Endogenous phospholipase D2 localizes to the plasma membrane of RBL-2H3 mast cells and can be distinguished from ADP ribosylation factor-stimulated phospholipase D1 activity by its specific sensitivity to oleic acid

Elisabeth SARRI, Raul PARDO, Amanda FENSOME-GREEN and Shamshad COCKCROFT¹

Department of Physiology, University College London, University Street, London WC1E 6JJ, U.K.

We have examined the specificity of oleate as an activator of phospholipase D2 (PLD2) and whether it can be used to study PLD2 localization and its involvement in cell function. Oleate stimulates PLD activity in intact RBL-2H3 mast cells. Comparing PLD1- with PLD2-overexpressing cells, oleate enhanced PLD activity only in PLD2-overexpressing cells. Membranes were also sensitive to oleate and when membranes prepared from PLD1- and PLD2-overexpressing cells were examined, oleate further increased PLD activity only in membranes from PLD2-overexpressing cells. Overexpressed green fluorescent protein (GFP)-PLD2 fusion protein was localized at the plasma membrane and GFP-PLD1 was found in an intracellular vesicular compartment. Oleate was used to examine whether overexpressed PLD2 co-localized with endogenous PLD2. RBL-2H3 mast cell homogenates were fractionated on a linear sucrose gradient and analysed for both oleate-stimulated activity and ADP ribosylation factor 1-stimulated PLD1 activity. The oleate-stimulated activity co-localized with markers of the plasma

membrane including the β -subunit of the Fc ϵ RI and linker for activation of T cells. Fractionation of homogenates from PLD2-overexpressing cells demonstrated that the overexpressed PLD2 fractionated in an identical location to the endogenous oleate-stimulated activity and this activity was greatly enhanced in comparison with control membranes. Examination of membranes prepared from COS-7, Jurkat and HL60 cells indicated a relationship between oleate-stimulated PLD2 activity and PLD2 immunoreactivity. We examined whether oleate could be used to activate secretion and membrane ruffling in adherent RBL-2H3 mast cells. Oleate did not stimulate secretion but did stimulate membrane ruffling, which was short-lived. We conclude that oleic acid is a selective activator of PLD2 and can be used for localization studies, but its use as an activator of PLD2 in intact cells to study function is limited due to toxicity.

Key words: antigen, exocytosis, membrane ruffling, phosphatidic acid, PMA.

INTRODUCTION

Phospholipase D (PLD) is a ubiquitous enzyme stimulated by many cell surface receptors that catalyses the hydrolysis of phosphatidylcholine (PC) to generate the second messenger phosphatidate and choline (Cho) [1–3]. Phosphatidate has been implicated in many cellular functions, including exocytosis, endocytosis, remodelling of the actin cytoskeleton and membrane trafficking, and it may function by the direct activation of a range of target proteins including phosphatidylinositol-4-phosphate-5-kinase [1–7]. PLD activity was first detected in membranes prepared from mammalian tissues when unsaturated fatty acids such as oleate were present [8]. Oleate and palmitoleate were the most potent activators in comparison with linoleate and arachidonate. Subsequently an oleate-activated PLD was purified from lung but never cloned [9]. Cloning of the plant and yeast PLDs led ultimately to the cloning of two mammalian PLDs, PLD1 and PLD2 [1–3]. PLD1 is regulated by several cytosolic factors including small GTPases of the ADP ribosylation factor (ARF) family and RhoA family, and protein kinase C α . In comparison with PLD1, recombinant PLD2 exhibits high basal activity when expressed in Sf9 cells, immunopurified and assayed *in vitro*, as well as when expressed in COS-7 cells and assayed *in*

vitro [10]. PLD2 can be activated mildly by ARF, exhibiting 1.5–2-fold activation [11,12]; however, when the N-terminal residues 1–308 are removed the protein exhibits low basal activity and can be stimulated potently with ARF. PLD1 shares with PLD2 the requirement for PtdIns(4,5)P₂ for activity, at least *in vitro*.

Expression of PLD1 and PLD2 varies within tissues and between cell lines. A single cell type can express one, both or neither isoform, although most cells co-express PLD1 and PLD2 [13,14]. The majority of the data are derived from analysis of mRNA levels and measurements of activity have been confined to the sensitivity of activation using ARF and guanosine 5'-[γ -thio]triphosphate ('GTP[S]'), which provides an indicator of PLD1 activity. Antibodies to PLDs are available but detection of endogenous protein is often a problem due to a combination of low expression levels and low affinity of the antibody for the antigen.

The relationship between the oleate-stimulated PLD and the two cloned PLDs remains obscure. Prior to the cloning of the two PLDs, oleate-stimulated PLD activity and the ARF-stimulated PLD activity had been separated chromatographically [15]. The oleate-stimulated activity was first characterized in brain tissue [8] and purified from lung [9], but activity has since

Abbreviations used: ARF, ADP ribosylation factor; Cho, choline; DMEM, Dulbecco's modified Eagle's medium; DNP, dinitrophenol; GFP, green fluorescent protein; h, human; LAT, linker for activation of T cells; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PLD, phospholipase D.

¹ To whom correspondence should be addressed (e-mail S.Cockcroft@ucl.ac.uk).

been identified in a number of cell types including Jurkat cells [16], renal tissue [17], colon carcinomas [18], liver [19,20], rat pancreas [21], rat myometrium [22] and L1210 cells [23]. The oleate-stimulated activity requires low millimolar concentrations for optimal activity [18,19], but at these concentrations it has been reported to inhibit both PLD1 and PLD2 [11,24]. One recent report shows that the activity of recombinant human PLD2 (hPLD2) can be stimulated equally well by unsaturated fatty acids, including oleate. However, this stimulation requires much lower oleate concentrations (20 μ M) than those characterized for the oleate-stimulated PLD [23]. In this study we have examined whether PLD2 is the oleate-sensitive PLD, as a prelude to the use of oleate as a tool to specifically stimulate PLD2 activity in intact cells. We have characterized the stimulation by oleate in RBL-2H3 mast cells and demonstrate that PLD2 is selectively stimulated by oleate. We used oleate to localize endogenous and overexpressed PLD2 to the plasma membrane of the RBL-2H3 mast cells. Having established that oleate is a useful reagent for selectively activating PLD2, we examined the use of oleate as a tool for studying cell function. We report that oleate can stimulate membrane ruffling but not secretion. However, membrane ruffling stimulated by oleate is short-lived in comparison with that elicited by antigen.

MATERIALS AND METHODS

Materials

[³H]Myristic acid, [¹⁴C]acetate and [³H]PC were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Antibodies to CD63 (AD1) and the β -subunit of Fc ϵ RI were monoclonal antibodies obtained from Dr R. Siraganian (NIDCR, National Institutes of Health, Bethesda, MD, U.S.A.) [25]. PLD2 antibody was a gift from Dr C. C. Leslie (Department of Pediatrics, National Jewish and Medical Research Center, Denver, CO, U.S.A.) [26]. Linker for activation of T cells (LAT) antibodies were purchased from Upstate Biotechnology (rabbit polyclonal; catalogue no. 06-807). Oleic acid and the protease inhibitor cocktail (catalogue no. P8340) were purchased from Sigma. Recombinant ARF1 (myristoylated) was made as described previously [27].

Cell culture

RBL-2H3 mast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 50 units/ml penicillin and 50 μ g/ml streptomycin. HL60 cells and Jurkat cells were grown in suspension culture in RPMI 1640 medium with the same supplements as DMEM. COS-7 cells were grown in DMEM with the same supplements as above.

Measurement of PLD activity in intact cells

Adherent RBL-2H3 cells, which were seeded the day before at 1×10^5 /well in 24-well plates, were labelled with [³H]myristic acid for 1 h at 37 °C in Hepes buffer (137 mM NaCl, 3 mM KCl, 20 mM Hepes, 2 mM MgCl₂, 1 mM CaCl₂ and 5.6 mM glucose, pH 7.2). In experiments where dinitrophenol (DNP)-human serum albumin was used as the antigen for cross-linking IgE as a stimulus, cells were primed overnight with 0.5 μ g/ml anti-DNP-IgE. After the labelling period, [³H]myristic acid was removed and the cells incubated with the stimulus in Hepes buffer in the presence of 0.5% butanol for 30 min. Reactions were terminated by adding 0.5 ml of ice-cold methanol/HCl (98:2, v/v). Cells were then scraped and transferred to test tubes.

The wells were rinsed with 500 μ l of methanol and the volume was transferred to the tubes. Two phases were generated by adding 1 ml of chloroform and 1 ml of water. The lower organic phase was removed and the chloroform evaporated. The lipids were resuspended in 20 μ l of chloroform and spotted on to silica gel 60 TLC plates that were developed with chloroform/methanol/acetic acid/water (75:45:3:0.4, by vol.). The areas corresponding to [³H]phosphatidylbutanol ([³H]PBut) and [³H]PC were identified with authentic standards after iodine staining, scraped and counted for radioactivity.

Electroporation of RBL-2H3 cells for transient transfection and fluorescent imaging of green fluorescent protein (GFP) fusion proteins

RBL-2H3 mast cells were transiently transfected by electroporation. The cells were washed and then resuspended in 400 μ l of electroporation buffer (20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂ and 1 mg/ml glucose) with 30 μ g of plasmid DNA (GFP-hPLD2 and GFP-hPLD1b). Following electroporation (two pulses at 500 mV, 125 μ F) the cells were placed at 4 °C for 10 min, resuspended in DMEM and then plated on to 22 mm coverslips for imaging or 6- or 24-well plates for measurements of PLD activity as described above. The cells were then left to recover for approx. 6 h before the medium was replaced with fresh DMEM containing 0.5 μ g/ml anti-DNP-IgE and incubated overnight to sensitize the cells.

For imaging, the cells were washed 24 h after electroporation with warmed (37 °C) Hepes buffer and placed on a heated stage. Transfected cells expressing the GFP-tagged protein were identified through a $\times 100$ oil-immersion objective using an epifluorescent system which excited the cells at 490 nm. Localization of the GFP-tagged protein in single cells was captured by taking sequential laser confocal 0.5 μ m slices through the cells from the adhesion plane to the top of the cell.

Preparation of total membranes

Cells were harvested, washed in PBS and finally resuspended in buffer containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.5 mM EDTA and 1 mM dithiothreitol, pH 7.2. Cells were kept on ice and sonicated (two bursts of 20 s each) in the presence of protease inhibitors. The homogenate was centrifuged at 2100 *g* for 20 min at 4 °C and then the post-nuclear supernatant was ultracentrifuged (Centrikon T-2190 centrifuge and a Kontron TST 41.14 rotor) at 120 000 *g* for 1 h at 4 °C to pellet total membranes, which were resuspended in the same buffer.

Sucrose-density-gradient fractionation

Approx. (2–4) $\times 10^7$ RBL-2H3 mast cells were used for each gradient. Cells were harvested and washed twice with PBS, and finally resuspended in 1 ml/gradient of Harms buffer (250 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid and 1 mM EDTA, pH 7.45). Protease inhibitors were added to the cell suspension. After incubation on ice for 10 min, the cells were homogenized in a stainless steel ball-bearing homogenizer (H & Y Enterprise, Redwood City, CA, U.S.A.). Following centrifugation, the post-nuclear supernatant (1 ml) was layered on to a 10 ml continuous sucrose gradient (0.45–2 M), which was prepared on a 0.5 ml cushion of 2 M sucrose. The gradients were then ultracentrifuged in a swing-out rotor (details as in the previous section) at 100 000 *g* for 18 h at 4 °C. Afterwards, 20 fractions of 0.575 ml each were collected and kept at –20 °C until use.

Determination of subcellular markers

β -Hexosaminidase (secretory granule marker) was assayed as described previously [28]. Briefly, 50 μ l aliquots of subcellular fractions were incubated with an equal volume of 1 mM 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide in 0.2 M citrate (pH 4.5) in a black multi-well plate for 1 h at 37 °C. The reaction was then stopped by adding 150 μ l of 0.2 M Tris and the resulting fluorescence was measured with a Titertek Fluoroskan II fluorescence reader. Galactosyltransferase activity was used as a Golgi marker [29]. In brief, 50 μ l of subcellular fractions were incubated with 50 μ l of a reaction mixture containing 50 mg/ml trypsin inhibitor, UDP-D-[6- 3 H]galactose (330 000 d.p.m./sample), 50 mM Mes (pH 7.5), 30 mM MnCl₂, 2 mM MgATP²⁻, 0.2% Triton X-100 and 1 mg/ml BSA, at 37 °C for 1 h. The reaction was quenched with 1 ml of ice-cold 1% phosphotungstic acid in 10% trichloroacetic acid. Precipitates were washed twice with the same solution and once with ethanol/ether (1:1, v/v). The final pellet was resuspended in 0.25 ml of 2 M NaOH and counted for radioactivity.

Western blot analysis

Fractions (80 μ l) from the sucrose density gradient were separated by SDS/PAGE and analysed for immunoreactivity by using a mouse monoclonal antibody against the β subunit of the IgE receptor Fc ϵ RI (plasma membrane marker), a mouse monoclonal antibody against the cell-surface glycoprotein CD63 (plasma membrane and granule membranes) and a rabbit polyclonal antibody against the transmembrane adaptor protein LAT (plasma membrane). Membrane proteins (50 μ g) were analysed by Western blotting using a peptide-specific PLD2 antibody generated against amino acid residues 7–65 of hPLD2 [26].

Measurement of PLD activity in membrane preparations and sucrose-density-gradient fractions utilizing exogenous substrate

Tritiated didecanoyl PC was used as an exogenous substrate to monitor PLD activity in membranes as described previously [30]. Aliquots of total cellular membranes or subcellular fractions were incubated with 8.6 μ M [3 H]PC (approx. 300 000 d.p.m./assay), which was presented in phospholipid vesicles with the composition phosphatidylethanolamine/PC/PtdIns(4,5)*P*₂ (10:1:0.3, molar ratio), and [3 H]Cho release was monitored. Membranes were incubated in the presence or absence of various stimuli for 1 h at 37 °C in buffer comprised of 50 mM Na-Hepes, 3 mM EGTA, 80 mM KCl, 1 mM dithiothreitol, 0.5 mM MgCl₂ and 2 mM CaCl₂, pH 7.5. Incubations were quenched by adding 0.3 ml of chloroform/methanol (1:2, v/v) and two phases were separated after the addition of 0.12 ml each of chloroform and water. [3 H]Cho present in the aqueous phase was separated from other [3 H]Cho metabolites by cation-exchange column chromatography, and counted for radioactivity.

Measurement of PLD activity in membrane preparations utilizing endogenous substrate

Membrane preparations were prepared from RBL-2H3 mast cells that were grown in the presence of [14 C]acetate (0.5 μ Ci/ml) for 48 h to label the lipids. Incubations with oleate were performed in a solution containing 20 mM Pipes, 137 mM NaCl, 2.7 mM KCl, 1 mM MgATP²⁻, 2 mM MgCl₂, 3 mM EGTA and 2.7 mM CaCl₂ (Ca²⁺_{free}, 10 μ M), pH 6.8, in the presence of 0.5% butanol. Reactions were quenched and processed by TLC as described above.

Imaging of live cells using Nomarski optics

RBL-2H3 cells were plated on to 22 mm coverslips and primed overnight with anti-DNP-IgE. The cells were washed in warmed (37 °C) Hepes buffer and placed on a heated stage and kept at 37 °C throughout the experiment. Oleate was added to 3 mM final concentration. The cells were viewed on an Olympus microscope using a $\times$ 100 oil-immersion objective. Bright-field images of the cells were acquired over a 30 min period every 20 s using Nomarski phase-contrast optics with a Perkin Elmer charge-coupled device camera cooled to –35 °C. The sequences of images were exported as AVI files and individual frames selected as shown in Figure 7 (see below).

RESULTS

Oleate stimulates PLD activity in intact RBL-2H3 cells

To monitor PLD activation in intact cells we measured the transphosphatidyl reaction catalysed by PLD, which elicits the accumulation of [3 H]PBut in the presence of butanol. An increase in PLD activity was observed when adherent RBL-2H3

Figure 1 Stimulation of PLD activity by oleate in adherent and suspended RBL-2H3 mast cells

(A) Adherent cells were labelled with [3 H]myristic acid and incubated with different concentrations of oleate for 30 min in the presence of 0.5% butanol. BSA was excluded from the buffer as this reduced the stimulation by oleate. (B) RBL-2H3 mast cells were labelled in suspension with [3 H]myristic acid, washed and incubated with different concentrations of oleate.

Figure 2 Stimulation of PLD2 activity by oleate from RBL-2H3 mast cells overexpressing GFP-hPLD1b and GFP-hPLD2

RBL-2H3 cells were transiently transfected with DNA encoding GFP-tagged hPLD1b or hPLD2. Confocal images demonstrate the localization of (A) GFP-hPLD1b and (B) GFP-hPLD2. After transfection (24 h), the cells were labelled with [3 H]myristic acid for 1 h and incubated for 20 min with (C) PMA or (D) different concentrations of oleate in the presence of 0.5% butanol. The basal activity shown in (C) was subtracted from the data shown in (D). The data (means $\pm$ S.D.) are representative of three independent experiments performed in triplicate.

mast cells were incubated with increasing concentrations of oleate (Figure 1A). The highest concentration of oleate that was tested was 3 mM, as it was not possible to maintain higher concentrations in solution. We also examined the concentration

dependence of oleate in cells in suspension; Figure 1(B) illustrates that 1 mM oleate was maximal in the activation of PLD activity.

Oleic acid-stimulated PLD activity increases in RBL-2H3 cells overexpressing GFP-hPLD2 but not GFP-hPLD1b

To examine which PLD isozyme was activated by oleate in intact cells, we overexpressed GFP-tagged hPLD1b and hPLD2 in RBL-2H3 mast cells (Figures 2A and 2B). The GFP-tagged proteins localized to distinct membrane compartments, with PLD1b localizing to an intracellular vesicular compartment (Figure 2A) and PLD2 localizing at the plasma membrane (Figure 2B). To verify that both GFP-tagged PLD isoenzymes were active, we monitored PLD activity following stimulation with PMA (Figure 2C). In PLD2-transfected cells, basal activity was substantially increased. Following stimulation with PMA, both PLD1b- and PLD2-transfected cells showed enhanced activation. When oleate was used as a stimulus, the cells overexpressing PLD2 showed a dramatic increase in PLD activity (Figure 2D). In contrast, no difference in oleate-stimulated PLD activity between mock- and GFP-hPLD1b-transfected cells was apparent. These data suggested that oleate could selectively activate PLD2 and could therefore be used as a tool for monitoring PLD2 activity in membranes.

Stimulation of PLD2 activity in membranes by oleate

PLD activity was monitored from increasing amounts of RBL-2H3 mast cell membranes with increasing concentrations of oleate. A relatively narrow range of oleate concentrations was effective at stimulating PLD, and the maximum required shifted to the right as the total membrane protein in the assay was increased (Figure 3A). However, the range at which oleate was stimulatory increased as the protein concentration increased. To confirm the observations obtained using release of [3 H]Cho to monitor PLD activity, [14 C]PBuT accumulation stimulated by oleate was also determined (Figure 3B). Activation by oleate showed a similar bell-shaped curve to that observed for [3 H]Cho release. These results indicate that the concentration of oleate used to monitor PLD activity in membrane preparations is critical and dependent on the amount of membrane protein in the assay.

We also characterized oleate-stimulated PLD activity in membranes prepared from RBL-2H3 mast cells overexpressing PLD2 or PLD1b. The results obtained show that the concentration-effect curves for oleate-stimulated PLD activity in membranes overexpressing PLD1b and PLD2 were bell-shaped, as in membranes from mock-transfected RBL-2H3 mast cells. The concentration-effect curve for GFP-hPLD1b-containing membranes did not differ from that for control membranes, apart from the PLD activity values obtained at low concentrations of oleate, which reflect a small increase of basal PLD activity in membranes containing GFP-hPLD1b (Figure 3C). The concentration-effect curve for membranes containing GFP-hPLD2 was also bell-shaped (Figure 3D) and exhibited a high basal PLD activity, which was further increased by oleate. These results confirm that only PLD2 is stimulated by oleate.

Identification of endogenous PLD2 at the plasma membrane in RBL mast cells

We next used oleate as a tool to localize endogenous PLD2 using subcellular fractionation in RBL-2H3 mast cells. RBL-2H3 mast

Figure 3 Stimulation of PLD2 activity by oleate in membranes from control and transfected RBL-2H3 mast cells

(A) Membranes from untransfected cells were incubated with lipid vesicles containing $^3\text{H}]\text{PC}$ for 1 h in the presence of different concentrations of oleate and the indicated amounts of membrane protein. $^3\text{H}]\text{Cho}$ was separated by cation-exchange chromatography. (B) Membranes (10 μg of protein) from $^{14}\text{C}]\text{acetate}$ -prelabelled cells were incubated for 1 h with different concentrations of oleate in the presence of 0.5% butanol. $^{14}\text{C}]\text{PBut}$ and $^{14}\text{C}]\text{PC}$ were separated by TLC and the radioactivity in each phospholipid was determined. PLD activity is expressed as d.p.m. in $^{14}\text{C}]\text{PBut}$ as a percentage of d.p.m. in $^{14}\text{C}]\text{PC}$. Results are the means $\pm$ range from two experiments performed in duplicate. (C and D) Membranes were prepared from RBL-2H3 mast cells overexpressing (C) GFP-hPLD1b or (D) GFP-hPLD2. After transfection (24 h) PLD activity was assayed as in (A) in the presence of different concentrations of oleate and 5 μg of membrane protein. PLD activity is expressed as d.p.m. in released $^3\text{H}]\text{Cho}$. Results (means $\pm$ range) are representative of two independent experiments performed in triplicate.

cells were homogenized and the organelles were separated on a continuous linear sucrose gradient; 20 fractions were collected and assayed for PLD activity using exogenous $^3\text{H}]\text{PC}$. In the absence of any activators, the basal activity was insignificant (results not shown). When the fractions included oleate to stimulate PLD2, activity was clearly observed (Figure 4A). Oleate-stimulated PLD activity localized at different subcellular fractions to the ARF1-stimulated PLD, with some overlap. Oleate-stimulated PLD exhibited a broad peak (fractions 6–15) with maximum activity found in fractions 8–12. In contrast, ARF1-stimulated PLD exhibited a narrower peak (fractions 11–16) with maximum activity found in fractions 13 and 14. Of note, some 20% of the total ARF1-stimulated PLD could also be detected in fractions 6–10. Each fraction was also tested for oleate-stimulated transphosphatidyltransferase reaction, which is specifically catalysed by PLD in a subcellular fractionation obtained after pre-labelling the RBL-2H3 mast cells with

$^{14}\text{C}]\text{acetate}$. The results of this experiment indicated that those fractions which contained oleate-stimulated $^{14}\text{C}]\text{PBut}$ accumulation matched those exhibiting oleate-stimulated $^3\text{H}]\text{Cho}$ release (results not shown). In the experiment shown in Figure 4(A), the concentration of oleate used to stimulate PLD activity was 100 μM . Because the activation by oleate is sensitive to protein content, we analysed the fractions at different oleate concentrations and the profile remained unaltered. The range of protein in the fractions used in the PLD assays was calculated to lie between 4 and 8 μg of protein.

We next investigated the presence of plasma membrane markers in the fractions using antibodies against the IgE receptor Fc ϵ RI, CD63, a transmembrane glycoprotein present at the plasma membrane and on granule membranes in RBL-2H3 mast cells [25,31], and the adapter protein LAT, which is expressed in mast cells [32] and is a member of the signalling machinery present in the plasma membrane lipid domains or

Figure 4 Separation of endogenous oleate- and ARF1-stimulated PLD by subcellular fractionation of RBL-2H3 mast cells

Subcellular fractions were prepared on a linear sucrose gradient as described in the Materials and methods section. (A) PLD activity in subcellular fractions was determined by incubating the fractions with lipid vesicles containing [3 H]PCo for 1 h in the presence of 0.1 mM oleate (▲) or 5 μ M ARF1 and 30 μ M guanosine 5'-[γ -thio]triphosphate (GTP γ S; ●), and afterwards measuring the release of [3 H]Cho. PLD activity in each fraction is expressed as a percentage of the maximum value obtained for each stimulus. Protein concentration is denoted by a dotted line. (B) Galactosyl-transferase (Golgi marker; □) and β -hexosaminidase (secretory granule marker; ◇) were determined in subcellular fractions as described in the Materials and methods section. The linearity of the sucrose gradient is also represented in the same graph (▽), the maximum concentration (100%) of sucrose being 2 M. (C) Subcellular fractions were analysed by Western blotting with specific antibodies to IgE receptor Fc ϵ RI, CD63 and LAT. Results shown are determinations performed in one representative fractionation of at least four separate fractionations.

rafts. Western blot analysis indicated the presence of Fc ϵ RI in fractions 5–15, CD63 in fractions 7–14 and LAT in fractions 7–15 (Figure 4C). These results demonstrate that the maximal oleate-stimulated PLD activity and plasma membrane markers co-localize in fractions 8–12.

To determine the localization of secretory granules and Golgi membranes in the gradient fractions, β -hexosaminidase and galactosyl-transferase activities were measured (Figure 4B). β -Hexosaminidase activity exhibited two peaks: a small broad peak comprising fractions 6–10 and a higher and sharper one comprising fractions 11–13. This profile does not exactly match the profile for ARF1-stimulated PLD activity (presumably PLD1), suggesting that PLD1 is not exclusively localized to

secretory granules. Galactosyl-transferase activity exhibited a broad peak comprising fractions 7–13.

Overexpressed GFP-hPLD2 localizes to the same subcellular fractions as endogenous oleate-stimulated PLD activity

To compare the subcellular localization of overexpressed GFP-hPLD1b and GFP-PLD2 with that for endogenous oleate-stimulated PLD, we fractionated RBL-2H3 cells overexpressing GFP-hPLD1 or GFP-hPLD2. PLD activity was tested by using exogenous [3 H]PC and measuring the release of [3 H]Cho in basal conditions as well as after stimulation with oleate. In subcellular fractions from GFP-hPLD2-overexpressing cells, we

Figure 5 Oleate stimulates overexpressed PLD2 but not PLD1b in subcellular fractions

Cells were transiently transfected with DNA encoding GFP-tagged hPLD2 and hPLD1b and after 24 h the cells were homogenized and fractionated on a sucrose gradient. Subcellular fractions were incubated with lipid vesicles containing [3 H]PC for 1 h in the (A) absence or (B) presence of 0.1 mM oleate. [3 H]Cho was separated by cation-exchange chromatography and radioactivity determined. PLD activity is expressed as d.p.m. in released [3 H]Cho. Results are representative of two subcellular fractionations.

observed that basal PLD activity was enhanced in the range of fractions that contained endogenous oleate-stimulated PLD (fractions 6–16; Figure 5A), and that this basal PLD activity could be further increased by oleic acid (Figure 5B). On the other hand, basal PLD activity in subcellular fractions from GFP-hPLD1b-overexpressing cells was slightly enhanced in fractions 14–16 compared with controls (Figure 5B), but none of the fractions tested showed higher oleate-stimulated PLD than the controls (Figure 5B). These results demonstrate that overexpressed GFP-hPLD2 is targeted to the same subcellular compartments as the endogenous oleate-stimulated PLD.

Oleate-stimulated PLD activity correlates with PLD2 expression levels in different mammalian cell lines

To test whether oleate could be used to identify the presence of PLD2 activity, we chose three mammalian cell lines, COS-7,

Figure 6 Oleate can be used to identify cells expressing endogenous PLD2

Membranes were prepared from COS-7, HL60 and Jurkat cells. (A) Protein (50 μ g) was used for immunoblotting with an anti-PLD2 antibody. (B) PLD activity was determined in the presence of 100 μ M oleate using 10 μ g of membrane protein.

HL60 and Jurkat cells. Western blots with antibodies against hPLD2 showed that expression of the PLD2 isoenzyme was high in COS-7 cells, slight in Jurkat cells and non-existent in HL60 cells (Figure 6A). In line with these results, the PLD activity stimulated by different concentrations of oleic acid in membranes from these three cell lines was high for COS-7 cells, slightly increased for Jurkat cells and insignificant in HL60 cells. The lack of a suitable antibody against rat PLD2 subtype has prevented us from directly determining the expression of endogenous PLD2 isoenzyme in RBL-2H3 mast cells.

Can oleate be used to study cell function?

RBL-2H3 mast cells can be stimulated to secrete their lysosomally derived secretory granules and to undergo extensive changes in their cytoskeleton when incubated with antigen, and PLD activity has been implicated as a regulator [6,33–35]. To examine whether selective activation of PLD2 by oleate was sufficient to stimulate any of these processes, we monitored secretion and membrane ruffling. Adherent cells were stimulated with oleate and the release of β -hexosaminidase monitored. Concentrations of oleate up to 3 mM were incapable of stimulating secretion (results not shown).

Antigen stimulates a dramatic reorganization of the cytoskeleton by the continual formation and dissolution of membrane ruffles, which can be observed over 25 min (Figure 7B). We examined whether oleate was capable of stimulating membrane ruffles. Oleate was added to the cells and the cells viewed with Nomarski optics to visualize membrane ruffling. No changes were observed when oleate was added up to 1 mM. At 3 mM oleate, 8 out of 11 cells examined demonstrated the formation of membrane filopodia observed at 5 min. Unlike the response to antigen, the cells did not continue ruffling; moreover, the cells

Figure 7 Comparison of membrane ruffling by phase-contrast, time-lapse recording of RBL-2H3 mast cells stimulated with oleate and antigen

(A) Oleate causes the appearance of membrane ruffles at 5 min, which is followed by shrinking of the cells and cessation of ruffling. The cells are refractory to subsequent stimulation with antigen. (B) Antigen stimulates continual membrane ruffling for at least 25 min. Arrows indicate the sites of membrane ruffles.

failed to respond to a subsequent addition of antigen. This suggested that the cells were damaged. Following the initial response (5–10 min), ruffling ceased and the morphology of the cells began to change on continued exposure to oleate with the cells becoming shrunken as they withdrew all their processes (Figure 7A).

Membrane ruffling by oleate in GFP-hPLD2-overexpressing cells was also examined. It was anticipated that low concentrations of oleate would be able to trigger membrane ruffling. GFP-PLD2-overexpressing did not ruffle spontaneously and concentrations of oleate below 3 mM did not stimulate ruffling either. In the presence of 3 mM oleic acid, the GFP-hPLD2-

overexpressing cells behaved like the non-expressing cells except that these cells had a tendency to exhibit extensive blebbing on prolonged exposure to 3 mM oleic acid (results not shown).

DISCUSSION

The work that is described here was carried out with three objectives in mind. The main impetus was to identify a way of selectively activating PLD2 activity in cells to analyse the specific function of this PLD. The second major goal was to establish a methodology that could be used to localize endogenous PLD2 activity. The third major goal was to identify the PLD isozyme that was activated by oleate in cells that expressed both PLD1 and PLD2. Unsaturated fatty acids including oleate have been used to unmask the presence of PLD activity in mammalian cells for over two decades. However, following the identification of cytosolic regulators for PLD, the focus of subsequent studies changed to the GTPases of the ARF and Rho families and protein kinase C as activators of PLD. The cloning of the two PLDs, PLD1 and PLD2, has certainly confirmed that ARF and protein kinase C α can both regulate these enzymes, while PLD1 is also regulated by the Rho family of GTPases. We have examined the possibility that oleate-stimulated PLD is none other than PLD2.

The results presented in this paper allow us to conclude that PLD2 is the oleate-sensitive PLD. This conclusion is based on the following observations. PLD activity can be stimulated in adherent RBL-2H3 mast cells with concentrations of oleate in the millimolar range. Overexpression of PLD2 but not PLD1 causes a dramatic increase in the sensitivity to oleate so that at 100 μ M oleate the stimulation observed in transfected cells is greater than the maximum stimulation observed in untransfected cells. PLD2-overexpressing cells, when stimulated with 1 mM oleate, responded by converting as much as 20 % of its cellular PC to PBut. This enhancement of sensitivity to oleate was also observed in cell membranes prepared from cells overexpressing PLD2 but not from PLD1b-overexpressing cells. When cells were fractionated on a linear sucrose gradient, the basal PLD activity increased due to the overexpression of PLD2 in fractions enriched with plasma membrane markers. Furthermore, the activity in these fractions from cells overexpressing PLD2 but not PLD1 could be further activated with oleate. Finally, HL60 cells could not be activated by oleate and showed no PLD2 proteins, while Jurkat cells could be stimulated by oleate and had some PLD2. In comparison, COS-7 cells had high basal activity, which could be further stimulated with oleate, and this correlated with the presence of a high concentration of PLD2 immunoreactivity. Thus our conclusion reinforces the study by Kim et al. [23], who concluded that PLD2 was the target for oleate, but differs from the study of Kodaki and Yamashita [24], in which rat PLD2 was inhibited by oleate at 0.4–1 mM. The discrepancy probably lies in the assay conditions; both the concentration of oleate used and the amount of protein in the assay appears to determine activation. Higher concentrations of oleate are inhibitory and this is highly dependent on protein concentration. Thus the inhibition observed by Kodaki and Yamashita [24] may relate to the range of oleate concentrations used. In a recent study it was reported that human peripheral blood mononuclear cells express PLD2 at the mRNA and protein levels but were devoid of oleate-dependent activity [36]. In this study, a single concentration of oleate (2 mM) was used to examine the presence of PLD activity in cell lysates. From our studies it is clear that oleate has to be titrated carefully, taking into consideration the protein concentration used in the assay.

In the present work we also show that oleate is a useful tool to study the cellular localization of both endogenous and overexpressed PLD2. The data obtained with subcellular fractionation confirms that the overexpressed GFP-hPLD2 localizes in the same compartment as the endogenous PLD2 [34,35]. Basal and oleate-sensitive PLD activity were enhanced for PLD2- but not for PLD1-overexpressing cells in those fractions where endogenous oleate-stimulated PLD was detected. The membrane fractions that exhibit endogenous oleate-stimulated PLD activity matched with plasma membrane markers, and together these observations give support to the confocal images, where overexpressed GFP-tagged PLD2 is in the plasma membrane. Thus overexpression of PLD2 does not mis-localize the protein. To our knowledge, there are only a few studies that have examined the localization of PLD2 [3]. In PC12 cells, HeLa cells and rat embryo fibroblasts, GFP- or haemagglutinin-tagged PLD2 was localized to the plasma membrane [5,10,37], whereas in HT29-cl19A epithelial cells both GFP-tagged PLD2 and endogenous PLD2 were localized at the Golgi compartment [38]. In PAE cells, PLD2 was localized in a submembrane vesicular compartment [39]. Since localization is key to understanding function, further studies will be required to determine what precisely is responsible for the localization of PLD2 in specific cell types. At least in the mast cell, endogenous PLD2 is localized at the plasma membrane.

The mechanism of oleate stimulation of PLD2 is not yet clear but recently a plant PLD that is responsive to oleate has been identified and cloned [40]. In plants such as *Arabidopsis*, four types of PLD have been identified; PLD α , PLD β , PLD γ and PLD δ . PLD α is the most common plant PLD, does not require PtdIns(4,5) P_2 for activity and is normally assayed at millimolar levels of calcium. In contrast, PLD β and PLD γ are PtdIns(4,5) P_2 -dependent enzymes and are most active at micromolar calcium concentrations. The latest addition to the *Arabidopsis* family of PLDs is PLD δ , which was identified on the basis of activation by oleate in the absence of PtdIns(4,5) P_2 and in the presence of 50 μ M Ca^{2+} . The other PLDs (α , β and γ) were not activated by oleate under the same conditions. The concentration of oleate required to maximally stimulate the activity was 0.5 mM and increasing concentrations inhibited activity. Other fatty acids were tested and only unsaturated fatty acids, such as linoleate and linolenate were active, but not palmitate or stearate. Arg-399, located approx. 30 amino acid residues after the first HKD motif, was mutated and this led to loss of stimulation by oleate. However, this residue is conserved in both mammalian PLD2 and PLD1 suggesting that loss of oleate sensitivity may be due to other factors. Like mammalian PLD2, plant PLD δ is also localized at the plasma membrane.

Can oleate be used to study cell function? Firstly, the concentration of oleate required to stimulate intact cells is much higher than that required to stimulate membranes, indicating that oleate has to gain access to the cytosolic face of the cell where PLD2 is located. PLD has been implicated in both secretory function and in changes in the actin cytoskeleton in RBL-2H3 mast cells [6,35,41]. Both PLD1 and PLD2 are implicated in exocytosis in RBL-2H3 cells [34] and PLD2 has been implicated in membrane ruffling [35]. We therefore analysed whether oleate could stimulate secretion, bearing in mind that triggering of secretion is dependent on a rise in cytosolic Ca^{2+} . Not surprisingly, oleate was unable to stimulate secretion in adherent RBL-2H3 mast cells. In contrast to secretion, membrane ruffling is not dependent on a rise in Ca^{2+} , and therefore membrane ruffling was examined. An increase in membrane ruffles was observed but the response was short-lived. After 10 min, with oleate, the cells appeared to shrink and were not

responsive to subsequent stimulation by antigen. In contrast to oleate, membrane ruffling triggered by antigen was sustained for 25 min. Thus although oleate can selectively activate PLD2, the concentrations of oleate required to activate intact cells makes the use of oleate complicated due to the toxic effect it has on the cells. However, the ability of oleate and other unsaturated fatty acids to stimulate PLD2 activity may be physiologically important, when released intracellularly. An imbalance between uptake and oxidation of fatty acids, disturbances in triglyceride accumulation or changes in phospholipase A₂ activity will increase the cellular concentration of unsaturated fatty acids [42]. For example in myocardial cells, PLD2 localizes to the sarcolemma [42,43], and can be activated by hormonal stimulation of phospholipase A₂; this is also found in heart diseases characterized by phospholipase A₂ dysfunction. PLD has been suggested to play an important role in several different aspects of cardiac physiology and PLD2 is likely to be responsible [42]. We conclude from our studies that oleate is a useful tool with which to localize PLD2 activity and to identify whether a particular cell has PLD2 activity. Since the availability of PLD2 antibodies still remains a major problem, this is probably the most sensitive way to determine the presence of this enzyme.

We thank the Human Frontiers Programme for support for E.S. The Wellcome Trust funds the Cockcroft Laboratory and we thank them for their support. R.P. is the recipient of a Doctoral Fellowship from the Universitat Autònoma de Barcelona (Spain). We thank Alison Skippen for making the ARF proteins and Michelle Li for technical assistance.

REFERENCES

- Exton, J. H. (1997) Phospholipase D: enzymology, mechanisms of regulation, and function. *Physiol. Rev.* **77**, 303–320
- Liscovitch, M., Czarny, M., Fiucci, G., Lavie, Y. and Tang, X. (1999) Localization and possible functions of phospholipase D isozymes. *Biochim. Biophys. Acta* **1439**, 245–263
- Cockcroft, S. (2001) Signalling roles of mammalian phospholipase D1 and D2. *Cell Mol. Life Sci.* **58**, 1674–1687
- Fensome, A., Cunningham, E., Prosser, S., Tan, S. K., Swigart, P., Thomas, G., Hsuan, J. and Cockcroft, S. (1996) ARF and PIP₂ restore GTP γ S-stimulated protein secretion from cytosol-depleted HL60 cells by promoting PIP₂ synthesis. *Curr. Biol.* **6**, 730–738
- Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A. and Kanaho, Y. (1999) Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521–532
- Way, G., O'Luanigh, N. and Cockcroft, S. (2000) Activation of exocytosis by cross-linking of the IgE receptor is dependent on ADP-ribosylation factor 1-regulated phospholipase D in RBL-2H3 mast cells: evidence that the mechanism of activation is via regulation of phosphatidylinositol 4,5-bisphosphonate synthesis. *Biochem. J.* **346**, 63–70
- Jones, D. H., Morris, J. B., Morgan, C. P., Kondo, H., Irvine, R. F. and Cockcroft, S. (2000) Type I PIP 5-kinase directly interacts with ARF1 and is responsible for PI(4,5)P₂ synthesis in the Golgi compartment. *J. Biol. Chem.* **275**, 13962–13966
- Chalifour, R. and Kanfer, J. N. (1982) Fatty acid activation and temperature perturbation of rat brain microsomal phospholipase D. *J. Neurochem.* **39**, 299–305
- Okamura, S. and Yamashita, S. (1994) Purification and characterisation of phosphatidylcholine phospholipase D from pig lung. *J. Biol. Chem.* **269**, 31207–31213
- Colley, W. C., Sung, T.-C., Roll, R., Jenco, J., Hammond, S. M., Altschuller, Y., Bar-Sagi, D., Morris, A. J. and Frohman, M. A. (1997) Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol.* **7**, 191–201
- Lopez, I., Arnold, R. and Lambeth, J. D. (1998) Cloning and initial characterization of a human phospholipase D2 (hPLD2). *J. Biol. Chem.* **273**, 12846–12852
- Sung, T.-C., Altschuller, Y. M., Morris, A. J. and Frohman, M. A. (1999) Molecular analysis of mammalian phospholipase D2. *J. Biol. Chem.* **274**, 494–502
- Meier, K. E., Gibbs, T. C., Knoepp, S. M. and Ella, K. M. (1999) Expression of phospholipase D isoforms in mammalian cells. *Biochim. Biophys. Acta* **1439**, 199–213
- Gibbs, T. C. and Meier, K. E. (2000) Expression and regulation of phospholipase D isoforms in mammalian cell lines. *J. Cell. Physiol.* **182**, 77–87
- Massenburg, D., Han, J.-S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J. and Vaughan, M. (1994) Activation of rat brain phospholipase D by ADP-ribosylation factors 1, 5, and 6: separation of ADP-ribosylation factor-dependent and oleate-dependent enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11718–11722
- Kasai, T., Ohguchi, K., Nakashima, S., Ito, Y., Naganawa, T., Kondo, N. and Nozawa, Y. (1998) Increased activity of oleate-dependent type phospholipase D during actinomycin D-induced apoptosis in Jurkat T cells. *J. Immunol.* **161**, 6469–6474
- Zhao, Y., Ehara, H., Akao, Y., Shamoto, M., Nakagawa, Y., Banno, Y., Deguchi, T., Ohishi, N., Yagi, K. and Nozawa, Y. (2000) Increased activity and intranuclear expression of phospholipase D2 in human renal cancer. *Biochem. Biophys. Res. Commun.* **278**, 140–143
- Yoshida, M., Okamura, S., Kodaki, T., Mori, M. and Yamashita, S. (1998) Enhanced levels of oleate-dependent and Arf-dependent phospholipase D isoforms in experimental colon cancer. *Oncol. Res.* **10**, 399–406
- Banno, Y., Tamiya-Koizumi, K., Oshima, H., Morikawa, A., Yoshida, S. and Nozawa, Y. (1997) Nuclear ADP-ribosylation factor (ARF)- and oleate-dependent phospholipase D (PLD) in rat liver cells. Increases of ARF-dependent PLD activity in regenerating liver cells. *J. Biol. Chem.* **272**, 5208–5213
- Siddiqui, R. A. and Exton, J. H. (1992) Oleate stimulation of diacylglycerol formation from phosphatidylcholine through effects on phospholipase D and phosphatidate phosphohydrolase. *Eur. J. Biochem.* **210**, 601–607
- Laine, J., Bourgoin, S., Bourassa, J. and Morisset, J. (2000) Subcellular distribution and characterization of rat pancreatic phospholipase D isoforms. *Pancreas* **20**, 323–336
- Le Stunff, H., Dokhac, L., Bourgoin, S., Bader, M. F. and Harbon, S. (2000) Phospholipase D in rat myometrium: occurrence of a membrane-bound ARF6 (ADP-ribosylation factor 6)-regulated activity controlled by $\beta\gamma$ subunits of heterotrimeric G-proteins. *Biochem. J.* **352**, 491–499
- Kim, J. H., Kim, Y., Lee, S. D., Lopez, I., Arnold, R. S., Lambeth, J. D., Suh, P. G. and Ryu, S. H. (1999) Selective activation of phospholipase D2 by unsaturated fatty acid. *FEBS Lett.* **454**, 42–46
- Kodaki, T. and Yamashita, S. (1997) Cloning, expression and characterization of a novel phospholipase D complementary DNA from rat brain. *J. Biol. Chem.* **272**, 11408–11413
- Nishikata, H., Oliver, C., Mergenhagen, S. E. and Siraganian, R. P. (1992) The rat mast cell antigen AD1 (homologue to human CD63 or melanoma antigen ME491) is expressed in other cells in culture. *J. Immunol.* **149**, 862–870
- Siddiqui, A. R., Strajer, G. E. and Leslie, C. C. (2000) Regulation of human PLD1 and PLD2 by calcium and protein kinase C. *Biochim. Biophys. Acta* **1497**, 103–114
- Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Troung, O. and Hsuan, J. J. (1994) Phospholipase D: a downstream effector of ARF in granulocytes. *Science* **263**, 523–526
- Stutchfield, J. and Cockcroft, S. (1993) Correlation between secretion and phospholipase D activation in differentiated HL60 cells. *Biochem. J.* **293**, 649–655
- Kim, Y. S., Perdomo, J. and Nordberg, J. (1971) Glycoprotein biosynthesis in small intestinal mucosa. *J. Biol. Chem.* **246**, 5466–5476
- Vinggaard, A. M., Jensen, T., Morgan, C. P., Cockcroft, S. and Hansen, H. S. (1996) Didecanoylphosphatidylcholine is a superior substrate for assaying mammalian phospholipase D. *Biochem. J.* **319**, 861–864
- Kitani, S., Berenstein, E., Mergenhagen, S., Tempst, P. and Siraganian, R. P. (1991) A cell surface glycoprotein of rat basophilic leukemia cells close to the high affinity IgE receptor (Fc ϵ RI). Similarity to human melanoma differentiation antigen ME491. *J. Biol. Chem.* **266**, 1903–1909
- Saitoh, S., Arudchandran, R., Manetz, T. S., Zhang, W., Sommers, C. L., Love, P. E., Rivera, J. and Samelson, L. E. (2000) LAT is essential for Fc(ϵ)RI-mediated mast cell activation. *Immunity* **12**, 525–535
- Powner, D. J., Hodgkin, M. N. and Wakelam, M. J. O. (2002) Antigen-stimulated activation of phospholipase D1b by Rac1, ARF6 and PKC α in RBL-2H3 cells. *Mol. Biol. Cell* **13**, 1252–1262
- Choi, W. S., Kim, Y. M., Combs, C., Frohman, M. A. and Beaven, M. A. (2002) Phospholipases D1 and D2 regulate different phases of exocytosis in mast cells. *J. Immunol.* **168**, 5682–5689
- O'Luanigh, N., Pardo, R., Jones, D., Fensome, A., Allen-Baume, V., Jones, D., Holt, M. R. and Cockcroft, S. (2002) Continual production of PA by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. *Mol. Cell. Biol.* **13**, 3730–3746
- Diaz, O., Berquand, A., Dubois, M., Di Agostino, S., Sette, C., Bourgoin, S., Lagarde, M., Nemoz, G. and Prigent, A. F. (2002) The mechanism of docosahexaenoic acid-induced phospholipase D activation in human lymphocytes involves exclusion of the enzyme from lipid rafts. *J. Biol. Chem.* **277**, 39368–39378
- Han, J. M., Kim, J. H., Lee, B. D., Lee, S. D., Kim, Y., Jung, Y. W., Lee, S., Cho, W., Ohba, M., Kuroki, T., Suh, P. G. and Ryu, S. H. (2002) Phosphorylation-dependent regulation of phospholipase D2 by protein kinase C δ in rat pheochromocytoma PC12 cells. *J. Biol. Chem.* **277**, 8290–8297

- 38 Denmat-Ouisse, L.-A., Phebidias, C., Honkavaara, P., Robin, P., Geny, B., Min, D. S., Bourgoin, S., Frohman, M. A. and Raymond, M.-N. (2001) Regulation of constitutive protein transit by PLDs in HT29-c119A cells. *J. Biol. Chem.* **276**, 48840–48846
- 39 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
- 40 Wang, C. and Wang, X. (2001) A novel phospholipase D of *Arabidopsis* that is activated by oleic acid and associated with the plasma membrane. *Plant Physiol.* **127**, 1102–1112
- 41 Brown, F. D., Thompson, N., Saqib, K. M., Clark, J. M., Powner, D., Thompson, N. T., Solari, R. and Wakelam, M. J. O. (1998) Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane located on cellular stimulation. *Curr. Biol.* **8**, 835–838
- 42 Liu, S. Y., Tappia, P. S., Dai, J., Williams, S. A. and Panagia, V. (1998) Phospholipase A2-mediated activation of phospholipase D in rat heart sarcolemma. *J. Mol. Cell. Cardiol.* **30**, 1203–1214
- 43 Park, J. B., Kim, J. H., Kim, Y., Ha, S. H., Kim, J. H., Yoo, J. S., Du, G., Frohman, M. A., Suh, P.-G. and Ryu, S.-H. (2000) Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by α -actinin in an ADP-ribosylation factor-reversible manner. *J. Biol. Chem.* **275**, 21295–21301

Received 28 August 2002/2 October 2002; accepted 9 October 2002

Published as BJ Immediate Publication 9 October 2002, DOI 10.1042/BJ20021347