DISCUSSION

The actin cytoskeleton mediates a variety of essential processes in all eukaryotic cells, including cell motility, cell shape, phagocytosis, and cytokinesis. Three distinct kinds of actin-based structures have been identified, which are regulated by the Rho family of GTPases: Cdc42 induces filopodia, Rac regulates membranes ruffles and lamellipodia, and Rho regulates stress fiber formation (Hall, 1998). These GTPases exert their effects via specific effectors, some of which may have direct or indirect effects on lipid metabolism. In this study we provide evidence that in addition to the well established role of Rac GTPase in membrane ruffling, there is an absolute requirement for the lipid-modifying enzyme PLD in mast cells upon stimulation with antigen. Interestingly, previous studies with endothelial cells had indicated that a PLD activity was required for Rho-dependent stress fiber formation by using lysophosphatidic acid as agonist (Cross *et al.*, 1996). Additionally, PLD2, when overexpressed in rat embryo fibroblasts, was localized exclusively to the plasma membrane and induced irregular projections at the cellular edges and when stimulated with serum, PLD2 accumulated in restricted regions of the cell edge and redistributed to submembranous particles (Colley *et al.*, 1997). More recently, PLD1 activity was required for actin stress fiber formation in fibroblasts (Kam and Exton, 2001). Furthermore, previous studies have suggested an interplay between Rho family and ARF family GTPases, e.g., the formation of stress fibers and focal adhesions in fibroblasts (RhoA and ARF1) (Norman *et al.*, 1998) and the identification of an ARF6/Rac1 binding protein, POR1, involved in cytoskeletal rearrangements (D'Souza-Scharey *et al.*, 1997).

Engagement of the high-affinity IgE receptor in mast cells elicits a rapid activation of PLD activity in addition to a number of other signaling events, including PLCγ activation and phosphoinositide 3-kinase activation. The only inhibitors of PLD-stimulated PA production that have been identified are primary alcohols, which compete with water in the transphosphatidylation reaction to make the corresponding phosphatidylalcohol. Secondary alcohols such as butan-2-ol are unable to participate in transphosphatidylation and therefore serve as a control for nonspecific effects of alcohols. A prominent feature of mast cell activation is the formation of lamellipodia and membrane ruffles, which we report to be exquisitely inhibited by butan-1-ol, but not butan-2-ol. Butan-1-ol blocks membrane

ruffling at any time after stimulation, indicating that continual PLD activity is essential for the dynamics of membrane transformations. Blockade by butan-1-ol was completely reversible as demonstrated by removal of butan-1-ol. Membrane ruffling was maintained for at least 30 min, and this ruffling was accompanied by continual PLD activity. Antigen stimulation increased the activity of both PLD1 and PLD2 when overexpressed in RBL mast cells. PLD1 localized to intracellular vesicles, whereas PLD2 localized to the plasma membrane in resting cells. This pattern of PLD1 and PLD2 localization in RBL mast cells has also been described recently (Choi *et al.*, 2002). In this study both PLD1 and PLD2 were required for exocytosis of hexosaminidase-containing secretory granules because overexpression of both PLD1 and PLD2 enhanced secretion and catalytically inactive PLD1 and PLD2 both blocked secretion stimulated by thapsigargin.

To confirm that the localization of the overexpressed PLD proteins was similar to the endogenous PLDs, we analyzed the distribution of endogenous PLDs by activity measurements and confirmed that the pattern observed for overexpressed PLD proteins was similar to the endogenous PLD proteins. PLD2 activity, monitored by stimulation with oleic acid, was localized at the plasma membrane, whereas the ARF-stimulated activity was localized intracellularly, showing partial overlap with hexosaminidase-containing secretory granules (Sarri, Pardo, Fensome, and Cockcroft; unpublished data). Overexpressed PLD2 was identified at the plasma membrane in resting cells and was found in membrane ruffles as well as the pinosomes that accompanied the membrane ruffles. In contrast, PLD1 was localized to an intracellular vesicular compartment, which did not change dramatically upon antigen addition. It has been reported previously that in stimulated mast cells, PLD1 translocates to the plasma membrane (Brown *et al.*, 1998a; Choi *et al.*, 2002; Powner *et al.*, 2002). Our inability to monitor the translocation of PLD1 could be due to differences in methodology or to the degree of exocytosis triggered by antigen. In our hands, only 25-35% secretion could be triggered and mixing of the PLD1-containing granule membranes with the plasma membrane during fusion may have led to the dispersal of the GFP signal.

Membrane ruffling is accompanied by pinocytosis, and ARF6 has been implicated in coordinating the dynamics of pinosome or endocytic traffic, which accompanies membrane ruffle formation and its dissolution (Honda *et al.*, 1999; Radhakrishna *et al.*, 1999). Expression of a constitutively activated form of ARF6 induces actin assembly, resulting in

the movement of vesicle-like particles, some of which contain markers for pinosomes (Schafer *et al.*, 2000). The ARF6 exchange factor, EFA6, which has a pleckstrin homology domain, has also been shown to coordinate membrane recycling and the actin cytoskeleton during membrane ruffling (Franco *et al.*, 1999). After stimulation with antigen, PLD2 was also seen in the pinosomes but PI(4,5)P2 was not. Thus, PLD2 in the pinosomes may be a means of shutting down PLD2 activity because PI(4,5)P2 was never seen in such structures.

What is the function of PLD activity during membrane ruffling? In the membrane ruffles, both PLD2 and ARF6 are found, and our data suggest that localized availability of PA via PLD2 and ARF6 coordinate the activity of PIP5K and, therefore, PIP2 production (Fensome *et al.*, 1996; Honda *et al.*, 1999; Divecha *et al.*, 2000). This conclusion is supported by both in vitro studies published previously and the studies in permeabilized cells reported herein. Because antigen-stimulated PI(4,5)P2 synthesis is both ARF dependent and is inhibited by butan-1-ol, both PA and ARF proteins are required simultaneously to regulate PIP5K (Honda *et al.*, 1999; Jones *et al.*, 2000). During antigen stimulation, we anticipate that both ARF and PA are rate-limiting components compared with the situation when GTPγS is used as a stimulus (Skippen *et al.*, 2002). In the case of the antigen, both pathways are required and could function as coincidence detectors. The observation that GTPγS can use both pathways for PI(4,5)P2 synthesis is most likely due to the irreversible nature of G protein activation by GTPγS compared with when antigen is used as a stimulus. Responses to GTPγS are much larger and longer sustained due to the near irreversible activation of the GTPases.

One of the most interesting facets of PLD activation that has emerged from this study is the interpretation of data when PLD activation is monitored by the formation of PBut. The majority of studies use transphosphatidylation as a means of monitoring PLD activity, and herein we demonstrate that results can be misleading. Measurements of PA as a monitor of PLD activation are also equally fraught with difficulty because PA is readily metabolized. Another difficulty in using biochemical measurements of PA is that it measures global PA rather than the PA that is topologically restricted to the site of PLD activation. We have attempted to monitor PA in living cells by using a PA-binding region of Raf-1 tagged with GFP (Rizzo *et al.*, 2000). However, this domain localized intracellularly in a punctate

staining pattern and remained so in the antigen-stimulated cells. Clearly, the availability of such a reagent would provide the ability to examine the production of PA in a topologically restricted region, and kinetics of PA production can then be directly compared with membrane ruffling.

We postulate the following sequence of events: In phase I, antigen stimulates a robust activation of PLD2 (and possibly also PLD1), generating PA. Antigen also stimulates ARF6 and together with PA stimulates the activity of PIP5K, leading to a burst of PI(4,5)P2, all within the membrane ruffle. The increase in PIP2 leads to further activation of PLD2 (phase II). Our data on PLD activity measurements provide evidence for this positive feedback model whereby ongoing local PLD activity is maintained, provided that the PA is made and can participate in a downstream event most likely stimulating the levels of PI(4,5)P2. This conclusion is deduced from the anomaly of the time course of PLD activation. An apparent plateau of PLD activity is observed after 10 min of antigen stimulation (Figure 2C), despite the demonstration that PLD is continually active over a 30-min period (Figure 2D). Butanol, by preventing phase I, would therefore prevent phase II of PLD activity. Butanol is widely used to measure PLD activity, but its effects on PA production mask the events that PA subsequently regulates, in this case the positive feedback loop. This is dependent on PA-dependent increase in PIP5K activity and thus increased PI(4,5)P2 level, leading to a further increase in PLD2 activity. A similar conclusion was suggested by recent studies overexpressing PLD2 with PIP5K (Divecha *et al.*, 2000). Membrane ruffling and lamellipodia formation are extremely dynamic processes, and it is expected that a local ARF6 GTPase cycle operates in addition to the well-established Rac cycle. The local buildup of high levels of PA together with PI(4,5)P2 can then allow for ARF-GTPase–activating protein to deactivate ARF6, a characteristic of the ASAP and ACAP family of ARF-GTPase–activating proteins (Brown *et al.*, 1998b; Jackson *et al.*, 2000). In addition, PIP 5-phosphatase is also found in membrane ruffles, which suggests that turnover of PI(4,5)P2 is also taking place (Mochizuki and Takenawa, 1999).

Membrane ruffles are regions of intense actin polymerization and contain many actin-binding proteins, in particular, gelsolin and profilin, which are also PI(4,5)P2-binding proteins. Gelsolin acts to sever existing actin filaments. Profilin acts to concentrate G-actin

monomer to sites of actin polymerization. PI(4,5)P2 plays an important role in regulating these two activities. We now provide evidence for a new enzymatic component, PLD2 (and possibly PLD1), whose continual activity is required for the formation and dissolution of membrane ruffles. Our results suggest that one function of PA is to modulate the activity of PIP5K together with ARF6. Local production of PI(4,5)P2 together with Rac1 regulates the activity of proteins such as gelsolin and profilin. It is therefore interesting to note that a physical interaction between PLD2 and gelsolin has been reported (Steed *et al.*, 1996). Furthermore, it was apparent that gelsolin increased markedly the activity of PLD. Fibroblasts lacking gelsolin do not ruffle and this is assumed to be due to loss of severing activity (Azuma *et al.*, 2000). We speculate that gelsolindependent membrane ruffling is due to enhanced PLD activity and subsequent increases in PI(4,5)P2 via PA-stimulated PIP5K.

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Appendix

Dictionary notes:

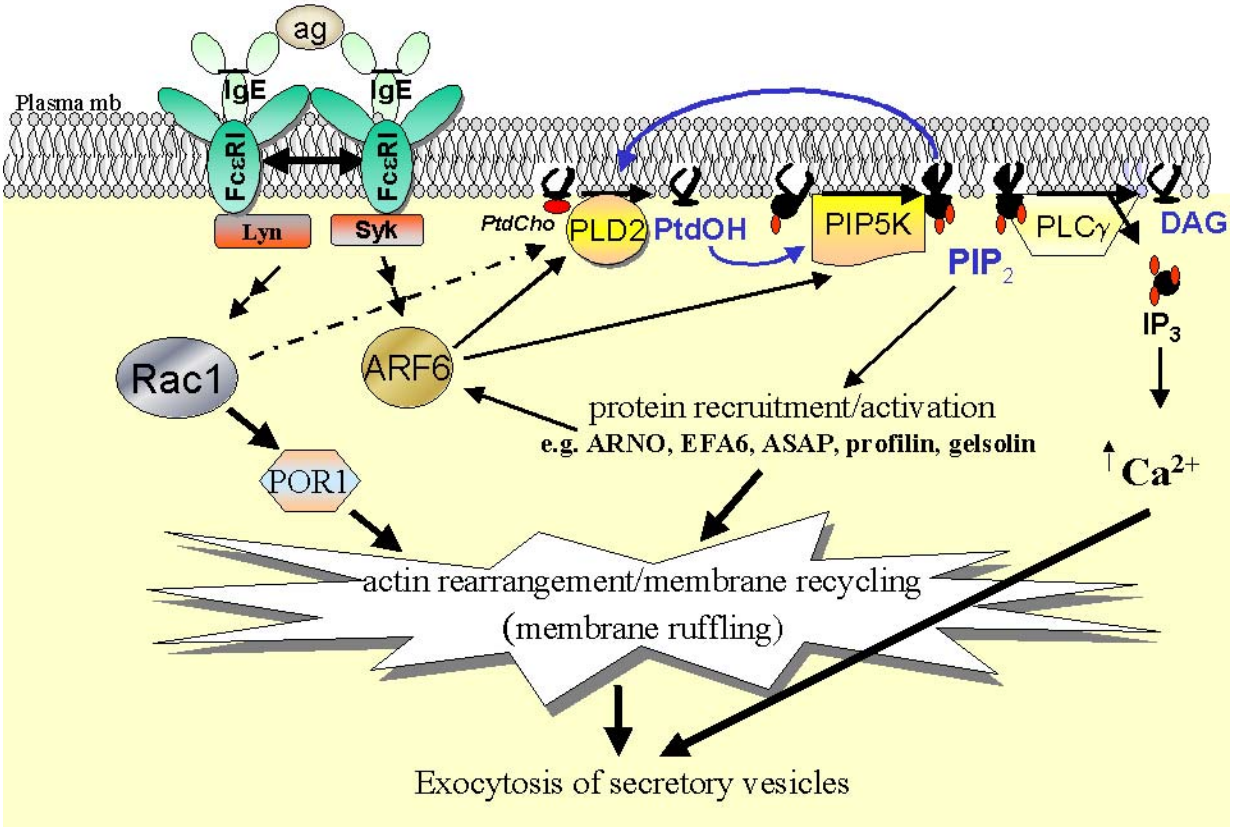
Priming.

Priming is a phenomenon in which initial treatment of cells with substimulatory concentrations of an agonist renders them hyperresponsive to subsequent stimulation. It is a characteristic of cells from the immune system. For exocytosis of secretory granules to take place, mast cells require the occupacy of IgE receptors (FcεRI) of the plasma membrane with IgE molecules. This is considered the **priming process**. When an antigen that is recognised by the IgE molecules is provided to primed cells, spatial approach of two Fc receptors can occur, by means of a single antigen being recognised by two IgE molecules. When two receptors come together, they become phosphorylated and cytosolic proteins are recruited to the site (Lyn, Syk). They trigger the signaling cascade that rises Ca^{2+} cytosolic levels, activates PLD, and that ends up with the fusion of the secretory granules with the plasma membrane.

Robust stimulation of neutrophils by the chemoatractant fMet-Leu-Phe requires also priming. In this case, the priming process requires fusion of secretory vesicles (where PLD is localised) with the plasma membrane, and can be achieved by incubation of the cells with nanomolar concentrations of the same fMet-Leu-Phe. Any further stimulation of primed cells will result in full activation.

Reconstitution experiments.

Reconstitution experiments are based on the use of permeabilising toxins (e.g. streptolysin O) which generate holes on the plasma membrane, thus allowing the difusion of cytosolic proteins. Cytosol-depleted cells often lose the ability to undergo a specific physiological function (i.e. PtdIns(4,5)P_2 synthesis), that can be restored following the addition of purified proteins.



Proposed model for antigen-stimulated membrane ruffle formation in RBL mast cells. We propose that membrane ruffling is dependent on PtdOH and PtdIns(4,5)P_2. Antigen crosslinking of IgE receptors (FcεRI) result in the recruitment of the Src family kinases Lyn and Syk. The mechanims that connect Lyn and Syk activation to the activation of the small GTPases Rac and ARF are not well understood. Rac1, ARF6, and PKC isoforms would be involved in the activation of PLD2 at the plasma membrane (PKC is not included in the picture for clarity). The resultant formation of PtdOH along with GTP-ARF6 would lead to activation of PtdIns 5-kinase (PIP5K) and to a local buildup in PtdIns(4,5)P_2 levels. This would contribute to a second phase of PLD2 activation (positive feedback denoted as blue arrows) which in turn would further activate PIP5K. The rise in PtdIns(4,5)P_2 levels in restricted sites of the plasma membrane would permit the recruitment of PH domain-containing proteins to the site. Recruitment of profilin (a protein with severing activity towards actin filaments) and gelsolin (which concentrates G-actin

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monomers to growing actin filaments) would contribute to the remodelling of the cortical actin cytoskeleton. The recruitment ARNO (an ARF-GEF for ARF6), EFA6 (a proposed GEF for both ARF6 and Rac1), and members of ASAP family of GAPs would permit the maintenance of a dynamic ARF cycle of activation/deactivation. A similar cycle for Rac1 may also take place. Thus, ARF and Rac1 would serve as molecular swiches that control the dynamic changes in the actin cytoskeleton acting in cooperation with downstream lipid-modifying enzymes (PLD2 and PIP5K). POR1 is a Rac1-binding protein that might participate in cytoskeletal rearrangements.

Ruffling and secretion should be considered independent phenomena, as secretion but not ruffling is Ca^{2+}-dependent.

PIP_2 stands for PtdIns(4,5)P_2.

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

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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

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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 FcϵRI 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 Cα. 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)P_2 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.

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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 μM) 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

[^{3}H]Myristic acid, [^{14}C]acetate and [^{3}H]PC were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Antibodies to CD63 (AD1) and the β-subunit of FcεRI 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 μg/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 [^{3}H]myristic acid for 1 h at 37 °C in Hepes buffer (137 mM NaCl, 3 mM KCl, 20 mM Hepes, 2 mM $MgCl_2$, 1 mM $CaCl_2$ 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 μg/ml anti-DNP-IgE. After the labelling period, [^{3}H]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 μl 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 μl 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 [^{3}H]phosphatidylbutanol ([^{3}H]PBut) and [^{3}H]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 μl of electroporation buffer (20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$ and 1 mg/ml glucose) with 30 μg of plasmid DNA (GFP-hPLD2 and GFP-hPLD1b). Following electroporation (two pulses at 500 mV, 125 μF) 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 μg/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 μm 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 Na_2HPO_4, 1.5 mM KH_2PO_4, 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 120000 ***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 100000 ***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 μl 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 μl 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 μl of subcellular fractions were incubated with 50 μl of a reaction mixture containing 50 mg/ml trypsin inhibitor, UDP-D-[6-^{3}H]galactose (330000 d.p.m./sample), 50 mM Mes (pH 7.5), 30 mM $MnCl_2$, 2 mM $MgATP^{2-}$, 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 μl) 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 FcϵRI (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 μg) 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 μM [^{3}H]PC (approx. 300000 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 $MgCl_2$ and 2 mM $CaCl_2$, 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 μCi/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 $MgATP^{2-}$, 2 mM $MgCl_2$, 3 mM EGTA and 2.7 mM $CaCl_2$ (Ca^{2+}_{free}, 10 μM), 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 transphosphatidylation 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

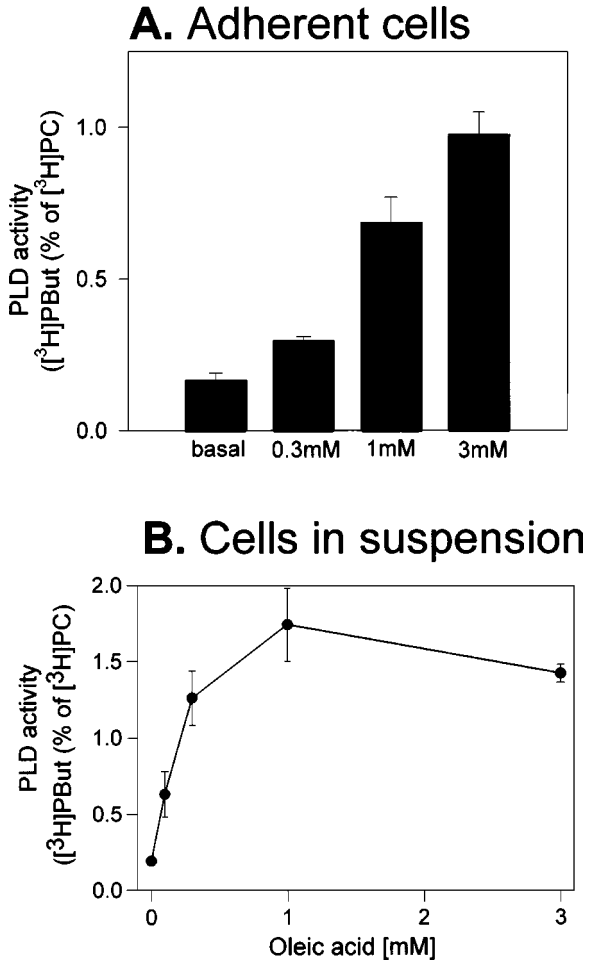

Figure 1 Stimulation of PLD activity by oleate in adherent and suspended RBL-2H3 mast cells

(**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.

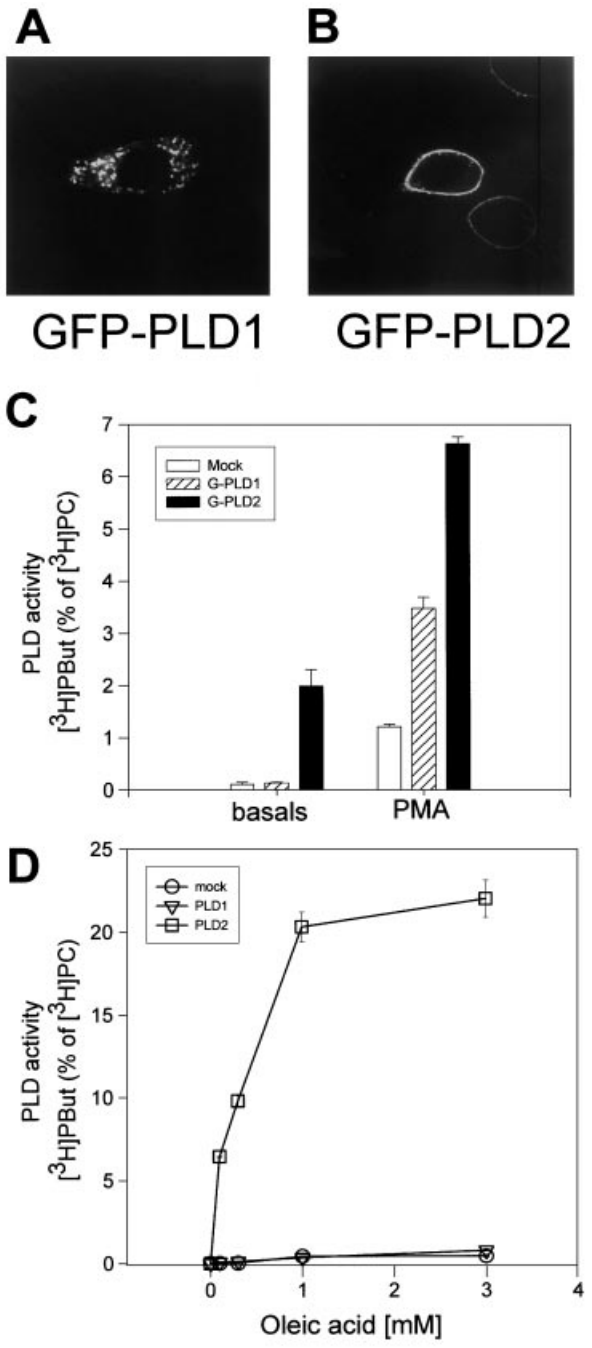

Figure 2 Stimulation of PLD2 activity by oleate from RBL-2H3 mast cells overexpressing GFP-hPLD1b and GFP-hPLD2

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 ± 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

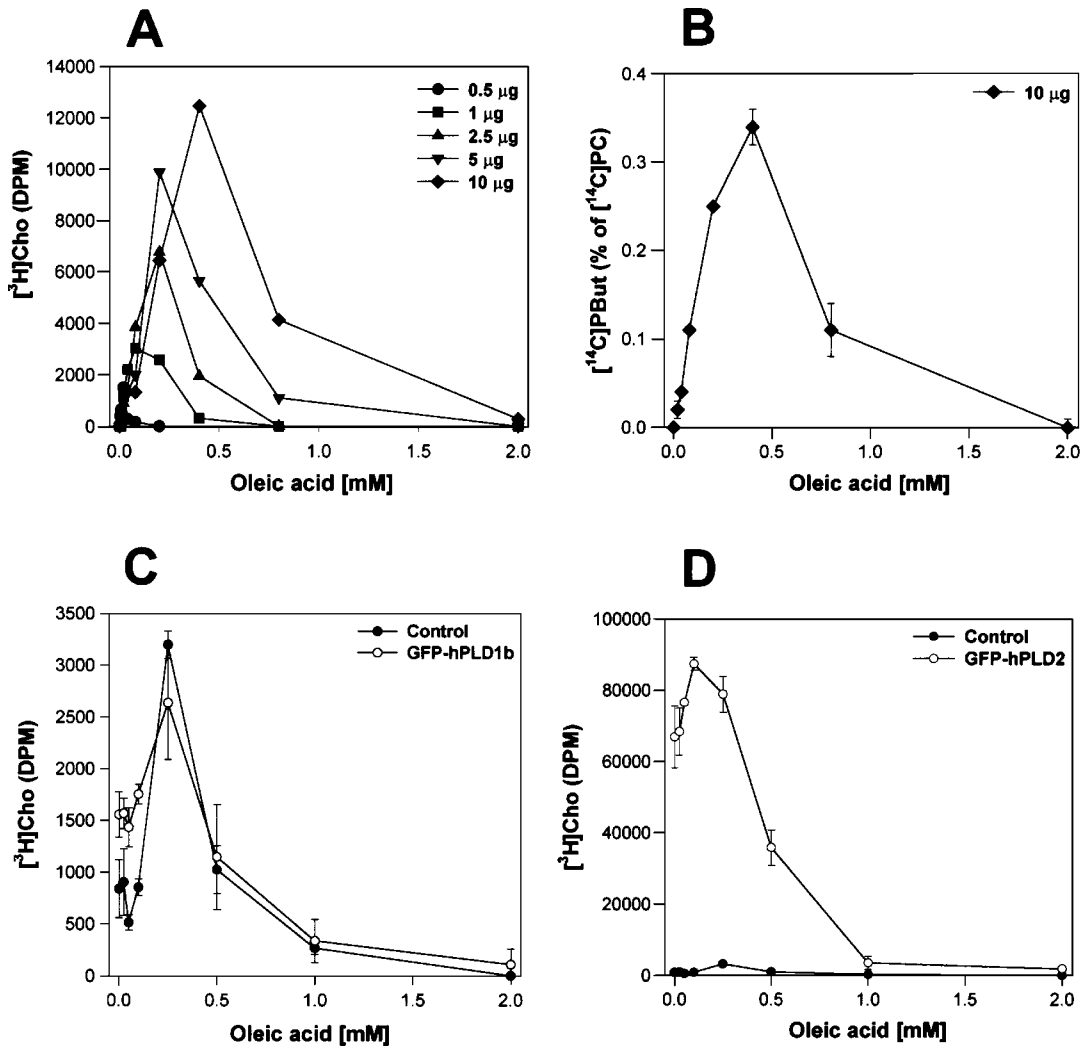

Figure 3 Stimulation of PLD2 activity by oleate in membranes from control and transfected RBL-2H3 mast cells

(**A**) Membranes from untransfected cells were incubated with lipid vesicles containing [^{3}H]PC for 1 h in the presence of different concentrations of oleate and the indicated amounts of membrane protein. [^{3}H]Cho was separated by cation-exchange chromatography. (**B**) Membranes (10 μg of protein) from [^{14}C]acetate-prelabelled cells were incubated for 1 h with different concentrations of oleate in the presence of 0.5% butanol. [^{14}C]PBut and [^{14}C]PC were separated by TLC and the radioactivity in each phospholipid was determined. PLD activity is expressed as d.p.m. in [^{14}C]PBut as a percentage of d.p.m. in [^{14}C]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}H]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}H]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 transphosphatidylation reaction, which is specifically catalysed by PLD in a subcellular fractionation obtained after pre-labelling the RBL-2H3 mast cells with [^{14}C]acetate. The results of this experiment indicated that those fractions which contained oleate-stimulated [^{14}C]PBut accumulation matched those exhibiting oleate-stimulated [^{3}H]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 FcϵRI, 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

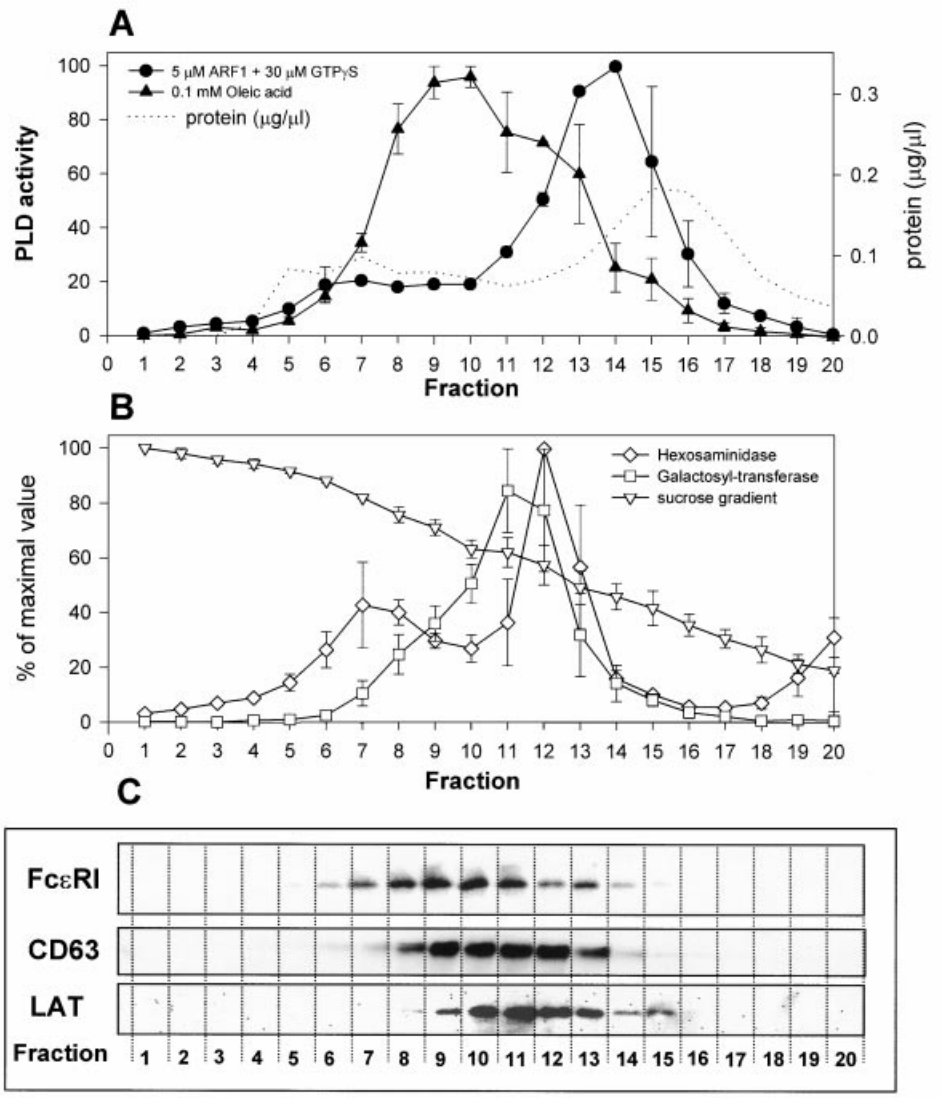

Figure 4 Separation of endogenous oleate- and ARF1-stimulated PLD by subcellular fractionation of RBL-2H3 mast cells

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]PCho for 1 h in the presence of 0.1 mM oleate (▲) or 5 μM ARF1 and 30 μM guanosine 5′-[γ-thio]triphosphate (GTPγS; ●), 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 FcεRI, 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 FcεRI 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

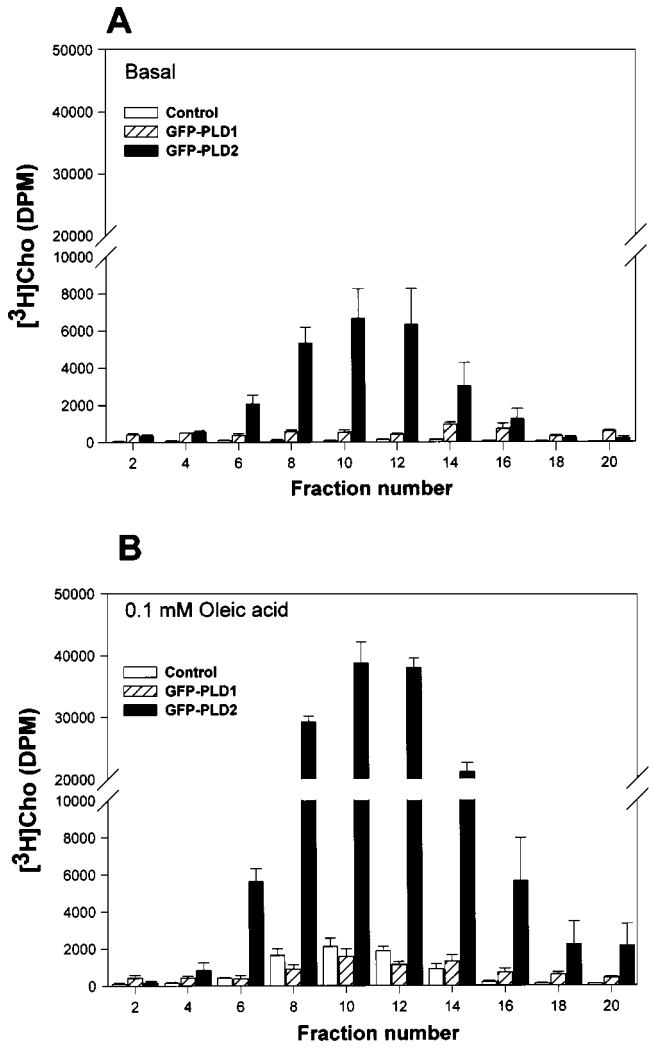

Figure 5 Oleate stimulates overexpressed PLD2 but not PLD1b in subcellular fractions

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, 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.

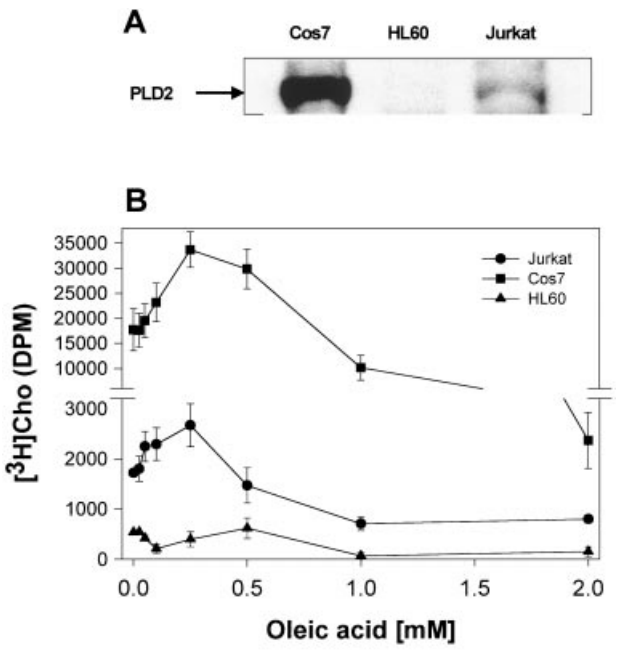

Figure 6 Oleate can be used to identify cells expressing endogenous PLD2

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

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

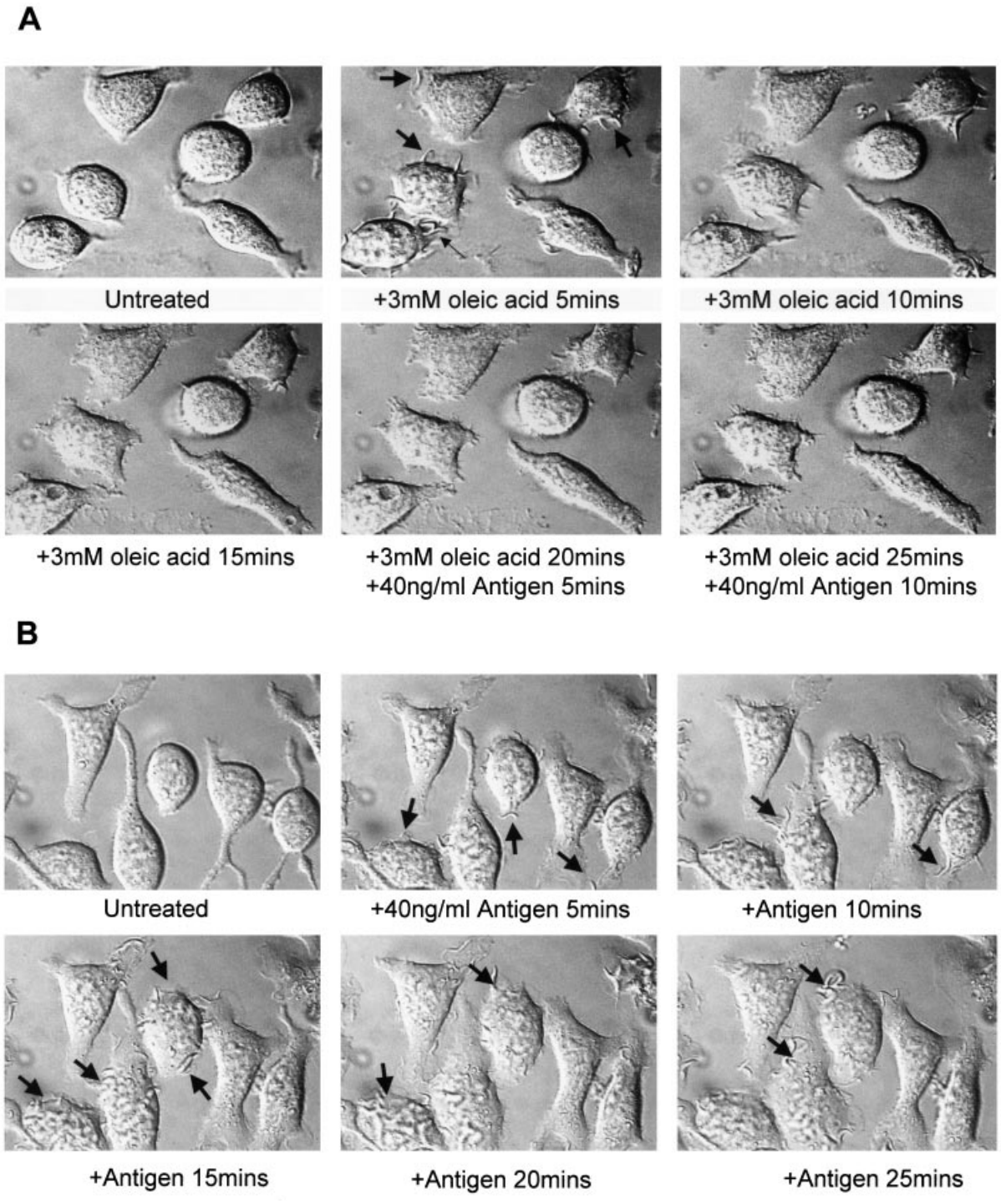

Figure 7 Comparison of membrane ruffling by phase-contrast, time-lapse recording of RBL-2H3 mast cells stimulated with oleate and antigen

(**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 Cα 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 μM 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 submembraneous 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; PLDα, PLDβ, PLDγ and PLDδ. PLDα 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, PLDβ and PLDγ 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 PLDδ, 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 μM 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 PLDδ 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 A_2 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 A_2; this is also found in heart diseases characterized by phospholipase A_2 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.

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