligible conversion of PC16:0/16:0 to PtdBut, and PtdBut was enriched in the late-eluting species. The low mass of the this component in substrate PtdCho precluded its conclusive identification. The sphingomyelin component in the PC analysis (Fig. 1B, peak S) was a contaminant and was not a substrate for PLD.

3.3. *Modification of phosphatidyl[^{3}H]butanol synthesis*

Supplementation of culture medium with 30 μM fatty acid/albumin mixtures for four days caused characteristic modifications to the molecular species compositions of cellular phospholipid. Supplementation with either 18:2n-6 (Fig. 2A) or 20:4n-6 (Fig. 3A) increased the concentrations of both diacyl and alkylacyl PtdCho molecular species containing the respective fatty acid at the *sn*-2 position. There was little evidence for any significant further metabolism of added fatty acids; for instance, 18:2n-6 decreased rather than increased the PC16:0/20:4 content previously measured in control cells. One consistent observation was the virtual elimination by exogenous fatty acids of PC18:1/18:1 (Fig. 1B). The pattern of Ptd[^{3}H]But species synthesised by supplemented cells in response to PMA in both cases qualitatively reflected these changes to PtdCho composition, with increased amounts of PUFA-containing species (Fig. 2A and B). However, quantitatively there was apparent selectivity for the production of unsaturated Ptd[^{3}H]But species with 18:0 rather than 16:0 esterified at the *sn*-1 position. For instance, the 16:0/18:2 (Fig. 2A) and 16:0/20:4 (Fig. 3A) species were predominant in PtdCho from respectively 18:2n-6 and 20:4n-6 supplemented cells, but the 18:0/18:2 and 18:0/20:4 species were more evident in synthesised Ptd[^{3}H]But (Fig. 2A and B). The 4-fold extent of this apparent substrate selectivity of PLD is summarised in Table 2, which compares relative distributions of *sn*-1 16:0 and *sn*-1 18:0 species in both PtdCho and Ptd[^{3}H]But. As with control cells, there was no significant synthesis of PB16:0/16:0. There was increased production of the late-eluting unidentified Ptd[^{3}H]But species in cells supplemented with 20:4n-6.

4. Discussion

There are two novel aspects to the results presented in this paper. First, HPLC analysis of PtdBut molecular species, cou-

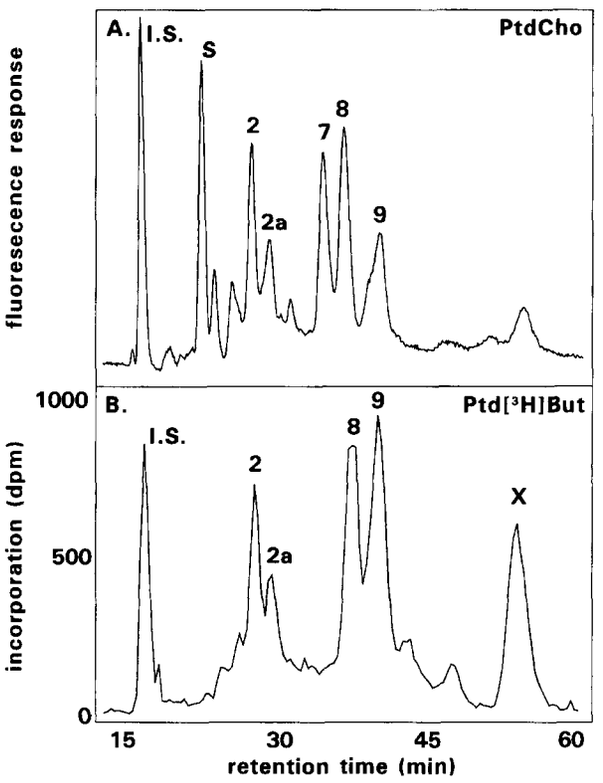

Fig. 3. The molecular species compositions of (A) cellular PtdCho and (B) synthesised Ptd[^{3}H]But from HL60 granulocytes supplemented in culture with 30 μM 20:4n-6. The peak numbering and identifications refer to Fig. 1 and Table 1, except for peak 2a which was the *sn*-1-*alkyl*16:0/*sn*-2 20:4 species.

Table 2
Selectivity of phospholipase D in HL60 cells

Molecular species	PtdCho (% total)	Ptd[^{3}H]But (% total)	Ptd[^{3}H]But / PtdCho
Cells supplemented with 18:2n-6.....			
16:0/18:2	19.1 ± 2.7	12.8 ± 0.2	0.67
18:0/18:2	8.6 ± 1.7	21.0 ± 0.7	2.44
Cells supplemented with 20:4n-6.....			
16:0/20:4	12.9 ± 2.3	11.3 ± 0.7	0.88
18:0/20:4	5.7 ± 3.7	22.6 ± 0.8	3.96

The fractional concentrations of selected molecular species of PtdCho from HL60 cells cultured in the presence of either 18:2n-6 or 20:4n-6 (30 μM) are compared with the radioactivity distributions of the same species of Ptd[^{3}H]But synthesised in the presence of [^{3}H]butanol in response to PMA. Results are expressed as mean ± S.D., n = 3 experiments.

pled with the incorporation of [^{3}H]butanol into Ptd[^{3}H]But, provided a sensitive and convenient technique to assess the composition of the immediate products of PLD activation in intact cells. Second, application of this analytical technique to cells incubated with exogenous fatty acid supplements demonstrated, for the first time, a molecular selectivity for PMA-activated PLD.

Our HPLC method for analysis of the products of PLD activation possesses significant advantages over alternative analytical approaches. The use of high specific activity [^{3}H]butanol in the assay diverts only a low proportion of synthesised PtdOH to Ptd[^{3}H]But, and so does not materially alter intracellular signalling mechanisms [7]. Although incubation times of 30 min were used in the study reported above, separate experiments have confirmed that adequate incorporations can be obtained over shorter time periods. This ability is important for analysis of cellular responses involving rapid activation of PLD over a short duration, for instance FMLP activation of neutrophils [11]. By contrast, the one previous analysis of the molecular selectivity of PLD, which used fast atom bombardment mass spectrometry to resolve phosphatidylethanol species synthesised by PC12 cells in response to PMA or bradykinin in the presence of 1% ethanol [12], required a 90 min incubation to generate sufficient material for analysis, at the expense of endogenous PtdOH formation. Analysis of the molecular species composition of PtdOH [13] cannot provide a direct assessment of PLD selectivity, as PtdOH is synthesised by a number of additional pathways, including phospholipid synthesis [14] and phosphorylation of diacylglycerol by diacylglycerol kinase [15].

Previous studies of PMA-activated PLD in many cell types have concluded that PtdCho was the primary, if not exclusive, substrate for hydrolysis [2], and our results detailing the molecular composition of newly synthesised PtdBut support this view. For both control and fatty acid-treated cells, the identities of Ptd[^{3}H]But, and presumably PtdOH, species synthesised closely resembled the molecular species present in PtdCho, and were not characteristic, for instance, of PtdEth (Table 1). By comparison, PIP_2 composition in cultured cells is even more unsaturated than PtdEth, comprising molecular species containing largely 20:4n-6 fatty acids [5,13]. These compositions of PtdCho and PtdEth were very similar to previous reports of phospholipid molecular species compositions of a variety of cell types cultured in medium containing fetal calf serum [13,16]. There was some suggestion of limited selectivity for PLD in these control cells, shown by the relatively greater incorporations of [^{3}H]butanol into both PB18:0/18:2 and the Ptd[^{3}H]But species of unknown composition, and lack of synthesis of PB16:0/16:0 (Table 1).

This suggestion of selectivity of PLD under control culture conditions was confirmed in the experiments which supplemented culture medium with low concentrations of either 18:2n-6 or 20:4n-6. Such manipulation readily modulated the PtdCho species composition of HL60 cells (Figs. 2A and 3A), with increased contents of PUFA-containing species and the virtual elimination of PC18:1/18:1. The identities of Ptd[^{3}H]But species synthesised by supplemented cells in response to PMA closely resembled these altered PtdCho compositions, but there was additional evidence for molecular selectivity of PLD hydrolysis. For both 18:2n-6 and 20:4n-6 treated cells, there was considerably greater incorporation of [^{3}H]butanol into PB18:0/18:2 (Fig. 2B) and PB18:0/20:4 (Fig. 3B) respectively, than suggested by the contents of the relevant species in PtdCho (Table 2). Again, there was no conversion of PC16:0/16:0 into PB16:0/16:0. It is probable that previous analyses of the products of PLD activation [5,12,13,16] have not demonstrated such a selectivity because of the limited distribution of molecular species of PtdCho of cells cultured under control conditions (Fig. 1) [13,16]. In our study, apparent PLD selectivity for unsaturated PtdCho species was greatly enhanced after manipulation of cellular phospholipid composition. Our results imply that, although analysis of cell PtdCho species can predict the general nature of the products of PLD activation, it cannot determine the precise composition of PtdOH derived from PLD hydrolysis of PtdCho. They suggest that PLD either acts on a discrete pool of substrate PtdCho separate from the bulk of cellular PtdCho, or that the enzyme exhibits a degree of substrate specificity for selected PtdCho molecular species.

Modification of dietary fatty acid intake can profoundly alter many cellular responses, such as lymphocyte proliferation [6] and neutrophil chemotaxis [17], effects largely unattributable to eicosanoid generation or membrane fluidity changes. It is possible that part of these dietary changes is modulated by an altered spectrum of products of receptor-mediated phospholipase activation, leading to a modified PKC response. The ability to characterise directly the composition of the products of PLD activation in intact cells, by analysis of Ptd[^{3}H]But molecular species, should prove valuable for assessment of the role of such changes to second messenger molecular species compositions in regulating PLD and PKC-mediated cellular responses.

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