Phospholipase D1b and D2a generate structurally identical phosphatidic acid species in mammalian cells

Trevor R. PETTITT, Mark McDERMOTT, Khalid M. SAQIB, Neil SHIMWELL and Michael J. O. WAKELAM¹

CRC Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TA, U.K.

Mammalian cells contain different phospholipase D enzymes (PLDs) whose distinct physiological roles are poorly understood and whose products have not been characterized. The development of porcine aortic endothelial (PAE) cell lines able to over-express PLD-1b or -2a under the control of an inducible promoter has enabled us to characterize both the substrate specificity and the phosphatidic acid (PtdOH) product of these enzymes under controlled conditions. Liquid chromatography–MS analysis showed that PLD1b- and PLD2a-transfected PAE cells, as well as COS7 and Rat1 cells, generate similar PtdOH and, in the presence of butan-1-ol, phosphatidylbutanol (PtdBut) profiles, enriched in mono- and di-unsaturated species, in particular 16:0/18:1. Although PtdBut mass increased, the species profile did not change in cells stimulated with ATP or PMA. Overexpression of PLD made little difference to basal or stimulated PtdBut

formation, indicating that activity is tightly regulated *in vivo* and that factors other than just PLD protein levels limit hydrolytic function. *In vitro* assays using PLD-enriched lysates showed that the enzyme could utilize both phosphatidylcholine and, much less efficiently, phosphatidylethanolamine, with slight selectivity towards mono- and di-unsaturated species. Phosphatidylinositol was not a substrate. Thus PLD1b and PLD2a hydrolyse a structurally similar substrate pool to generate an identical PtdOH product enriched in mono- and di-unsaturated species that we propose to function as the intracellular messenger forms of this lipid.

Key words: lipid signalling, mass spectrometry, PAE cells, phosphatidylbutanol, phosphatidylcholine.

INTRODUCTION

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PtdCho), generating phosphatidic acid (PtdOH) and choline. Activation of this pathway has been demonstrated in most cell types in response to ligands acting via heterotrimeric GTP-binding protein-coupled receptors and those that activate polypeptide growth factor receptors. Currently, two mammalian PLD genes have been cloned, each producing several splice variants [1]; however, despite the range of agonists shown to activate PLD, there is no indication of agonist-specific selective PLD isoform activation.

The PtdOH produced by PLD, but probably not the majority of PtdOH generated via other routes, is proposed to have intracellular messenger functions, however, there is a paucity of information about its nature. This contrasts with the wealth of data on the phospholipase C (PLC)-generated lipid messenger, diacylglycerol (DAG), and its primary target, protein kinase C (PKC). There is evidence that PLD-generated PtdOH can activate a number of physiological events [1,2], including secretion, neutrophil superoxide generation and cytoskeletal reorganization, but in most cases defined molecular targets remain to be identified. A characterized role for this lipid is in the neutrophil respiratory burst where it is an essential cofactor for the NADPH oxidase complex, activating a protein kinase that phosphorylates p47 phox [3]. Other putative targets include several other protein kinases, such as Raf1 [4] and p21-activated kinase [5]. It has also been suggested that the lipid regulates cAMP phosphodiesterase

activity [6]. Additionally we, and others, have shown that PtdOH is involved in actin stress fibre formation [1,7].

We previously suggested that endogenous PLD generates PtdOH containing primarily saturated and mono-unsaturated fatty acids, and that this lipid, when hydrolysed to generate DAG, *in vivo*, was a very poor activator of PKC [8]. From the evidence available we proposed a model whereby the PLC and PLD pathways act in parallel to produce the intracellular messengers polyunsaturated DAG and saturated/mono-unsaturated/di-unsaturated PtdOH [9]. Removal of these signals by the actions of diacylglycerol kinase (DAGK) and phosphatidic acid phosphohydrolase (PAP) interconvert these lipids. However, because of the differences in fatty acyl composition, these structures are inactive as messengers and serve only as intermediates in the re-synthesis of the original phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] and PtdCho.

Most studies of PLD in mammalian systems have focused on the PtdCho-hydrolysing enzymes; however, a small number of reports have suggested additional enzymes capable of hydrolysing other lipids. These include reports of PLDs specific for phosphatidylethanolamine (PtdEtn) [10–13] and phosphatidylinositol (PtdIns) [14,15], together with several of its more highly phosphorylated forms, including phosphatidylinositol 3,4,5-trisphosphate [16]. While no enzymes capable of specifically catalysing these reactions have been cloned (although there are some partial purifications), we would presume that the resultant PtdOH would need to be saturated/mono-unsaturated/di-unsaturated if it were to have messenger functions. This appears unlikely if

Abbreviations used: DAG, diacylglycerol; DAGK, diacylglycerol kinase; ESI, electrospray ionization; HA, haemagglutinin; IPTG, isopropyl β -D-thiogalactoside; LC–MS, liquid chromatography–MS; PAE, porcine aortic endothelial (cells); PAP, phosphatidic acid phosphohydrolase; PIP kinase 1 α , phosphatidylinositol 4-phosphate 5-kinase 1 α ; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine.

¹ To whom correspondence should be addressed (e-mail M.J.O.Wakelam@bham.ac.uk).

phosphoinositides act as substrates, since these lipids have predominantly polyunsaturated acyl structures in mammalian cells.

With the identification and cloning of mammalian PLD-1 and -2 [1,17] (existing as the splice variants 1a, 1b, 2a and 2b) it has become possible to overexpress particular PLDs under controlled conditions. Coupled with the increased availability of highly sensitive and discriminatory techniques, such as liquid chromatography–MS (LC–MS), this has enabled us to investigate directly both the substrates and the products generated by these mammalian PLDs. We show in the present study that both PLD1b and PLD2a hydrolyse a structurally similar phospholipid pool to generate a PtdOH rich in mono- and di-unsaturated species. This provides the final piece of evidence for our model that PLC generates primarily polyunsaturated signalling DAG, whereas PLD generates primarily mono-unsaturated/di-unsaturated signalling PtdOH species. This prevents DAG/PtdOH interconversion as active messengers, thus limiting the potential for a futile signalling loop. It also shows that spatial and regulatory differences between PLD-1 and -2 must determine their downstream targets rather than generation of different PtdOH species.

EXPERIMENTAL

Cells and their stimulation

Porcine aortic endothelial (PAE) cells were transfected with a p3'SS plasmid (Stratagene) modified by replacement of the F9-1 promoter with a cytomegalovirus promoter (kindly donated by Dr P. Hawkins, The Babraham Institute, Babraham, Cambridge, U.K.). Clones were selected which demonstrated high levels of nuclear lac repressor expression upon immunocytochemistry. Transfection of these cells with a recombinant construct containing three lac repressor binding sites (donated by Dr P. Hawkins) encoding Myc-tagged human PLD1b or haemagglutinin (HA)-tagged human PLD2a allowed the selection of cell lines expressing isopropyl β -D-thiogalactoside (IPTG)-inducible, Myc-tagged PLD1b or HA-tagged PLD2a. These were grown to 70% confluency in 175 cm² flasks containing Ham's F12 medium, 10% (v/v) foetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. Medium was replaced with serum-free Ham's F12, incubated for 18 h then induced with 500 μ M IPTG for a further 6 h. To analyse the PLD product, it was trapped as phosphatidylbutanol (PtdBut) by incubating cells in the presence of 0.3% butan-1-ol (30 mM) for 10 min prior to stimulation with buffer (control), 100 μ M ATP or 100 nM PMA for 15 min. Following aspiration of the medium, cells were killed with ice-cold methanol (5 ml) and scraped into glass screw-capped tubes. To extract the lipids, chloroform (10 ml) was added, left to stand for 5 min then mixed with 0.88% KCl in 0.1 M HCl (5 ml). After allowing the phases to separate, the upper phase was discarded while the lower organic phase containing the lipids was washed with 3 ml of methanol/0.88% KCl in 0.1 M HCl (1:1, v/v), dried under a stream of nitrogen, resuspended in 25 μ l of chloroform/methanol (2:1, v/v) and stored at -20°C .

COS7 cells were cultured in RPMI medium containing 10% (v/v) foetal calf serum and were transfected for 24 h with plasmids containing PLD2a or phosphatidylinositol 4-phosphate 5-kinase 1 α (PIP kinase 1 α) using FuGENE 6 transfection reagent (Roche Diagnostics Ltd). Rat1 fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. Sf9 cells infected with baculoviruses encoding human PLD1b or PLD2a were cultured in TC100 medium containing 10% (v/v) foetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin.

HPLC analysis

Total lipid extracts (5 μ l aliquots) were separated and characterized by LC–MS (QP8000alpha; Shimadzu) using a Luna silica column (3 μ m, 2 mm $\times$ 150 mm; Phenomenex) with a solvent gradient of 100% chloroform/methanol/water/ammonia solution (90:9.5:0.5:0.32, by vol.) changing to 100% chloroform/methanol/water/ammonia solution (50:48:2:0.32, by vol.) over 40 min at 0.35 ml/min. Detection in negative electrospray ionization (ESI) mode (N_2 flow, 4 l/min; curved desolvation line, 300 $^{\circ}\text{C}$; Probe, -5 kV) allowed characterization of PtdBut, PtdOH, PtdEtn, PtdIns and phosphatidylserine (PtdSer) while detection in positive ESI mode (N_2 flow, 4 l/min; curved desolvation line, 300 $^{\circ}\text{C}$; Probe, 5 kV) enabled characterization of PtdCho and sphingomyelin.

Preparation of cell lysates

After washing with ice-cold PBS, 50 μ l of ice-cold lysis buffer (137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.7 mM KCl, 2.5 mM EDTA, 1.15 mM KH_2PO_4 and Roche Complete EDTA-free protease inhibitor cocktail, pH 7.5) was added to PLD-transfected PAE cells (induced for 6 h with 500 μ M IPTG where required) in 6-well dishes, which were then scraped into Eppendorf tubes and lysed using two 10 s pulses with a probe sonicator.

In vitro PLD assay

Cell lysate (20 μ l), 5 μ l of 4 M NaCl and 5 μ l of 300 μ M GTP[S] in 20 mM CaCl_2 and 30 mM MgCl_2 (CaCl_2 and MgCl_2 only present in the GTP[S]-free assays) were placed in a glass tube. To start the assay 20 μ l of vesicle mixture {8.6 μ M egg PtdCho, 8.6 μ M brain PtdIns, 12 μ M PtdIns(4,5) P_2 and 137 μ M egg PtdEtn in 125 mM Hepes, 200 mM KCl, 7.5 mM EGTA and 2.5 mM dithiothreitol, pH 7.5; where required [^3H]PtdCho plus [^{14}C]PtdEtn or [^3H]PtdIns were incorporated into the vesicles at approx. 5×10^6 d.p.m./ml} was added with vortex mixing. For PtdBut formation 0.3% butan-1-ol was included in the assay. Following incubation for 2 h at 37 $^{\circ}\text{C}$ the reaction was stopped by addition of 750 μ l of chloroform/methanol (1:2, v/v). A further 250 μ l of chloroform was added followed by 400 μ l of 0.1 M HCl in 0.88% KCl (with mixing) to form two phases. For PtdBut species determinations, the lower phase containing the lipids was removed and analysed by LC–MS. For radiolabel release, a 100 μ l aliquot of the upper phase was taken for scintillation counting. For [^3H]choline and [^{14}C]ethanolamine release the remaining upper phase was diluted into 5 ml of water then passed on to a 1.5 ml Dowex 50W 50X8-200 column, washed with a further 20 ml of water, eluted with 8 ml of 1 M KCl and then taken for scintillation counting.

In vivo PLD assay

Inducible PLD1b- and PLD2a-transfected PAE cells grown to 50% confluency in 24-well dishes were labelled with [9,10- ^3H]palmitic acid (0.5 μ Ci/ml) in medium containing 1% (v/v) foetal calf serum for a further 24 h. PLD expression was induced with 500 μ M IPTG for 6 h and then butan-1-ol was added to give a final concentration of 0.3%. Following incubation for a further 5 min, the cells were stimulated with or without 100 μ M ATP. Incubations were stopped by aspiration of medium, followed by addition of ice-cold methanol. Cells were scraped into glass vials, unlabelled PtdBut (5 μ g) was added to each vial as a carrier and the total lipids extracted. Following separation on Whatman LK5DF silica TLC plates using the solvent upper phase obtained from mixing ethylacetate/2,2,4-trimethylpen-

tane/water/acetic acid (110:50:100:20, by vol.), the PtdBut band was detected by exposure to iodine vapour, scraped and [^3H]PtdBut generation determined by scintillation counting.

Other techniques

Expression of Myc-tagged PLD1 and HA-tagged PLD2 was confirmed by Western-blot analysis. Briefly, cell lysates were boiled in 75 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 4 M urea, 5% (v/v) 2-mercaptoethanol and Bromophenol Blue for 5 min, separated on 4–12% gradient SDS/polyacrylamide gels (Novex; 20 μg of protein/lane), transferred by semi-dry blotting on to PVDF membranes (Immobilon-P; Millipore), blocked [5% (w/v) non-fat milk powder in Tris-buffered saline containing 0.1% Tween 20], probed with anti-Myc 9E10 (1:500 dilution; Santa Cruz Biotechnology) or anti-HA 12CA5 (1:2000 dilution; Roche Diagnostics) antibodies and detected using enhanced chemiluminescence.

For confocal microscopy, PAE cells were grown on glass slides then, at 20 °C, fixed [4% (w/v) paraformaldehyde, 5% (w/v) sucrose, 0.1% Triton X-100, 2 mM MgCl_2 and 0.5 mM CaCl_2 in PBS] for 15 min, washed four times with PBS (each wash lasting 5 min), blocked [20% (v/v) heat-inactivated goat serum in PBS] for 1 h, probed with anti-Myc 9E10 or anti-HA 12CA5 antibodies (1:200 dilution in blocking solution) for 1 h, washed four times with PBS (each wash lasting 5 min), detected with Texas Red-conjugated goat anti-mouse IgG (1:20 dilution in blocking solution) for 1 h, washed four times with PBS (each wash lasting 5 min), air dried, mounted with ProLong Antifade (Molecular Probes), photographed with an upright Zeiss Axioscope 50 and the images processed using ImproVision Openlab 2.2 software.

RESULTS

Attempts to generate stable cell lines constitutively overexpressing PLD1 or PLD2 were unsuccessful, probably because the phospholipase is highly regulated and when consistently overexpressed will degrade cellular membranes, resulting in cell death. Therefore inducible cell lines were generated using the LacSwitch inducible system. Transfection of lac repressor-expressing PAE cells with the plasmids for Myc-tagged human PLD1b or HA-tagged human PLD2a generated a number of cell lines expressing the phospholipase. Western-blot analysis, PLD activity measurements and immunohistochemistry were used to select cell lines in which PLD1b and PLD2a were rapidly induced by IPTG. In the non-induced cells no Myc- or HA-tagged PLD protein was found by Western blotting or immunofluorescence, indicating no detectable 'leakage' from the inducible promoter. However, when incubated for 6 h with 500 μM IPTG a large production of both Myc-tagged PLD1b (124 kDa) and HA-tagged PLD2a (106 kDa) was observed (Figure 1). PLD induction did not cause any obvious changes in overall cell morphology. Confocal microscopy demonstrated that the intracellular distribution of each induced protein was similar to that seen in transient expression experiments. Under basal conditions the Myc-tagged PLD1b localized primarily to endosomal/lysosomal compartments, whereas HA-tagged PLD2a was found at the plasma membrane and in vesicular compartments (Figure 2). ATP stimulation for 5 min caused translocation of PLD1b to the plasma membrane. PLD2a showed a modest translocation to the plasma membrane together with an increased association along cytoskeletal elements.

In vitro PLD assays, performed in the presence of GTP[S], using lysates from unstimulated PAE cells showed that PLD1b induction caused an approx. 5-fold increase in the rate of PtdCho hydrolysis (changing from 1.0 to 5.1 nmol/h per mg of lysate

Figure 1 PLD expression in IPTG-inducible PAE cells expressing Myc-tagged PLD1b or HA-tagged PLD2a

Proteins were detected with anti-Myc (9E10) or anti-HA (12CA5) antibodies. Data are representative of three separate experiments.

protein). PLD2a induction caused an approx. 2.5-fold increase (changing from 0.2 to 0.5 nmol/h per mg of lysate protein). In the absence of GTP[S], PLD1b induction failed to raise phospholipase activity above that seen in the non-induced cells (0.1 nmol/h per mg of lysate protein). In contrast, lysates from non-induced PLD2a clones showed essentially no PLD activity, but upon induction, an increase to 0.1 nmol/h per mg of lysate protein was observed in the absence of GTP[S].

With a functional system able to overexpress selectively PLD1b or PLD2a it was now possible to characterize the PtdOH and PtdBut molecular species formed by each of these enzymes *in vivo*. Initially, total lipid extracts were separated into individual phospholipid classes by HPLC then each phospholipid was independently analysed by running on a C_{18} column with mass spectrometric detection; however, this time consuming, multi-step process resulted in variable recoveries, particularly for minor components. Further experimentation revealed that prior separation was unnecessary and that most phospholipids could be resolved and analysed by MS at the same time in a single run on a silica HPLC column using a chloroform/methanol/water/ammonia gradient (Figure 3). The retention times for acidic phospholipids, particularly PtdOH, were critically dependent on the ammonia concentration; too low and they eluted too fast (PtdOH merged into the preceding PtdCho), too high and they eluted too slowly (PtdIns and PtdSer merged into PtdCho, and PtdOH merged into sphingomyelin). Retention times for the zwitterionic lipids, such as PtdCho, PtdEtn and sphingomyelin, were largely unaffected by the ammonia concentration. A 0.32% ammonium solution (50 mM) proved to be optimal for the Luna silica column used, with most phospholipid classes resolving essentially to baseline. Under these conditions there was also partial resolution of the component molecular species within each phospholipid class, which, in conjunction with the MS analysis, aided identification. Longer chain species (e.g. 18:0/18:0) eluted slightly ahead of shorter chain species (e.g. 16:0/18:0),

Figure 2 Confocal microscopy of induced Myc-tagged PLD1b and HA-tagged PLD2a in PAE cells under basal and ATP stimulated conditions

Cells were grown on slides for 24 h, induced with 500 μ M IPTG for 6 h, stimulated with buffer (control) or 100 μ M ATP for 5 min, fixed, permeabilized and detected with anti-Myc or anti-HA antibodies. Data are representative of three separate experiments.

Figure 3 PAE phospholipids

HPLC separation of total phospholipids from ATP-stimulated PAE cells (10 min pre-incubation with 0.3% butan-1-ol prior to 15 min stimulation with 100 μ M ATP) on a Luna silica column with a solvent gradient of chloroform/methanol/water/ammonia solution (90:9.5:0.5:0.32, by vol.) changing to 50:48:2:0.32 (by vol.) over 40 min at 0.35 ml/min using evaporative light-scattering detection.

whereas the more unsaturated species (e.g. 18:0/20:4) eluted slightly ahead of the more saturated species (e.g. 18:0/20:3). Most acidic phospholipids, such as PtdBut (Figure 4a), were

Figure 4 Representative mass spectra for PtdBut (negative ionization) and PtdCho (positive ionization) from PAE cells preincubated with 0.3% butan-1-ol prior to stimulation with 100 μ M ATP for 15 min

Arrowed peaks are sodiated ions (m/z 22 greater than non-sodiated form). See Table 1 for the identity of the PtdBut molecular ions. The labelled PtdCho ions have the same acyl structures as the corresponding PtdBut ions.

Figure 5 Diacyl species profile for PtdOH from unstimulated, non-induced PAE cells

Data are expressed as mol% $\pm$ S.D. ($n = 4$). Minor species have been omitted.

readily detected as their molecular ions in negative ESI mode, PtdCho (Figure 4b) and sphingomyelin were best detected in positive ESI mode, while PtdEtn could be detected in both modes. In positive ESI mode the ion traces were more difficult to interpret, since, under these conditions, the MS detects a mixture of free ions together with their sodiated and potassiated (normally minor) counterparts.

Initial experiments to analyse cellular PtdOH only detected low levels of this lipid, even when PLD was overexpressed.

Stimulation with ATP or PMA caused little change to total PtdOH mass, which is in agreement with earlier data from PAE cells where only a small, transient and variable PtdOH elevation was seen following stimulation [7]. This, combined with a poorer MS sensitivity for PtdOH compared with other phospholipids, made it difficult to perform accurate structural characterizations, however, the major species was identified as 16:0/18:1 (Figure 5). The profile did not appear to change following PLD induction and/or stimulation.

Since PtdOH can be generated through several pathways and its rapid metabolism normally prevents substantial accumulation, all subsequent studies of PLD specificity were based on the analysis of PtdBut. This lipid is only formed by PLD in the presence of butan-1-ol, making it a much better indicator of PLD activity. Also, with its much greater metabolic stability, PtdBut accumulates to a greater extent than PtdOH, thus making analysis easier. Under resting conditions, the addition of butan-1-ol had little effect on PtdOH mass and had no effect on species profiles (results not shown).

LC-MS characterization showed that the major diacyl PtdBut structures formed *in vivo* by endogenous PLDs in parental or uninduced PAE cells were saturated, mono-unsaturated and di-unsaturated species, particularly 16:0/18:1, 18:0/18:1 and 18:1/18:1+18:0/18:2 (Table 1). This was similar to the species profiles obtained for PtdOH, although the latter contained slightly more polyunsaturated species, particularly 18:0/20:4 and 18:0/20:3. Alkyl,acyl structures represented 15–20% of total PtdBut species, with the major form being *sn*1-O-16:0/18:1 (results not shown). Stimulation of serum-starved PAE cells with either ATP or PMA increased PtdBut generation 4–5-fold, however, the species profile did not change, remaining enriched

Table 1 PtdBut molecular species from unstimulated PLD-transfected PAE cells

Following serum-starvation for 22 h cells were incubated for a further 6 h with (Induced) or without (Non-induced) 500 μ M IPTG. Results are expressed as mol% of total diacyl PtdBut species $\pm$ S.D. (where $n = 3$ –6).

PtdBut		PLD1b		PLD2a	
Diacyl species	<i>m/z</i>	Non-induced	Induced	Non-induced	Induced
14:0/16:0	675.2	3.3 $\pm$ 1.5	2.9 $\pm$ 1.6	2.7 $\pm$ 1.1	2.5 $\pm$ 1.1
16:1/16:1, 14:0/18:2	699.2	1.4 $\pm$ 0.7	1.3 $\pm$ 0.6	1.3 $\pm$ 0.4	1.4 $\pm$ 0.3
16:0/16:1, 14:0/18:1	701.2	8.0 $\pm$ 1.2	7.2 $\pm$ 1.5	6.4 $\pm$ 1.2	6.6 $\pm$ 1.3
16:0/16:0, 14:0/18:0	703.2	7.7 $\pm$ 1.4	7.4 $\pm$ 1.3	5.4 $\pm$ 0.8	5.6 $\pm$ 0.4
16:1/18:1, 16:0/18:2	727.2	5.8 $\pm$ 0.7	5.3 $\pm$ 0.6	5.9 $\pm$ 0.8	5.9 $\pm$ 0.9
18:0/16:1, 16:0/18:1	729.2	20.1 $\pm$ 1.9	20.8 $\pm$ 2.8	20.5 $\pm$ 2.0	20.5 $\pm$ 2.4
16:0/18:0	731.2	9.6 $\pm$ 1.6	9.2 $\pm$ 1.5	8.8 $\pm$ 1.4	8.4 $\pm$ 1.7
16:0/20:4	751.3	1.4 $\pm$ 0.5	1.2 $\pm$ 0.3	0.9 $\pm$ 0.1	1.1 $\pm$ 0.3
18:1/18:2, 16:0/20:3	753.3	3.1 $\pm$ 0.6	3.1 $\pm$ 0.6	3.5 $\pm$ 0.3	3.2 $\pm$ 0.5
18:1/18:1, 18:0/18:2	755.3	10.8 $\pm$ 1.3	10.9 $\pm$ 0.9	13.2 $\pm$ 1.7	12.8 $\pm$ 1.4
18:0/18:1, 16:0/20:1	757.3	8.9 $\pm$ 1.0	9.6 $\pm$ 1.0	11.7 $\pm$ 1.7	11.8 $\pm$ 1.3
18:0/18:0, 16:0/20:0	759.3	2.5 $\pm$ 0.2	2.8 $\pm$ 0.5	3.5 $\pm$ 0.6	3.5 $\pm$ 0.4
16:0/22:6	775.3	0.9 $\pm$ 0.2	1.0 $\pm$ 0.2	0.7 $\pm$ 0.3	0.8 $\pm$ 0.2
18:1/20:4, 16:0/22:5	777.3	1.8 $\pm$ 0.3	1.9 $\pm$ 0.3	1.5 $\pm$ 0.5	1.5 $\pm$ 0.3
18:0/20:4	779.3	2.8 $\pm$ 0.6	2.9 $\pm$ 0.6	2.4 $\pm$ 0.4	2.5 $\pm$ 0.5
18:0/20:3	781.3	2.9 $\pm$ 0.5	2.7 $\pm$ 0.6	2.7 $\pm$ 0.6	2.8 $\pm$ 0.6
18:0/20:2, 18:1/20:1	783.3	1.9 $\pm$ 0.5	1.9 $\pm$ 0.5	1.8 $\pm$ 0.6	1.8 $\pm$ 0.4
18:0/20:1	785.3	1.0 $\pm$ 0.3	0.9 $\pm$ 0.2	1.0 $\pm$ 0.2	1.1 $\pm$ 0.3
18:1/22:6	801.4	0.8 $\pm$ 0.4	1.0 $\pm$ 0.4	1.0 $\pm$ 0.4	0.7 $\pm$ 0.3
18:0/22:6	803.4	1.1 $\pm$ 0.3	1.4 $\pm$ 0.4	1.2 $\pm$ 0.4	1.0 $\pm$ 0.3
18:0/22:5	805.4	1.5 $\pm$ 0.6	1.5 $\pm$ 0.4	1.3 $\pm$ 0.5	1.3 $\pm$ 0.7
18:0/22:4	807.4	1.0 $\pm$ 0.4	1.0 $\pm$ 0.3	0.9 $\pm$ 0.4	1.0 $\pm$ 0.5
18:0/22:3	809.4	0.7 $\pm$ 0.1	0.8 $\pm$ 0.4	0.6 $\pm$ 0.4	0.8 $\pm$ 0.7
18:0/22:2	811.4	0.6 $\pm$ 0.2	0.6 $\pm$ 0.3	0.6 $\pm$ 0.1	0.7 $\pm$ 0.3
18:0/22:1	813.4	0.6 $\pm$ 0.3	0.7 $\pm$ 0.3	0.6 $\pm$ 0.3	0.6 $\pm$ 0.3
Total (nmol/10 ⁷ cells)		0.20 $\pm$ 0.03	0.25 $\pm$ 0.03	0.30 $\pm$ 0.04	0.30 $\pm$ 0.02

Figure 6 Comparison of the diacyl species profiles from the major glycerophospholipids with that for PtdBut in unstimulated, non-induced PAE cells

Data are expressed as mol% $\pm$ S.D. ($n = 4-6$). Minor species (< 0.5 mol%) have been omitted.

in mono- and di-unsaturated species, particularly 16:0/18:1, under all conditions. Induction of Myc-tagged PLD1b or HA-tagged PLD2a made little difference to either basal or stimulated PtdBut production and had no effect on the species profile.

Molecular species analysis of other bulk membrane phospholipids showed that the PtdBut profile most closely matched that for total PtdCho, the presumed substrate for both PLDs, although there were some clear differences. These were most notably the lower proportions of 14:0/16:0, 14:0/18:1 + 16:0/16:1 and 14:0/18:0 + 16:0/16:0, and the higher proportions of 16:0/18:1 in PtdBut (Figure 6). The diacyl profile of PtdEtn also showed some similarities to that for PtdBut, however, the PtdIns and PtdSer profiles were very different.

The basal, *in vivo*, PtdBut profile obtained from monkey COS7 epithelial cells transiently transfected with human PLD2a for 24 h (Figure 7) was similar to that seen in PAE cells. The PtdBut profiles from COS7 cells transfected with HA-vector control, PLD2a, PIP kinase type 1 α or PLD2a + PIP kinase type 1 α

Figure 7 Species profile for PtdBut from unstimulated, vector control COS7 cells

Data are expressed as mol% $\pm$ S.D. ($n = 4$). The profile remains essentially identical for cells transiently transfected for 24 h with HA-vector control, PIP kinase 1 α , PLD2a or PLD2a + PIP kinase 1 α , yet total PtdBut formation after 30 min of incubation with butan-1-ol is elevated in the PLD2 transfectants (inset; data are representative of two separate experiments).

Figure 8 Time course for PtdBut formation in PAE cells

Cells expressing inducible PLD1b pre-labelled with [9,10- ^{3} H]palmitic acid stimulated with 100 μ M ATP in the presence of 0.3% butan-1-ol. [^{3} H]PtdBut is expressed as the percentage of total [9,10- ^{3} H]palmitic acid $\pm$ S.D. ($n = 3$).

were essentially identical, even though total PtdBut mass was doubled in the latter (Figure 7, inset). This indicates that the transfected PLD2a had the same substrate specificity as the endogenous PLD. The PtdBut profile generated by endogenous PLD from a third cell type, Rat1 fibroblasts, was also similar to that seen in COS7 and PAE cells (results not shown).

Since maximal stimulated PtdBut formation appeared largely unaffected by the amount of PLD present in the PAE cells, it raised the possibility that elevated enzyme levels may, instead, alter the initial rate of agonist-stimulated PtdBut formation. Thus the kinetics of ATP-stimulated PtdBut formation *in vivo* was compared in induced and non-induced PAE PLD1b cells (Figure 8). This showed that stimulated PtdBut formation reached a maximum in approximately 2 min and then remained

Figure 9 PLD *in vitro* substrate specificity

Lysates from Sf9 cells (parental cells and cells overexpressing PLD1b or PLD2a) were assayed with mixed lipid vesicles containing [^3H]PtdCho, [^{14}C]PtdEtn or [^3H]PtdIns (all labelled in the water soluble headgroup). No hydrolysis of PtdIns was detected, and so it is omitted from the Figure. Data are expressed as pmol of substrate hydrolysed/h (assuming a lipid vesicle composed of a single substrate).

close to this level for at least a further 10 min. There was only a small difference in the maximum PtdBut level between induced and non-induced cells; however, measurement at short time points (< 120 s) indicated that the initial rate of formation was slightly greater for the induced cells. A similar experiment with PLD2a did not detect any consistent differences in the kinetics of ATP-stimulated PtdBut formation between induced and non-induced cells (results not shown).

To determine if PtdCho is the sole PLD substrate, lysates prepared from control (uninfected) Sf9 cells and Sf9 cells overexpressing PLD1b or PLD2a were used for *in vitro* assays where the enzymes were presented with [^3H]PtdCho, [^{14}C]PtdEtn or [^3H]PtdIns in mixed lipid vesicles. Release of the radiolabelled polar headgroup ([^3H]choline, [^{14}C]ethanolamine or [^3H]inositol) was measured. These experiments showed that, *in vitro*, lysates from cells overexpressing PLD had substantially higher phospholipase activity than the endogenous activity detected in the control lysates. Both PLD1b and PLD2a preferentially hydrolysed PtdCho, however, they could also utilize PtdEtn, though at a much lower rate (Figure 9). PtdIns was not a substrate. Experiments using lysates from transiently transfected COS7 cells gave similar results (results not shown). Under these experimental conditions PLD2a had a consistently greater specificity for PtdCho over PtdEtn (approx. 15-fold) than did PLD1b (approx. 7-fold).

The Sf9 cell lysates were also used to examine PLD1b diacyl species specificity *in vitro*. The assay, performed in the presence of 0.3% butan-1-ol, used non-radiolabelled vesicles containing either mixed species egg-derived PtdCho + 12:0/12:0-PtdEtn or 12:0/12:0-PtdCho + mixed species egg-derived PtdEtn. Since 12:0/12:0 species are absent from the egg-derived lipids, the formation of 12:0/12:0-PtdBut could only result from the transphosphatidylation of the appropriate synthetic PtdCho or PtdEtn. Conversely, other species could only derive from the egg lipid. LC-MS analysis of the resultant PtdBut showed that both PtdCho and PtdEtn could be substrates for PLD1b, since 12:0/12:0 species were formed when using either vesicle

Figure 10 PLD1b *in vitro* species specificity: comparison of substrate (egg PtdCho or egg PtdEtn) and product (PtdBut) profiles

Data are representative of two separate experiments. While synthetic 12:0/12:0-PtdCho and 12:0/12:0-PtdEtn are both substrates for PLD1b *in vitro*, they are not present in egg-derived PtdCho or PtdEtn and are thus omitted from the Figure.

composition. The analysis also showed that, *in vitro*, PLD1b will hydrolyse all egg-derived PtdCho and PtdEtn species with only a slight preference for mono- and di-unsaturated structures compared with polyunsaturated structures (Figure 10). The data also showed that neither PtdIns nor PtdIns(4,5) P_2 (both present in the vesicles) were substrates for PLD, since polyunsaturated PtdBut structures, which would be expected from phosphoinositide hydrolysis, were not observed.

DISCUSSION

The data in the present paper represent the first comprehensive study of human PLD1 and PLD2 substrate specificity and the resultant PtdOH/PtdBut structures formed. Although not examined in the present study, PLD1a and PLD2b are expected to behave similarly, with regard to their substrate specificity, to PLD1b and PLD2a respectively. The results show that the description of PLD as being absolutely PtdCho-specific is incorrect, since PtdEtn can serve as a substrate *in vitro*; however, the enzyme does show a considerably greater selectivity (some 7–15-fold) for the former lipid (Figure 9). In contrast, *in vivo*, there was no evidence for PtdEtn hydrolysis, since we consistently failed to detect ethanolamine release from PAE cells or any other cell type, indicating that the PLDs show greater specificity *in vivo* than *in vitro*. This is probably due to the highly regulated and structured environment within biological membranes compared with the artificial vesicles used for *in vitro* studies. There are a few reports, most notably from Kiss and co-workers [10,11], of *in vivo* ethanolamine release under certain conditions, although whether this is an indication of cell type or species differences or of another class of PLD (perhaps the putative mitochondrial PtdEtn-PLD [13]) is unclear. There have also been reports suggesting that inositol lipids can be hydrolysed by PLD [14–16]; however, the data presented here demonstrate that this reaction cannot be catalysed by PLD1 or PLD2.

PLD-1 and -2 appear able to hydrolyse all presented diacyl PtdCho species both *in vivo* and *in vitro* (Figures 6 and 10), although there was a bias towards mono- and di-unsaturated structures. *In vivo* the major PtdOH and PtdBut structures formed are 16:0/18:1, 18:0/18:1 and 18:1/18:1+18:0/18:2. These are major diacyl structures in both PtdCho and PtdEtn. *In vitro* the major products depend on the phospholipid substrate. From the present study it is clear that apparent phospholipase specificities *in vitro* do not necessarily correspond to those *in vivo* and should be interpreted with care. Similar observations have been noted for various PKC isoforms that can be activated by essentially any DAG *in vitro*, but *in vivo* show little or no response to saturated/mono-unsaturated structures [8], but are highly activated by polyunsaturated DAG species [18]. The observation that the same mono- and di-unsaturated PtdBut species are the major PLD-derived structures in three different cell types (COS7, PAE and Rat1) representing three different cell types (epithelial, endothelial and fibroblast) from three different organisms (monkey, pig and rat) strongly implies that the corresponding PtdOH structures have an essential and conserved signalling role.

PtdOH levels did not change much in the presence of butan-1-ol compared with its absence, indicating that most PtdOH in resting cells is generated through non-PLD pathways, such as DAG phosphorylation by DAGK or acylation of glycerol 3-phosphate. Although this basal PtdOH has a similar species profile to that generated by PLD it is not expected to have a significant signalling role. Indeed, the presence of polyunsaturated PtdOH species is probably a reflection of DAGK-catalysed removal of the DAG generated by PLC-catalysed PtdIns(4,5) P_2

hydrolysis. It is thus possible that localized increases in mono- and di-unsaturated PtdOH as a result of PLD activation raise the levels above a threshold, thereby initiating their role as intracellular signals.

Since cellular PLD1 and PLD2 protein levels are increased upon IPTG induction and the lysates prepared from these cells have substantially elevated *in vitro* PtdCho hydrolytic activity, it could reasonably be expected that the *in vivo* PLD activity should also be raised. However, only a very limited effect on stimulated PtdBut formation was observed *in vivo* (see Figure 8). This makes it probable that there is a limit to the maximum possible activation of PLD *in vivo*, and that this limit is only slightly above that which can be reached by the endogenous enzyme alone following appropriate (e.g. ATP or PMA) stimulation. Increasing the total amount of cellular PLD alone is insufficient to exceed this maximum, probably since the availability of one or more regulators, such as ADP-ribosylation factor, Rho and PKC with PLD1 [19], or PIP kinase 1 α with PLD2 (Figure 7 and [20]), essential for activation becomes limiting. Recently co-transfection of cells with PLD1 plus ADP-ribosylation factor 6, Rac1 or PKC α was found to increase *in vivo* PLD activity compared with any component transfected alone (D. J. Powner, M. N. Hodgkin and M. J. O. Wakelam, unpublished work). This supports the idea of limiting cofactors restricting maximal activity. Similarly, in COS7 cells transiently co-expressing PLD2a and PIP kinase 1 α we observed an increase in generated PtdBut mass which was much larger than that seen with PLD2a alone (Figure 7) and, significantly, the relative proportions of the individual species remained unchanged with the same profile as that seen in untransfected cells and also very similar to that in PAE cells (Figure 6) whether or not overexpressing PLD1b or PLD2a. These observations reflect the need for the cell to exert tight regulation on PLD activity since its over-activation is toxic. *In vitro* assays do not reflect these limitations on maximal PLD activation probably because compartmentalization, which *in vivo* would normally restrict cofactor–PLD interactions, is no longer an issue and hence all cofactor molecules become available to interact with PLD. Thus if total cofactor availability exceeds PLD availability then increased PLD expression should lead to increased *in vitro* PtdBut formation.

The small changes in total PtdBut formation following 15 min stimulation in induced PAE cells compared with non-induced cells (Table 1) raises the question as to whether the induced PLD is active. However, since *in vitro* assays show increased PLD activity, and immunofluorescence microscopy detects what we believe to be normal translocation (since, in our hands, no suitable antibodies for detection of endogenous PLDs are currently available) in response to agonist stimulation, then we fully expect the transfected PLDs to be active *in vivo*. Transient transfections with the same PLD DNA sequences in COS7 cells and other cell types show that the resultant enzymes are active *in vivo*. As outlined above, significantly increased PLD activity *in vivo* is unlikely to be observed following increased PLD expression without corresponding increased expression of appropriate cofactors. Thus it is probable that the phospholipase activity derived from the induced PLD becomes a significant proportion of the total observed PLD activity. Consequently, any differences in species specificity between the endogenous and induced PLDs would cause an alteration in the PtdBut profile compared with that seen with non-induced cells. No such changes were observed, which strongly implies that the induced PLD-1b and -2a have identical species specificities to the endogenous PLDs and to each other.

The data in the present study show that *in vivo* PLD-1 and -2 generate primarily mono- and di-unsaturated PtdOH, in par-

ticular 16:0/18:1, which can reasonably be expected to be the signalling forms of this lipid. This is thus in full agreement with our model of parallel PLC and PLD pathways producing polyunsaturated DAG and mono/di-unsaturated PtdOH messengers respectively, whose signalling is terminated through conversion by DAGK ϵ or PAP into the corresponding inactive polyunsaturated PtdOH and mono/di-unsaturated DAG structures ready for re-synthesis of the starting PtdIns(4,5)P₂ and PtdCho. While different DAGK isoforms undoubtedly play a variety of roles [21], certain forms, such as α in CTLL-2 cells, also produce what are probably signalling forms of PtdOH [22], but with subtly different molecular structures to those generated by PLD, thus it is now important to identify specific targets for these signalling PtdOH species.

We thank Dr Phil Hawkins (The Babraham Institute, Babraham, Cambridge, U.K.) for the gift of plasmid constructs and Dr Tony Postle (University of Southampton, Southampton, U.K.) for advice on the application of MS to lipid analysis. M.M. is supported by a Medical Research Council studentship and we thank the Wellcome Trust for funding this work.

REFERENCES

- Liscovitch, M., Czarny, M., Ficuci, G. and Tang, X. (2000) Phospholipase D: molecular and cell biology of a novel gene family. *Biochem. J.* **345**, 401–415
- Jones, D., Morgan, C. and Cockcroft, S. (1999) Phospholipase D and membrane traffic. Potential roles in regulated exocytosis, membrane delivery and vesicle budding. *Biochim. Biophys. Acta* **1439**, 229–244
- McPhail, L. C., Waite, K. A., Regier, D. S., Nixon, J. B., Qualliotine-Mann, D., Zhang, W.-X., Waillin, R. and Sergeant, S. (1999) A novel protein kinase target for the lipid second messenger phosphatidic acid. *Biochim. Biophys. Acta* **1439**, 277–290
- Daniel, L. W., Sciorra, V. A. and Ghosh, S. (1999) Phospholipase D, tumor promoters, proliferation and prostaglandins. *Biochim. Biophys. Acta* **1439**, 265–276
- Bokoch, G. M., Reilly, A. M., Daniels, R. H., King, C. C., Olivera, A., Spiegel, S. and Knaus, U. G. (1998) A GTPase-independent mechanism of p21-activated kinase activation. Regulation by sphingosine and other biologically active lipids. *J. Biol. Chem.* **273**, 8137–8144
- Grange, M., Sette, C., Cuomo, M., Conti, M., Lagarde, M., Prigent, A. F. and Nemoz, G. (2000) The cAMP-specific phosphodiesterase PDE4D3 is regulated by phosphatidic acid binding. Consequences for cAMP signaling pathway and characterization of a phosphatidic acid binding site. *J. Biol. Chem.* **275**, 33379–33387
- Cross, M. J., Roberts, S., Ridley, A. J., Hodgkin, M. N., Stewart, A., Claesson-Welsh, L. and Wakelam, M. J. O. (1996) Stimulation of actin stress fibre formation mediated by activation of phospholipase D. *Curr. Biol.* **6**, 588–597
- Pettitt, T. R., Martin, A., Horton, T., Liossis, C., Lord, J. M. and Wakelam, M. J. O. (1997) Diacylglycerol and phosphatidate generated by phospholipases C and D, respectively, have distinct fatty acid compositions and functions. Phospholipase D-derived diacylglycerol does not activate protein kinase C in porcine aortic endothelial cells. *J. Biol. Chem.* **272**, 17354–17359
- Hodgkin, M. N., Pettitt, T. R., Martin, A., Michell, R. H., Pemberton, A. J. and Wakelam, M. J. O. (1998) Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? *Trends Biochem. Sci.* **23**, 200–204
- Mukherjee, J. J., Chung, T., Ways, D. K. and Kiss, Z. (1996) Protein kinase C α is a major mediator of the stimulatory effect of phorbol ester on phospholipase D-mediated hydrolysis of phosphatidylethanolamine. *J. Biol. Chem.* **271**, 28912–28917
- Kiss, Z., Tomono, M. and Anderson, W. B. (1994) Phorbol ester selectively stimulates the phospholipase D-mediated hydrolysis of phosphatidylethanolamine in multidrug-resistant MCF-7 human breast carcinoma cells. *Biochem. J.* **302**, 649–654
- Kobayashi, M., Kidd, D., Hutson, E., Grafton, J., McNulty, S. and Rumsby, M. (2001) Protein kinase C activation by 12-O-tetradecanoylphorbol 13-acetate in CG-4 line oligodendrocytes stimulates turnover of choline and ethanolamine phospholipids by phospholipase D and induces rapid process contraction. *J. Neurochem.* **76**, 361–371
- Madesh, M. and Balasubramanian, K. A. (1997) Metal ion stimulation of phospholipase D-like activity of isolated rat intestinal mitochondria. *Lipids* **32**, 471–479
- Huang, C., Wykle, R. L., Daniel, L. W. and Cabot, M. C. (1992) Identification of phosphatidylcholine-selective and phosphatidylinositol-selective phospholipases D in Madin–Darby canine kidney-cells. *J. Biol. Chem.* **267**, 16859–16865
- Balsinde, J., Diez, E., Fernandez, B. and Mollinedo, F. (1989) Biochemical characterization of phospholipase D activity from human neutrophils. *Eur. J. Biochem.* **186**, 717–724
- Ching, T.-T., Wang, D.-S., Hsu, A.-L., Lu, P.-J. and Chen, C.-S. (1999) Identification of multiple phosphoinositide-specific phospholipases D as new regulatory enzymes for phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **274**, 8611–8617
- Frohman, M. A. and Morris, A. J. (1999) Phospholipase D structure and regulation. *Chem. Phys. Lipids* **98**, 127–140
- Pettitt, T. R. and Wakelam, M. J. O. (1999) Diacylglycerol kinase epsilon, but not zeta, selectively removes polyunsaturated diacylglycerol, inducing altered protein kinase C distribution *in vivo*. *J. Biol. Chem.* **274**, 36181–36186
- Hodgkin, M. N., Clark, J. M., Rose, S., Saqib, K. M. and Wakelam, M. J. O. (1999) Characterization of the regulation of phospholipase D activity in the detergent-insoluble fraction of HL60 cells by protein kinase C and small G-proteins. *Biochem. J.* **339**, 87–93
- Divecha, N., Roefs, M., Halstead, J. R., D'Andrea, S., Fernandez-Borga, M., Oomen, L., Saqib, K. M., Wakelam, M. J. O. and D'Santos, C. (2000) Interaction of the Type I α PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4,5-bisphosphate in the regulation of PLD2 activity. *EMBO J.* **19**, 5440–5449
- van Blitterswijk, W. J. and Houssa, B. (2000) Properties and functions of diacylglycerol kinases. *Cell. Signalling* **12**, 595–605
- Jones, D. R., Pettitt, T. R., Sanjuán, M. A., Mérida, I. and Wakelam, M. J. O. (1999) Interleukin-2 causes an increase in saturated monounsaturated phosphatidic acid derived from 1,2-diacylglycerol and 1-O-alkyl-2-acylglycerol. *J. Biol. Chem.* **274**, 16846–16852

Received 14 May 2001/12 October 2001; accepted 23 October 2001