Didecanoyl phosphatidylcholine is a superior substrate for assaying mammalian phospholipase D

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Phospholipase D (PLD) activity 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′-[γ-thio]-triphosphate (GTP[S]) and a cytosolic factor [usually ADP-ribosylation factor (Arf)]. In this paper we report that the use of didecanoyl phosphatidylcholine (C_{10}-PC) in mammalian PLD assays considerably increases the detection limit. C_{10}-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_{10}-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.

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 high amounts of radioactive substrate and long incubation times are employed [2] and/or ammonium sulphate is added [3]. Thus membranes from mammalian brain and several other tissues (e.g. rat lung and bovine kidney) showed very low PLD activities in both the presence and the absence of Arf (ADP-ribosylation factor) and guanosine 5′-[γ-thio]triphosphate (GTP[S]) [4]. In general, the PLD activity of a number of tissues is not detectable until membranes are extracted with detergent and subjected to an initial chromatography step [5]. In addition, the PLD activity of membranes from cell lines (including HL-60 cells) is not readily observed (even in the presence of GTP[S]) unless a cytosolic factor such as Arf is added [4,6,7]. The low activities observed in tissues and some cell lines have been suggested to be due to factors that interfere with the assay of PLD [4]. Furthermore, it was found that the inclusion of phosphatidylinositol 4,5-bisphosphate (PIP_2) was an absolute requirement for effective measurement of Arf-stimulated PLD activity [5].

In this paper we report on the benefits of a new substrate for PLD, i.e. phosphatidylcholine (PC) containing two C_{10} fatty acids. Didecanoyl PC (C_{10}-PC) is superior to long-chain PCs by a factor of 2–28 depending on assay conditions and tissue. The use of this substrate allows for easy screening of tissues and cell lines for PLD activity without the need to add GTP[S], cytosolic factors such as Arf or Rho, or high salt concentrations. Even addition of PIP_2 may be omitted when measuring Arf-stimulated PLD. The use of C_{10}-PC as an exogenous substrate in PLD assays may greatly facilitate the characterization and purification of this enzymic activity.

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-[^{14}C]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

Abbreviations used: Arf, ADP-ribosylation factor; mArf1, recombinant myristoylated Arf; rArf1, recombinant non-myristoylated Arf; GTP[S], guanosine 5′-[γ-thio]triphosphate; PC, phosphatidylcholine; C_{10}-PC, didecanoyl PC; C_{16}-PC, dipalmitoyl PC; PE, phosphatidylethanolamine; PEt, phosphatidylethanol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D.

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3 min in 2 vol. of buffer A (50 mM Hepes, pH 7.0, 1 mM EDTA, 1 mM PMSF and 1 mM dithiothreitol). After centrifugation at 500 ***g*** for 10 min at 4 °C, the supernatant was centrifuged at 109000 ***g*** for 60 min at 4 °C. The supernatant and pellet were designated 'cytosol' and 'membranes' respectively. The membrane fraction was suspended in buffer A plus 0.15 M NaCl and 1 % Triton X-100 (buffer B). The suspension was agitated for 60 min at 4 °C and then centrifuged at 109000 ***g*** for 60 min at 4 °C. The supernatant was diluted 10 times with buffer B with no Triton X-100 for column chromatography (giving a final concentration of 0.1 % Triton X-100) or for measurement of 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 % sucrose gradient with a 65 % 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 [^{3}H]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. [^{3}H]C_{10}-PC or [^{3}H]dipalmitoyl phosphatidylcholine ([^{3}H]C_{16}-PC) and C_{10}-PC or C_{16}-PC were added, and the solvents were evaporated under a stream of N_2. 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 ^{14}C-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, PIP_2 and PC (C_{10} or C_{16}) in the molar ratio 10:0.3:1. For vesicles not containing PIP_2, the vesicle composition was PE/PC (10:1, mol/mol). The final PC concentration was 8.6 μM. [^{3}H]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 $MgCl_2$ (0.5 mM) and $CaCl_2$ (2 mM) in the assay buffer. [^{3}H]Choline was extracted and measured as described previously [11].

RESULTS

Experiments were performed in order to compare [^{14}C]C_{10}-PC with three other molecular species of [^{14}C]PC containing the fatty acids $C_{16:0}/C_{18:1}$, $C_{16:0}/C_{20:4}$ and $C_{18:0}/C_{20:4}$. Incubations were run for 10 min with 1 mM phospholipid (0.05 μCi/assay) and 40 μM PIP_2. 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 C_{10}-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 3731 ± 481 (mean ± range, $n = 2$), 7754 ± 613 and 192 ± 25 pmol/min per mg of protein respectively.

Next, C_{10}-PC was compared with C_{16}-PC, the most commonly used exogenous substrate in PLD assays. Increasing concentrations of C_{10}-PC or C_{16}-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 PIP_2 level was kept constant at 3.8 mol %.

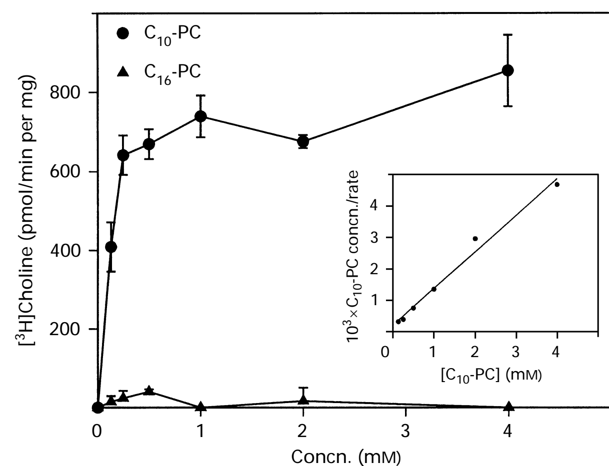

Figure 1 Effect of increasing concentrations of C_{10}-PC or C_{16}-PC on the activity of partially purified cytosolic PLD from human placenta

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 [^{3}H]C_{10}-PC (2000 d.p.m./nmol) or [^{3}H]C_{16}-PC (3600 d.p.m./nmol), 3.8 mol% PIP_2, 0.01% octyl glucoside, 50 mM Hepes, pH 7.5, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 3 mM EGTA, 80 mM KCl and 1 mM dithiothreitol for 15 min at 37 °C. Release of [^{3}H]choline was measured as described in the Materials and methods section. The insert shows a Hanes–Woolf transformation of the data obtained with C_{10}-PC. Data represent the means ± ranges of the results of one experiment in duplicate performed twice.

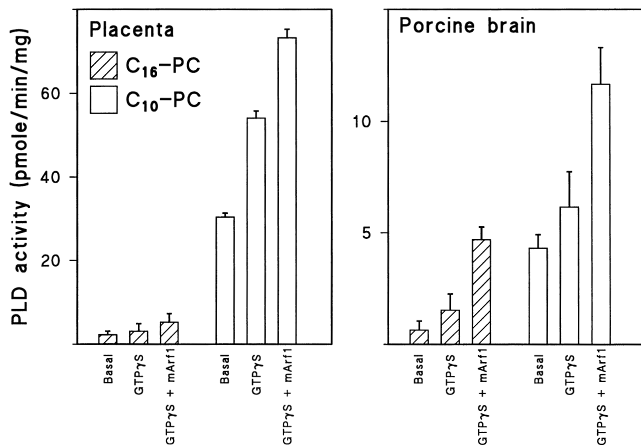

Figure 2 Comparison of C_{10}-PC and C_{16}-PC as substrates for PLD from membranes of human placenta and pig brain

Left panel: substrate vesicles of PE/PIP_2/PC (600:30:60 μM) containing [^{14}C]C_{10}-PC (18000 d.p.m./nmol) or [^{14}C]C_{16}-PC (21000 d.p.m./nmol) were incubated with Triton X-100-solubilized membrane proteins from human placenta (64 μg of protein) for 30 min. The vials contained 50 mM Hepes, pH 7.5, 3 mM EGTA, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM dithiothreitol, 80 mM KCl and 15 mM NaF in a final volume of 100 μl. The membrane proteins were incubated alone, with GTP[S] (GTPγS; 20 μM) or with GTP[S] plus mArf1 (2.5 μM). Lipids were extracted and assayed for [^{14}C]PEt formation by TLC. Data represent the means $\pm$ ranges of one experiment in duplicate performed twice. Right panel: solubilized membrane proteins from pig brain (85 μg of protein) were incubated and product formation measured as described for placental proteins.

Using C_{10}-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 C_{16}-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 C_{10}-PC was superior to C_{16}-PC in the concentration range 0.13–0.5 mM by a factor of 17–28.

In order to optimize the assay employing C_{16}-PC, the assay conditions were changed to those described in the legend to Figure 2. Comparison of C_{10}-PC and C_{16}-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 C_{10}-PC compared with C_{16}-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 μM) or mArf1 (2.5 μM) 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 C_{10}-PC compared with C_{16}-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, C_{10}-PC was excellent as an exogenous substrate compared with C_{16}-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 PIP_2 from the incubations resulted in lower, but still measurable, activity with C_{10}-PC, whereas C_{16}-PC was not a very useful substrate under these assay conditions (Figure 3).

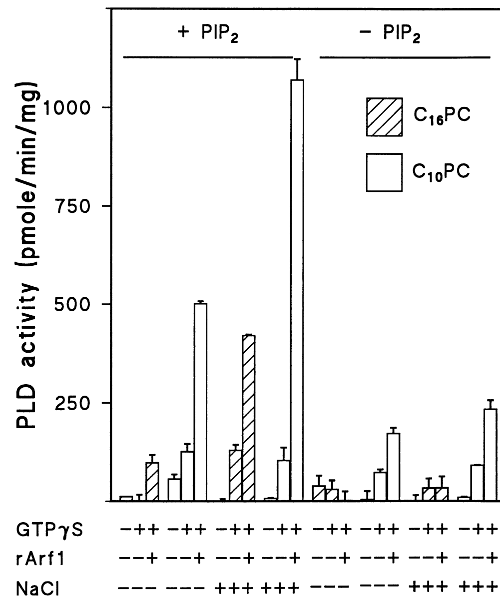

Figure 3 Comparison of C_{10}-PC and C_{16}-PC as exogenous substrates for a partially purified PLD fraction from human neutrophil membranes

Neutrophil plasma membranes were incubated with substrate vesicles composed of PE/PIP_2/PC (10:0.3:1 μM) or PE/PC (10:1 μM) containing [^{3}H]choline-labelled C_{10}-PC or C_{16}-PC. The final PC concentration was 8.6 μM. The vials were incubated with 1.5–2.0 μg of membrane protein for 90 min. The PLD fractions were incubated alone, with 30 μM GTP[S] (GTPγS) and/or with 40 μM rArf1, in the presence or absence of 0.5 M NaCl. Data represent the means $\pm$ ranges of one experiment in duplicate performed twice. A third experiment with PE/PIP_2/PC (86:2.6:8.6 μM) was performed for $t = 60$ min with similar results.

Current PLD assay techniques involve the use of C_{16}-PC together with PIP_2 and PE as substrate [2,5]. While the reason for adding PIP_2 is obvious, the reason for adding PE has not been clarified. Our experiments showed that the addition of PE (egg yolk), giving a phospholipid composition of PE/PIP_2/PC of 600:15:30 μM, resulted in a decrease in the basal placental membrane PLD activity purified on a DEAE-Sepharose column, measured in the absence of Arf and GTP[S] (results not shown). This was the case with either C_{16}-PC or C_{10}-PC as substrate (2-fold decrease in both cases; means for $n = 4$). However, using substrate compositions of 86:2.6:8.6 μM or 100:15:10 μM, the addition of PE with C_{16}-PC resulted in an almost unchanged response. Addition of phosphatidylserine, which is thought to have no effect on PLD activity, gave rise to a decreased PLD response in all cases.

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 C_{16}-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

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-chained PC containing two C_{10} fatty acids, the use of which results in a pronounced increase in sensitivity of the assay. Using optimum assay conditions (1 mM C_{10}-PC), 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 C_{16}-PC 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 C_{10}-PC in the absence of PIP_2. Previously we have found that membrane PLD activities in human placenta are also easily measured using our substrate without adding PIP_2 [9].

The assay conditions in the present paper were varied between the different experiments. As the time curve for cytosolic placental PLD measured with C_{10}-PC 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 C_{16}-PC can be measured under these conditions. Thus the optimum assay conditions for measuring PLD activity towards C_{10}-PC do not allow measurement of any activity with C_{16}-PC. We think that it is a strength of our results that, irrespective of which assay conditions are used, C_{10}-PC 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 C_{10}-PC will be superior under all of them. Furthermore, we have used C_{10}-PC 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 $PE/PIP_2/PC$ 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 C_{10}-PC or C_{16}-PC as substrate; however, C_{10}-PC was still superior to C_{16}-PC. 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 C_{16}-PC [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 C_{10}-PC is not known at present. C_{10}-PC 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 C_{10}-PC does not seem to be the substrate for PLD. Studies of phospholipid hydrolysis by phospholipase A_2 (*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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REFERENCES

1 Cockcroft, S. (1992) Biochim. Biophys. Acta **1113**, 135–160

2 Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R.-G., Symons, M. and Exton, J. H. (1995) J. Biol. Chem. **270**, 8466–8473

3 Nakamura, S., Shimooku, K., Akisue, T., Jinnai, H., Hitomi, T., Kiyohara, Y., Ogino, C., Yoshida, K. and Nishizuka, Y. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 12319–12322

4 Brown, H. A., Gutowski, S., Kahn, R. A. and Sternweis, P. C. (1995) J. Biol. Chem. **270**, 14935–14943

5 Brown, H. A. and Sternweis, P. C. (1995) Methods Enzymol. **257**, 313–325

6 Cockcroft, S. (1994) in Signal-Activated Phospholipases (Liscovitch, M., ed.), pp. 65–83, R. G. Landes, Austin

7 Bourgoin, S., Harbour, D., Desmarais, Y., Takai, Y. and Beaulieu, A. (1995) J. Biol. Chem. **270**, 3172–3178

8 Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O. and Hsuan, J. J. (1994) Science **263**, 523–526

9 Vinggaard, A. M. and Hansen, H. S. (1995) Biochim. Biophys. Acta **1258**, 169–176

9a Vinggaard, A. M., Provost, J. J., Exton, J. H. and Hansen, H. S. (1996) Cell. Signal., in the press

10 Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. C. (1993) Cell **75**, 1137–1144

11 Geny, B., Fensome, A. and Cockcroft, S. (1993) Eur. J. Biochem. **215**, 389–396

12 Hanes, C. S. (1932) Biochem. J. **26**, 1406–1421

13 Okamura, S. and Yamashita, S. (1994) J. Biol. Chem. **269**, 31207–31213

14 Hammond, S. M., Altschuller, Y. M., Sung, T-C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J. and Frohman, M. A. (1995) J. Biol. Chem. **270**, 29640–29643

15 Nakamura, S., Kiyohara, Y., Jinnai, H., Hitomi, T., Ogino, C., Yoshida, K. and Nishizuka, Y. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 4300–4304

16 Roberts, M. F. (1991) Methods Enzymology **197**, 95–112

17 Reynolds, J. A., Tanford, C. and Stone, W. L. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3796–3799

18 Gabriel, N. E. and Roberts, M. F. (1987) Biochemistry **26**, 2432–2440

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