Didecanoyl phosphatidylcholine is a superior substrate for assaying mammalian phospholipase D

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INTRODUCTION

Activation of phospholipase D (PLD) is involved in the signal transduction of both seven-membrane-spanning receptors activated by hormones and tyrosine kinase receptors activated by growth factors [1]. Generally, measurement of PLD activity has been done either by using cells endogenously radiolabelled in their phospholipids or by using exogenous radiolabelled phospholipid substrate. However, PLD in crude or solubilized membranes from mammalian tissues is difficult to detect with the current assay techniques, unless a high radioactive concentration of substrate and/or long incubation times are employed. Generally, the enzyme has to be extracted and partially purified on one column before easy detection of activity. Furthermore, PLD activity in cultured cells can only be detected by the available assay techniques in the presence of guanosine 5\(^{-}\gamma\)-thiotriphosphate (GTP\(^{S}\)) and a cytosolic factor [usually ADP-ribosylation factor (Arf)]. In this paper we report that the use of didecanoyl phosphatidylcholine (C\(_{16}\)-PC) in mammalian PLD assays considerably increases the detection limit. C\(_{16}\)-PC was compared with the commonly used dipalmitoyl phosphatidylcholine (C\(_{16}\)-PC) as a substrate for PLD activity from membranes of human neutrophils, human placenta and pig brain, and from placental cytosol. C\(_{16}\)-PC was superior to C\(_{16}\)-PC by a factor of 2–28 depending on assay conditions and tissue, and it allowed the detection of GTP[S]-and Arf-stimulated PLD activity without addition of phosphatidylinositol 4,5-bisphosphate.

MATERIALS AND METHODS

Materials

1,2-[\(^{14}\)C]Didecanoyl-sn-glycero-3-phosphocholine (67 mCi/mmol) and 1,2-didecanoyl-sn-glycero-3-phospho-[\(^{3}H\)]choline (85 Ci/mmol) (prepared by Amersham) were gifts from Novo Nordisk. 1-Stearoyl-2-[\(^{14}\)C]arachidonyl-sn-glycero-3-phosphocholine (54 mCi/mmol), 1-palmitoyl-2-[\(^{14}\)C]linoleoyl-sn-glycero-3-phosphocholine (57 mCi/mmol) and 1-palmitoyl-2-[\(^{14}\)C]palmitoyl-sn-glycero-3-phosphocholine were from Amersham, and 1-palmitoyl-2-[\(^{3}H\)]arachidonyl-sn-glycero-3-phosphocholine (57 mCi/mmol) was from Dupont–NEN. 1,2-Dipalmitoyl-sn-glycero-3-phospho-[\(^{3}H\)]choline was obtained from NEN (Figure 1) and Amersham (Figure 3). Glass-backed silica gel 60 TLC plates were from Merck (Darmstadt, Germany). Ecoscient scintillation fluid was from National Diagnostics. Dithiothreitol, Triton X-100 and octyl glucoside were from Boehringer (Mannheim, Germany). Phosphatidylethanolamine (PE; from egg yolk) and all other chemicals were from Sigma (St. Louis, MO, U.S.A.). Recombinant myristoylated Arf (mArf1) was kindly provided by Dr. S. Paris (CNRS, Valbonne, France), and recombinant non-myristoylated Arf (rArf1) was prepared as described previously [8].

Preparation of placental membranes, placental cytosol and pig brain membranes, and fractionation of placental proteins

Human placentae were homogenized as described previously [9]. Briefly, the tissue was homogenized with an UltraTurrax* for...
Phosphatidylcholine (° indicated time periods at 37 °C) was used for testing of the substrate. Markers were used for PLD activity. Solubilized proteins and cytosol were purified on DEAE-Sepharose columns as described [9,9a]. Fractionation of the cytosolic enzyme employed the same procedure, except that no Triton X-100 was added to the buffers. Solubilized proteins from pig brain membranes were prepared according to the above procedure. Protein was determined by the Bradford procedure, using IgG as a standard.

Preparation and fractionation of PLD activity from human neutrophil membranes

Human neutrophils were prepared from human blood. They were then allowed to swell in hypotonic buffer and cell-cracked. A post-nuclear supernatant was prepared by low-speed centrifugation and applied to a continuous 10.4–40 °C cushion. Fractions were assayed for PLD and marker enzymes. The peak activity associated with plasma membrane markers was used for testing of the substrate.

Determination of PLD activity in placental and brain tissues

PLD activity was measured by monitoring the release of [3H]choline according to [10] with minor modifications. Incubations in a total volume of 100 µl were carried out for the indicated time periods at 37 °C. [3H]C10-PC or [3H]dipalmitoyl phosphatidylcholine ([3H]C16-PC and C16-PC or C16-PC were added, and the solvents were evaporated under a stream of N2. Vesicle formation was carried out by sonication for 10 min with 50 mM Hepes and 0.07% octyl glucoside. The exact assay conditions, including substrate concentrations, added buffers and salts, are specified in the legends to the Figures. Incubations were stopped by addition of 50 µl of BSA (20 mg/ml) and 100 µl of trichloroacetic acid (20%), followed by centrifugation at 10000 g for 10 min at 4 °C. An aliquot (200 µl) of the supernatant was counted for radioactivity by liquid scintillation counting.

Measurement of phosphatidylethanol (PEt) formation using 13C-labelled PCs was done essentially as described previously [9]. The exact assay conditions, including substrate composition, added buffers and salts, are specified in the text and legend to the Figures. NaF was included as an inhibitor of phosphatidic acid phosphatase. The reactions were stopped by addition of 2 ml of chloroform/methanol/conc. HCl (200:100:0.5, by vol.). The lipids were extracted and separated on TLC plates as described [9]. The activity values in the Figures were obtained by subtracting the control value.

Determination of PLD activity in human neutrophil membranes

Substrate was prepared from PE, PIP2, and PC (C16 or C14) in the molar ratio 10:0.3:1. For vesicles not containing PIP2, the vesicle composition was PE/PC (10:1, mol/mol). The final PC concentration was 8.6 µM. [3H]Choline-labelled PC was included to give approx. 50000 d.p.m. per 10 µl of substrate. Assays were performed at 37 °C with 1.5–2.0 µg of protein for 60–90 min. A 10 µl aliquot of substrate was added in a final volume of 60 µl. mArf1 was used at 40 µM, and GTP[S] at 30 µM. Substrate and assay buffer was 50 mM Na/Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl and 1 mM dithiothreitol, with the inclusion of MgCl2 (0.5 mM) and CaCl2 (2 mM) in the assay buffer. [3H]Choline was extracted and measured as described previously [11].

RESULTS

Experiments were performed in order to compare [14C]C10-PC with three other molecular species of [14C]PC containing the fatty acids C16:0/C16:1, C16:0/C20:4 and C16:0/C20:5. Incubations were run for 10 min with 1 mM phospholipid (0.05 µCi/assay) and 40 µM PIP2. To our surprise, no PLD activity (measured by PEt formation) was detected using PCs containing relatively long-chain fatty acids, whereas the relatively short-chained C10-PC allowed easy detection of extracted/purified PLD from human placenta. Thus the PLD activities (PEt formation) of solubilized membranes (117 µg of protein) and of DEAE-Sepharose peak fractions of membranes (110 µg) and cytosol (168 µg) were 3751±481 (mean ± range, n = 2), 7754±613 and 192±25 pmol/min per mg of protein respectively. Next, C10-PC was compared with C16-PC, the most commonly used exogenous substrate in PLD assays. Increasing concentrations of C10-PC or C16-PC (0.1–4 mM) were added to cytosolic PLD from human placenta that had been partially purified on a DEAE-Sepharose column (Figure 1). Cytosolic PLD was chosen as the PLD source in these experiments. This PLD offers an advantage over membrane PLD as no detergents have to be added, and thus the mol % of substrate in the vesicles can be kept constant, which means that considerations of surface dilution can be ignored. The PIP2 level was kept constant at 3.8 mol %.

![Figure 1](image-url)

**Figure 1** Effect of increasing concentrations of C10-PC or C16-PC on the activity of partially purified cytosolic PLD from human placenta.

Cytosol from human placenta was partially purified on a DEAE-Sepharose column. A sample of the PLD peak fraction (216 µg of protein) was incubated with 0.13–4 mM [14C]C10-PC (2000 d.p.m./nmol) or [14C]C16-PC (3600 d.p.m./nmol), 3.8 mol % PIP2, 3.01% octyl glucoside, 50 mM Hepes, pH 7.5, 2 mM CaCl2, 3 mM MgCl2, 3 mM EGTA, 80 mM KCl and 1 mM dithiothreitol for 15 min at 37 °C. Release of [3H]Choline was measured as described in the Materials and methods section. The inset shows a Hanes–Woolf transformation of the data obtained with C10-PC. Data represent the means± ranges of the results of one experiment in duplicate performed twice.
Using C₁₀-PC the enzyme followed Michaelis–Menten kinetics, with a $K_m$ of approx. 0.1 mM. Transforming the data to a Hanes–Woolf plot [12], which ensures a more uniform distribution of the data points, resulted in a straight line. However, with C₁₀-PC, very low, if any, PLD activity was observed, and it was not possible under these assay conditions (see the legend to Figure 1) to obtain a Michaelis–Menten curve. In this experiment C₆-PC was superior to C₁₀-PC in the concentration range 0.13–0.5 mM by a factor of 17–28.

In order to optimize the assay employing C₆-PC, the assay conditions were changed to those described in the legend to Figure 2. Comparison of C₁₀-PC and C₆-PC under these assay conditions showed that PLD activities in membranes from placenta and brain were measurable with both substrates. However, pronounced differences in PLD activities obtained using C₁₀-PC compared with C₆-PC as substrate were observed with membranes both from human placenta (by a factor of 15) (Figure 2, left panel) and from pig brain (by a factor of 4) (Figure 2, right panel). The addition of GTP[S] (20 $\mu$M) or mArf1 (2.5 $\mu$M) did not change this pattern. Generally, the specific activity of PLD was higher in membranes from human placenta than in those from pig brain. However, this difference was more pronounced using C₁₀-PC compared with C₆-PC.

In order to extend the study, PLD activity in human neutrophils was measured. The PLD activity was from a highly enriched fraction of plasma membranes, purified on a continuous sucrose gradient. Results showed that, for this PLD also, C₁₀-PC was excellent as an exogenous substrate compared with C₆-PC, irrespective of whether GTP[S], rArf1 or 0.5 M NaCl was added (Figure 3). NaCl (0.5 M) was found to activate neutrophil PLD activity. Omitting PIP₂ from the incubations resulted in lower, but still measurable, activity with C₆-PC, whereas C₁₀-PC was not a very useful substrate under these assay conditions (Figure 3).

**DISCUSSION**

The ability to obtain a pure mammalian PLD by protein purification or molecular biological techniques is of major importance to the area of agonist-stimulated PC hydrolysis. Until now, only one report of the purification to homogeneity of a mammalian PLD has appeared, namely an oleate-activated form of PLD that was purified from pig lung [13]. Recently, an Arf-stimulated PLD was highly purified from pig brain to a specific activity of 28 nmol/min per mg of protein, as determined with C₁₀-PC in the presence of Arf [4]. There has also been a recent report on the cloning of Arf-stimulated PLD from HeLa cells [14]. However, the purification of Arf-stimulated PLD to homogeneity still remains to be done. The major obstacle to this

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**Figure 2.** Comparison of C₁₀-PC and C₆-PC as substrates for PLD from membranes of human placenta and pig brain

**Figure 3.** Comparison of C₁₀-PC and C₆-PC as exogenous substrates for a partially purified PLD fraction from human neutrophil membranes
project is that PLD is labile during purification, and that cytosolic factors have to be added for detection of PLD activity [4].

Here we describe PLD activity towards a short-chain PC containing two C₁₀ fatty acids, the use of which results in a pronounced increase in sensitivity of the assay. Using optimum assay conditions (1 mM C₄₀₋₆₀PC), specific PLD activities in human placental membranes can be measured in the range of nmol/min per mg. For comparison, the reported specific activities of both cytosolic and membrane-associated enzymes from brain determined in the presence of C₆₀₋₆₀PC are approx. 16 pmol/min per mg of protein [6]. In contrast, the PLD specific activity in agonist-stimulated cells such as neutrophils is around 4.5 nmol/min per mg [6]. Furthermore, we have shown that PLD activities in human neutrophil membranes can be measured with C₆₀₋₆₀PC in the absence of PIP₂. Previously we have found that membrane PLD activities in human placenta are also easily measured using our substrate without adding PIP₂ [9].

The assay conditions in the present paper were varied between the different experiments. As the time curve for cytosolic placental PLD measured with C₆₀₋₆₀PC is only linear for 15–20 min (A. M. Vinggaard, T. Jensen and H. S. Hansen, unpublished work), this incubation period was chosen for the Michaelis–Menten curve. In our hands, virtually no PLD activity towards C₆₀₋₆₀PC can be measured under these conditions. Thus the optimum assay conditions for measuring PLD activity towards C₆₀₋₆₀PC do not allow measurement of any activity with C₄₀₋₆₀PC. We think that it is a strength of our results that, irrespective of which assay conditions are used, C₆₀₋₆₀PC is under all circumstances the best substrate, even if the optimum conditions for measuring activity with this substrate have not been met. Thus the purpose of the application of different assay conditions is to show that C₆₀₋₆₀PC will be superior under all of them. Furthermore, we have used C₆₀₋₆₀PC in the standard assays of PLD from two independent laboratories (i.e. in Copenhagen and London).

The composition of the lipid vesicles has been reported to be crucial for observing guanine nucleotide-sensitive hydrolysis of PC by HL-60 membranes and partially resolved activities derived from these membranes [10]. Thus a substrate composed of PE/PIP₂/PC at 100:15:10 μM was recommended [5]. Our experiments with placental membrane PLD show that the addition of PE to the substrate vesicles in the absence of Arf and GTP[S] resulted in a lower or unchanged PLD activity, irrespective of using C₆₀₋₆₀PC or C₄₀₋₆₀PC as substrate; however, C₆₀₋₆₀PC was still superior to C₄₀₋₆₀PC. Recently it was reported that PE containing at least one unsaturated fatty acid was able to dramatically enhance the activity of partially purified PLD from bovine kidney membranes towards C₆₀₋₆₀PC [15]. Activity was measured in the presence of small G-proteins, GTP[S] and ammonium sulphate. Thus it may be that PE is important for G-proteins to stimulate PLD, whereas it may not affect basal PLD activity.

The kinetics of phospholipase action against aggregated substrates can be divided into two parts: (1) binding of the enzyme to the aggregated substrate (interfacial phenomena), and (2) kinetic processing of the substrate [16]. Determining whether an altered activity is the result of changed interfacial binding or kinetic processing can be extremely difficult [16]. The reason for the excellent substrate properties of C₆₀₋₆₀PC is not known at present. C₄₀₋₆₀PC is less hydrophobic than the longer-chain PCs, having a critical micellar concentration of 5.0 μM [17]. As the PLD activity increases with increasing substrate concentrations above the critical micellar concentration, where the monomeric concentration is constant, the free monomer can be expected to be the substrate for PLD. Studies of phospholipid hydrolysis by phospholipase A₁ (Naja naja naja) and phospholipase C (Bacillus cereus) have shown that short-chain PCs in a micellar matrix are excellent substrates for both of these enzymes [16]. This has been suggested to be due to relatively weak intermolecular interactions in the micelle. The greater mobility and lack of interlipid interactions may make the short-chain PCs more ‘accessible’ to phospholipases. Since less energy is required to disrupt phospholipid/phospholipid packing interactions, either easier extraction/removal of the PC from the vesicle or better binding of PC to the enzyme may be involved in the mechanism of action [18]. These mechanisms may also apply to the hydrolysis of short-chain PCs by PLD.

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