CMLS, Cell. Mol. Life Sci. 58 (2001) 1674–1687
1420-682X/01/111674-14 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Signalling roles of mammalian phospholipase D1 and D2

S. Cockcroft

Department of Physiology, Rockefeller Building, University College London, University St., London WC1E 6JJ (United Kingdom), Fax: + 44 71 387 6368, e-mail: ucgbsxc@ucl.ac.uk

Received 25 April 2001; received after revision 15 June 2001; accepted 15 June 2001

Abstract. Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine to generate the lipid second messenger, phosphatidate (PA) and choline. PLD activity in mammalian cells is low and is transiently stimulated upon activation by G-protein-coupled and receptor tyrosine kinase cell surface receptors. Two mammalian PLD enzymes (PLD1 and PLD2) have been cloned and their intracellular regulators identified as ARF and Rho proteins, protein kinase Cα as well as the lipid, phosphatidylinositol [4, 5] bisphosphate (PIP_2). I discuss the regulation of these enzymes by cell surface receptors, their cellular localisation and the potential function of PA as a second messenger. Evidence is presented for a role of PA in regulating the lipid kinase activity of PIP 5-kinase, an enzyme that synthesises PIP_2. A signalling role of phospholipase D via PA and indirectly via PIP_2 in regulating membrane traffic and actin dynamics is indicated by the available data.

Key words. Phospholipase D; ADP ribosylation factor; Rho protein; phosphatidic acid; phosphatidylinositol(4,5)bisphosphate.

Introduction

Phospholipase D (PLD) is now recognised as a receptor-regulated signalling enzyme, which may control many biological functions, including exocytosis, phagocytosis, actin dynamics and many aspects of membrane traffic. Considerable progress has been made in understanding the enzymology of the PLD enzymes and their regulators and for earlier studies I refer the reader to several excellent reviews [1–8], in particular a special issue devoted to phospholipase D [9]. This review focuses on recent developments in phosphatidylcholine (PC)-specific PLDs involved specifically in mammalian signal transduction, with a particular emphasis on the functional aspects of PLD activation.

Mammalian PLD catalyses the hydrolysis of the major membrane phospholipid, phosphatidylcholine (PC), to produce phosphatidic acid (PA) and choline (fig. 1). PA is the potential lipid 'second' messenger akin to diacylglycerol, derived from the canonical PLC signalling pathway. In addition, PA can be metabolised to other biologically important lipid metabolites including lyso-PA (LPA) and diacylglycerol (DG). PLD activity in mammalian cells is low and is transiently increased following occupation of many cell surface receptors including those of the heterotrimeric G-protein and tyrosine kinase families. Conceptually, PLD activation by cell surface receptors is akin to PLC and phosphoinositide 3-kinase (PI3K) activation where metabolism of a specific lipid is used to generate 'second messengers'. In the case of PLC and phosphoinositide 3-kinase (PI3K), the substrate (PIP_2) is used to generate inositol (1,4,5)triphosphate (IP_3) and DG (PLC), and phosphatidylinositol (3,4,5)trisphosphate (PIP_3) (PI3K). DG and PIP_3 reside in the membrane where they are produced and are known to translocate and activate specific proteins. PIP_3 recruits proteins with PH domains, whilst DG recruits and activates protein kinase C in conjunction with phosphatidylserine and Ca^{2+}. This review will discuss how similar paradigms may also apply to PA.

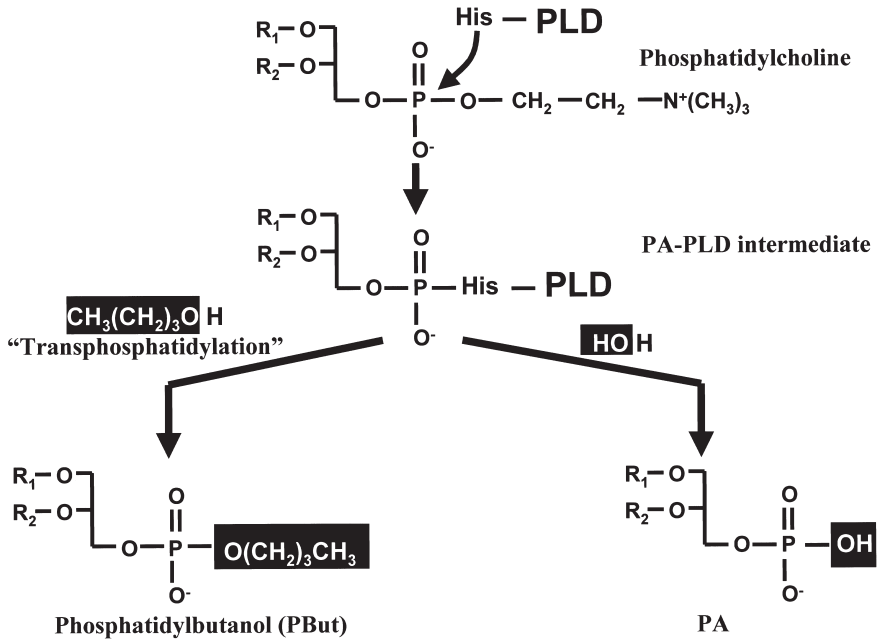

Figure 1. PLD-catalysed hydrolysis and transphosphatidylation reactions. The first part of the reaction involves the formation of a PA-PLD intermediate by covalent linkage of PA to a histidine. Either water or a primary alcohol can act as a nucleophile in the second stage of the reaction. In the presence of water, the reaction product is PA, and in the presence of alcohol, the reaction product is phosphatidylalcohol.

To examine the function of PLD activity in cells, many studies have relied on alcohols in the absence of specific PLD inhibitors. Primary alcohols participate in a transphosphatidylation reaction which is a hallmark of enzymes of the PLD superfamily. In the case of PLD enzymes, transphosphatidylation makes a phosphatidylalcohol at the expense of PA, the putative second messenger. This reaction is specific for primary alcohols, and therefore secondary alcohols are generally used as a control for the non-specific effects of such a pleiotrophic compound. The reaction mechanism utilised by PLDs for 'transphosphatidylation' is described below.

Enzymology of PLD

PLD activity was first identified in plants and PLD was the first PC-hydrolysing enzyme to be purified and successfully cloned [10]. The cloning of the first plant PLD and the subsequent realisation that the yeast sporulation gene called *SPO14* had sequence homology to plant *PLD* led to the identification of the yeast *SPO14* as the yeast *PLD* [11] (see fig. 2). The availability of the plant and yeast sequences opened the way for the subsequent cloning of mammalian *PLD*s [12–14]. Prior attempts at purifying mammalian PLDs had failed, due to lack of knowledge about their regulators and their low abundance, and only one enzyme, the oleate-dependent PLD from porcine lung was purified to homogeneity but never cloned [15].

Today, two mammalian *PLD*s have been cloned, *PLD1* and *PLD2* (fig. 2). Together, the plant, yeast and mammalian genes comprise a new gene family. The limited but significant similarity that they share defines a number of characteristic, highly conserved sequence motifs [6]. These *PLD* genes all belong to an extended gene superfamily that also includes bacterial *PLD*s, bacterial phosphatidylserine and cardiolipin synthases, bacterial endonucleases, a *Yersinia pestis* murine toxin, and vaccinia viral envelope proteins, K4 and Vp37 [16]. The *Y. pestis* murine toxin has PLD activity [17], unlike K4 and Vp37 [18, 19]. PLD superfamily members all share a conserved motif, $HxK(x)_4D(x)_6GSxN$, where x denotes any amino acid residue [16]. This sequence has been dubbed the HKD or PLDc motif, and with the exception of the bacterial endonucleases and one of the virus proteins, all members of the PLD superfamily contain two HKD motifs. Analysis of proteins containing the HKD (or PLDc as identified by SMART [20]) motif identifies three proteins each containing two PLDc domains in *Homo sapiens* (fig. 2). These are human PLD1, PLD2 and a Hu-K4. Homologues of PLD1 and PLD2 are found throughout the animal kingdom. Homologues of the Hu-K4 protein are found in *Caenorhabditis elegans, Dictyostelium discoideum* and mouse. The mouse *K4* gene (also called *SAM-9*) is expressed in mature neurons of the forebrain and appears to be turned on at late stages of neurogenesis [18]. However, the enzymatic activity catalysed by the K4 class of proteins remains to be characterised.

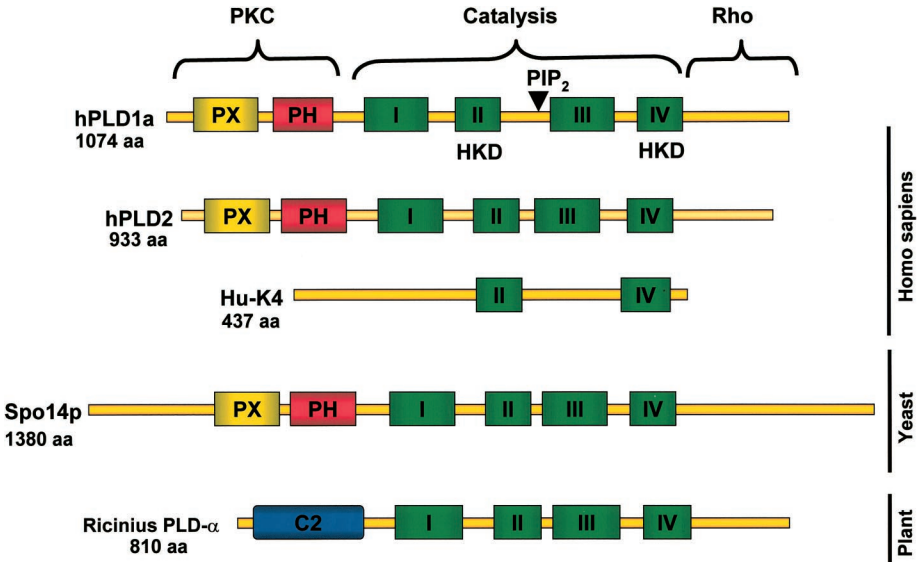

Figure 2. Domain structure of *PLD1*, *PLD2* and *Hu-K4* found in *Homo sapiens* and their relationship to plant and yeast *PLD*s. Regions of conserved sequence are shown. PX, phox homology domain; PH, pleckstrin homology domain; C2, calcium- and phospholipid-binding domain; motifs I, II, III and IV, regions of sequence conserved among all PLD isozymes. Motifs II and IV (also known as HKD or PLDc) are also found in Hu-K4. Regions of PLD1 identified in stimulation of catalytic activity by protein kinase C and Rho are indicated and the PIP_2-interacting site is also denoted.

The mammalian *PLD1* cDNA was cloned from a HeLa cell cDNA library and found to encode a 1074-amino acid protein [12], whilst *PLD2* was cloned following the identification of related sequences in expressed sequence tag (EST) databases. PLD2 is a 933-amino acid, 106-kDa protein that shares 55% identity with PLD1. PLD1 and PLD2 mainly differ in their N and C termini. In addition, PLD1 has a 116-amino acid 'loop' region inserted immediately following the first HKD motif. Otherwise, both PLDs show the existence of a putative PX domain followed by a PH domain (fig. 2). Both PX and PH domains bind to phosphoinositides [21]. In addition, four motifs, I–IV, can be identified which are shared with members of the PLD superfamily (fig. 2). PLD1 exists as two splice variants, PLD1a and PLD1b, which differ by the insertion of a 38-amino acid region in PLD1a [13, 22].

PLD and nuclease reactions proceed by a ping-pong mechanism in which a phosphoenzyme intermediate is formed and then hydrolysed in the second step of the reaction [23, 24] (fig. 1). From the structure of the bacterial endonuclease, residues from two HKD motifs form a single active site and a histidine residue from one motif serves as a nucleophile in the reaction forming the phosphoenzyme intermediate, while a histidine from the other motif is a general acid that functions in the hydrolysis of the substrate phosphodiester bond [25]. The first crystal structure of a 54-kDa bacterial PLD confirms that the HKD motif acts as a dimer and can bind to a phosphodiester bond [26]. This is also true for mammalian PLD where the two HKD motifs associate together to form a catalytic centre [27].

This mechanism of catalysis provides an explanation for the unique propensity of the PLD enzymes to function as phosphatidyltransferases in which primary alcohols serve as nucleophiles in the second step of the reaction hydrolysing the phosphoenzyme intermediate and generating a phosphatidylalcohol product [16]. The transphosphatidylation reaction is a unique property of enzymes of the PLD superfamily and the non-PLD enzymes (PS and cardiolipin synthases) use transphosphatidylation exclusively. Plant and mammalian PLDs greatly prefer alcohol to water and 0.2% butanol is sufficient for transphosphatidylation to take place. Secondary and tertiary alcohols are not substrates for either enzyme [28]. For branched alcohols, activity increases with distance from the alcohol to the branch point. Thus iso-butanol is active but secondary and tertiary butanol are not. This then is the basis of many studies where low concentrations of alcohols have been used to indicate the requirement for PA generated from hydrolysis of PC by PLD. Additionally, transphosphatidylation has been exploited as a means to measure PLD activation in intact cells, since phosphatidylalcohols are not rapidly metabolised in comparison to PA and it is a unique product of PLD activity.

Expression of PLD isoforms

Measurements of biochemical activity indicate that PLD is expressed in most cells. But to obtain insight into the isoforms expressed, success with antibodies for detection of endogenous PLD1 and PLD2 proteins has been only

partial. This problem is due to the lack of high-affinity antibodies coupled with the low level of expression of these proteins. Endogenous PLD isoforms have been identified in FRTL-5 thyroid cells which express PLD1 predominantly with some PLD2 [29]. An alternative way of detecting expression of endogenous PLD isoforms has ultilised measurements of mRNA levels, and from such an analysis, expression levels of *PLD1* and *PLD2* clearly vary within primary tissues and between cell lines, but in the majority of cases, most cells co-express *PLD1* and *PLD2* [30, 31]. A notable exception appears to be mature lymphocytes that are devoid of PLD1 or PLD2.

PLD activity has been identified in brain, and both PLD1 and PLD2 are enriched there. Studies with cell cultures or brain slices have demonstrated that PLD in the nervous system is activated in response to a variety of agonists. Analysis of mRNAs in the developing brain point to the astrocytes, for PLD2, and oligodendrocytes, for PLD1, as the main cell types enriched in PLDs [32]. In the adult rat, PLD1 was found to be upregulated in astrocytes in response to transient ischemia [33]. Considering that the mammalian brain is one of the organs with the highest specific activity of PLD, that neurons are not the major sites of PLD enzymes is rather unexpected.

There are reports that expression of mRNA for both *PLD1* and *PLD2* can be regulated at the transcriptional level by growth and differentiation factors in both primary and cultured cells. For example, in primary mouse keratinocytes, 1,25-dihydroxyvitamin D3 induces *PLD1* expression during differentiation [34]. In human myeloid cells such as HL60 cells, differentiation leads to a dramatic increase in both *PLD1* and *PLD2* expression [35, 36]. *PLD* activity has also been shown to be significantly elevated in human cancers including breast, renal, gastric and colon cancer suggesting that PLD might be implicated in tumorigenesis [37–41].

Regulation of PLD1 and PLD2 activity

A very large number of agonists increase the activity of PLD in many cells and tissues (see table 1 for selected examples and further information can be obtained from previous reviews [1, 5]). Because many of these agonists act through receptors coupled to the heterotrimeric G-proteins, signal(s) arising from the activation of G-proteins must be indirectly regulating PLD activity. Likewise, many of the agonists are growth factors and antigens that act through receptors that utilise intrinsic or extrinsic tyrosine kinase activity. Thus, signals generated downstream of tyrosine phosphorylation must also control PLD activation. Many of these receptors also stimulate PLC activity leading to an increase in cellular Ca^{2+} as well as DG, a regulator of protein kinase C (PKC). The phorbol ester, PMA, is a potent activator of PLD activity in most cell types, implying that in many instances PLC activation may be upstream to PLD activation. However, there is substantial evidence that receptor-mediated regulation of PLD enzymes is complex and is in part mediated by the small GTPases of the ARF and Rho family (Table 1, fig. 3). There are six ARF proteins, ARF1–ARF6, all of which activate PLD1. Rho family members, RhoA, Rac and Cdc42 are again all capable of activating PLD1. How activation of cell surface receptors regulates the activation of ARF and Rho proteins is not within the scope of this review. In general, activation of ARF and Rho

Table 1. Examples of PLD activation in primary tissues and cultured cell lines mediated by ARF, Rho and protein kinase C (PKC)

Cell type	Agonist	Comments	References
Neutrophils	FMLP; monosodium urate crystals;	dependent on ARF, PKCα, Rho	105–107
Human airway epithelial adenocarcinoma A549 cells	bradykinin	involves RhoA	109
fibroblasts	PDGF	dependent on ARF proteins but not Rho	109
vascular smooth muscle cells	angiotensin II, endothelin-1; PDGF	stimulation is ARF-dependent and is via PLD2	110
PC12 cells	bradykinin	activates PLD2 via PKCδ	111
HEK 293 cells expressing the M3 muscarinic receptor	carbachol	mediated by ARF and Rho	112–114
RBL-2H3 mast cells	antigen cross-linking of IgE receptor	dependent on ARF proteins	79
Avian DT40 B cells	antigen on B cell receptor	syk, Btk and PLCγ2 dependent	115
U937 cells (a human monocyte cell line)	IgG on FcγR1 receptor	dependent on ARF, PKCα and PLD1	
FRTL-5 thyroid cells	thyroid stimulating hormone	activation of PLD1 dependent on ARF and Rho	29
Mouse embryo fibroblasts	PDGF	deletion of PLCγ1 inhibits activation of PLD	116
Astrocytes	PMA; adrenalin	requires ARF, Rho and PKC for full activation	117, 118

Past reviews have compiled an extensive list of tissues where agonist-stimulated PLD activation has been demonstrated [1, 2, 5].

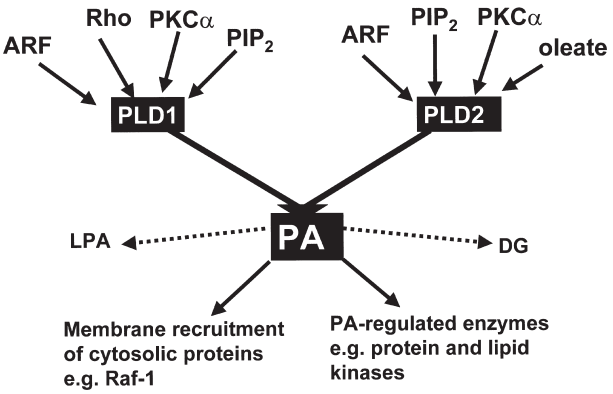

Figure 3. Direct activators of PLD1 and PLD and the potential function of PA. Broken lines indicate the lack of substantial evidence to support the possibility.

GTPases is mediated by appropriate guanine nucleotide exchange factors (GEFs), which constitute a large family of proteins and whose regulation by cell surface receptors is only partly understood.

The baculovirus-expressed recombinant hPLD1 enzyme is directly activated by ARF proteins in the presence of PIP_2 [13]. This confirmed numerous past observations of an ARF-activated PLD activity first identified in HL60 cells [42, 43]. In addition to ARF, this enzyme is also activated by Rho proteins and PKCα. The splice variants of PLD1 (a and b) do not show any marked differences in their ability to be activated by the numerous activators. Based on extensive structural analysis, the amino-terminal 325 amino acids are required for PKCα activation of PLD1 but not for ARF1 and RhoA [44]. In contrast, Rho interacts with a site in the amino acid sequence 873–1024 at the C terminus of PLD1 [45, 46]. Further analysis indicated that single or double mutations in specific regions were sufficient for abolishing Rho binding as well as activation by Rho [46, 47]. The site of ARF interaction has not yet been identified.

For some cell types, PKC has been suggested to be the major cellular activator of PLD by various agonists including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), thrombin, bradykinin, angiotensin II and vasopressin. Mutants of PLD1 which are only unresponsive to PKC have been generated and could not be activated by receptor regulation of bombesin, and muscarinic receptors 1 and 3 when expressed in HEK293 cells [48]. Some studies had shown that PKC can directly activate PLD1 in an ATP-independent manner, whilst others suggested that a phosphorylation-dependent mechanism was important. PLD1 is phosphorylated by PKCα at serine residue 2, threonine 147 (located in the PX domain) and serine 561 (located in the loop region) and mutation of any of these residues leads to significant reductions in PMA-induced PLD1 activity [49].

PLD2 when expressed in COS 7 cells was found to be constitutively active but this is not necessarily the case when it is transfected into other cell types [14]. Regulation of PLD2 is less well understood. Modest activation of PLD2 can be observed with ARF proteins and it can be robustly activated upon removal of the NH_2-terminal 308 amino acids [50, 51]. PLD2 also exhibits a modest response to phorbol ester stimulation, suggesting a positive role of PKCα in the regulation of PLD2 activity in cells. Co-expression of PKCα and PLD2 results in an increase in PLD2 activity supporting the possibility that PKCα can regulate PLD2 activity [52, 53]. Like PLD1, PLD2 has also been found to form a physical complex with PKCα [52, 53].

An oleate-sensitive PLD was first purified to homogeneity from lung. This enzyme was most likely PLD2 based on size [15] and on the recent analysis demonstrating that PLD2 can be activated by unsaturated fatty acids including oleate (18:1), linoleate (18:2) and arachidonate (20:4) [54]. Lymphocytic mouse leukaemic L1210 cells only express the oleate-sensitive PLD with no indication of an ARF-sensitive PLD activity. In contrast, HL60 cells have no oleate-stimulated PLD activity but contain a robust ARF-stimulated activity [55]. Thus, these two cell types are at extreme ends of the spectrum, with the PLD2 isoform predominating in lymphocytic cells whilst a PLD1-like activity is predominant in HL60 cells. In the rat heart, the major PLD has also been identified as PLD2 and can be activated by a PLA_2-dependent release of unsaturated fatty acids [56, 57]. Cardiac PLD2 was found to be associated with α-Actinin and the N-terminal 185 amino acids were responsible for this interaction. α-Actinin was found to inhibit PLD2 activity and the inhibition could be reversed by ARF. These data together with the observation that removal of the N terminus makes PLD2 responsive to ARF would indicate that under certain conditions when an interacting protein like actinin is present, ARF may be a regulator of PLD2.

PLD1 and PLD2 are apparently regulated by activation of cell surface receptors when examined in intact cells, e.g. HEK293 cells stimulated with EGF or insulin [53]. Additionally, PLD2 becomes tyrosine-phosphorylated and forms a physical complex with the EGF receptor.

Table 1 assembles the evidence that indicates the mechanism of PLD activation used by different cell surface receptors. ARF and RhoA have been implicated in mediating agonist-induced activation of PLD in several cell types. ARF and Rho proteins have also been shown to be activated as monitored by their translocation to membranes upon addition of agonist. Figure 3 summarises the input of several regulators impinging on the activity of PLD1 and PLD2 based on available data from both in vitro and cell-based studies. PLDs are not really unusual in having multiple regulators and this may allow receptor- and tissue-specific activation of this class of enzymes.

Regulation by PIP_2

Establishment of in vitro assays for mammalian PLD activity identified a requirement for PIP_2 [42] and this lipid has now been established as an allosteric regulator of both PLD1 and PLD2. Both PLD1 and PLD2 have a putative PH domain which is a potential candidate for PIP_2 regulation. In one study, point mutations within the PLD1 PH domain were reported to inhibit PLD1b enzyme activity, whereas deletion of the domain both inhibited enzyme activity and disrupted normal PLD1 localisation [58]. It is worthwhile noting that cysteine residues 240 and 241 which lie within the putative PH domain are palmitoylated and this also influences the localisation and association of PLD1 with membranes [59, 60]. A second site for phosphoinositide regulation has also been identified between motifs II and III (see fig. 2), a unique conserved region of basic amino acids which is a stretch of sequence (10–20 residues) loosely denoted as 'KR' motifs, rich in basic amino acids and often interspersed with aromatic and aliphatic amino acids. Mutational analysis of residues in this region in PLD2 reveals the loss of PIP_2-dependent activation, although the enzymes localise normally [61].

Location of PLD determines its function

To understand the function of PLD activity an insight into its location within cells is essential. The product of PLD activity is a lipid that can only function in the membrane compartment where it is generated. Table 2 summarises the published data that pertain to the localisation of PLD activity. The most detailed analysis of the location of endogenous PLD activity in a primary cell has been done with human neutrophils. Human neutrophils are terminally differentiated cells which are unable to proliferate and are scheduled to die after a couple of days. The biology of the neutrophil is well understood. Quiescent neutrophils in the circulation contain endocytically derived secretory vesicles which are a reservoir for signalling molecules such as integrins and fMetLeuPhe (FMLP) receptors [62]. Prior to activation, the neutrophil needs to be 'primed' and this is accomplished by very low concentrations of agonists, including the bacterially produced tripeptide FMLP. PLD is minimally activated during 'priming'. 'Priming' is effectively the insertion of signalling molecules including PLD into the plasma membrane by fusion of secretory vesicles [63]. Primed neutrophils show a robust production of PA which peaks at less than a minute after stimulation with FMLP, and initiates both exocytosis of secretory granules and the activation of NADPH oxidase. Low concentrations of alcohol block both responses. Translocation of ARF to membranes is also dependent on 'priming' [64]. Phosphatidylethanol production in stimulated cells is primarily localised at the plasma membrane as determined by subcellular fractionation. The data from the neutrophil studies clearly establish the plasma membrane as the main site of PLD activation.

HL60 cells have also been extensively analysed and widely used as a model system for neutrophils. They can be terminally differentiated towards a neutrophil-like lineage with a variety of differentiation agents and acquire many of the characteristics of a neutrophil including the expression of FMLP receptors. In contrast to the human neutrophil, much of the ARF-regulated PLD activity is found intracellularly with only a small amount at the plasma membrane [65]. Since cultured cell lines are often derived from patients suffering from cancer, and cancer cells have been shown to upregulate the PLD proteins, caution must be exercised when evaluating data from cell lines because they may not represent the situation in primary cells. This caveat is important, because the majority of studies have utilised cultured cell lines for the analysis of PLD localisation.

Which intracellular compartment has PLD activity? The identification of ARF as a regulator of PLD activity led to the speculation that PLD1 may be important for the formation of COP1-coated vesicles from the Golgi. An early study had suggested that ARF-stimulated PLD activity was localised in a Golgi-enriched fraction in cultured cells [66]. However, other studies in primary tissues indicate that activity at the Golgi (prepared from rat liver) is not substantial [67, 68]. Recent data indicate that the ability of ARF to participate in COP1-coated vesicles may be independent of PLD activity [69–71].

To overcome the ambiguities of subcellular fractionation (lack of purity of fractions) and the inability to distinguish between PLD1 and PLD2 isoforms, many investigators have resorted to the use of tagged PLDs to study localisation by microscopy. The following picture emerges. In most cases, PLD2 is found at the plasma membranes and also in submembranous vesicular structures which appear to be endocytic in origin [14, 72; O'Luanaigh et al., unpublished data]. Figure 4 illustrates an example of the localisation of PLD2 at the plasma membrane in RBL-mast cells. Upon stimulation, PLD2 is found in the ruffling membrane as well as in circular endocytic structures that are formed following stimulation [O'Luanaigh et al., unpublished data]. In a number of studies, where the localisation of PLD1 has been examined, the emerging picture indicates that it is localised in the endosomal/lysosomal compartment [73, 74]. Again, an example is shown from the RBL-mast cells where PLD1 is localised to the lysosomal compartment (fig. 4A). In RBL-mast cells, the lysosomal compartment functions as a secretory granule which can be exocytosed upon stimulation. The PLD1 localises to this compartment and is able to translocate to the plasma membrane upon stimulation [73].

Table 2. Localisation of PLD activity or isozymes.

Cell type	Stimulus	Cellular compartment	Measurements of PLD activity	References
Human neutrophils	FMLP	P.M.	PEt formation in cells	119
Human neutrophils	FMLP	Endocytic secretory vesicles which fuse with the P.M. upon 'priming'	ARF-dependent PLD activity	63
HL60 cells	FMLP	P.M. and endomembranes	PEt formation in cells	65
HL60 cells	N.A.	P.M. and endomembranes	ARF-stimulated PLD activity	65
HeLa cells	PMA	P.M. and E.R.	PEt formation in cells	120
3T3 Li cells	bradykinin	P.M. and E.R.	PEt formation in cells	120
Rat embryo fibroblasts (REF-52)	N/A	P.M.	HA-tagged PLD2	14
Rat embryo fibroblasts (REF-52)	N/A	Golgi, E.R., endosomes	HA-tagged PLD1	14
BHK cells	PMA	E.R.	PEt formation in cells	121
NRK, Vero, HeLa	N/A	late endosomes and lysosomes	FLAG-tagged PLD1a and HA-tagged PLD1b	75
RBL mast cells	N/A	plasma membranes	EGFP-tagged PLD2	73
RBL mast cells	N/A	secretory granule/ lysosomal markers	EGFP-tagged PLD1b (translocates to P.M. on antigen stimulation)	122
3Y1 fibroblast	N/A	P.M. (caveolae), E.R. and Golgi	Endogenous PLD1 immunore activity and GFP-PLD1	123, 124
U937 cells, HL60	N/A	detergent-insoluble cytoskeleton	PLD1 together with ARF and Rho	125, 126
U937 cells, human keratinocytes; CHO cells	N/A	detergent insoluble fraction /cavealoe	PLD2	127, 128

N/A, not applicable; P.M., plasma membranes; E.R., endoplasmic reticulum; PEt, phosphatidylethanol.

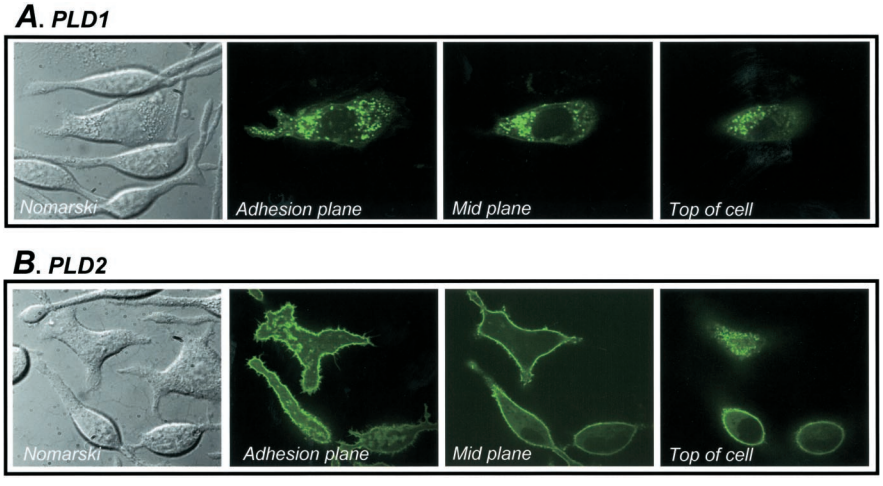

Figure 4. Localisation of PLD1 and PLD2 isoforms in RBL-mast cells following transfection with EGFP-tagged PLDs. PLD1 is localised to the lysosomally derived secretory granules and PLD2 at the plasma membrane. An image of the bright field (Normarski) is shown together with three confocal planes, the adhesion plane, the mid plane and the top of the cell.

The plasma membrane is thought to contain microdomains that are laterally segregated in the plane of the bilayer, whose unique lipid and protein composition may reflect specific functions including sites of signalling activity and regions where exocytic compartments fuse. These microdomains are characterised by their enrichment in phosphoinositides [75–77], a lipid which is required for PLD activity. Not surprisingly, PLD2 and in some cases PLD1 have been found associated with these microdomains (see table 2).

It is clear from table 2 that PLDs can be found at both the plasma membrane and in intracellular compartments including the endoplasmic reticulum, Golgi, endosomes and lysosomes. The availability of antibodies and studies of endogenous enzymes in primary tissues will be essential in confirming the above conclusions concerning lo-

calisation. The danger with transfection studies could be mislocalisation due to over-expression.

Functions of PLD

In the majority of studies discussed below, the initial evidence identifying PLD activity as important in a functional event relied on the use of alcohols. The concentration of alcohol required to give maximal transphosphatidylation has been examined and is 0.5–1.5% for ethanol and 0.2–0.5% for butanol [78, 79]. As a control for non-specific effects of butanol, the secondary and tertiary forms of butanol have often been employed. Furthermore, because water is still present at high enough concentrations, some PA will always be formed in the presence of alcohols [28].

As stated above, neutrophils and mast cells contain secretory granules which are really modified lysosomes. The first hint that PLD activation was necessary for exocytosis in neutrophils and mast cells came from the observation that alcohols blocked exocytosis. Further analysis has confirmed the importance of ARF and PLD in exocytosis in both primary neutrophils and mast cells and in their related cell lines, HL60 cells and RBL-mast cells [64, 70, 73, 79]. A reconstitution system has been employed to examine the requirement for ARF proteins in both PLD activation and exocytosis. In cytosol-depleted cells, both reactions are refractory to stimulation and can be restored upon addition of ARF proteins [64, 70, 79]. ARF-reconstituted secretion is blocked by ethanol, further substantiating the dependence on PA derived from the PLD pathway [79]. Mutational analysis has identified regions of ARF1 responsible for PLD1 activation, and the same regions were required for restoring exocytosis. ARF proteins have other effectors including COP1 and AP1, and ARF mutants have been identified that are selective for different effectors [70, 71].

Studies with chromaffin cells indicate that ARNO (an ARF exchange factor for ARF1 and ARF6), ARF6 and a plasma membrane-localised PLD1 are required for exocytosis. ARF6 was present on secretory granules and was required to activate a plasma membrane-associated PLD [80–82]. Secretion triggered by raising cytosol Ca^{2+} in permeabilised cells was blocked partially with ethanol or butanol. The concentration of butanol used was 1.5%, much higher than that required for maximal 'transphosphatidylation'.

PLD has also been found to play a role in controlling changes in the actin cytoskeleton, and stimulation of actin stress fibre formation [83] and membrane ruffling [72, 73] are both dependent on PLD activity. Membrane ruffling is exquisitely sensitive to 0.5% butanol but not butan-2-ol, and the PLD isoform found in membrane ruffles is PLD2. PLD2 has also been shown to associate with PIP 5-kinase [84], and this enzyme is activated in a co-ordinated manner by PA together with ARF6 [72; O'Luanaigh et al., unpublished data]. Actin stress fibre formation, on the other hand, is inhibited by expressing the catalytically inactive form of PLD1 but not PLD2, indicating specific functions of PLD isoforms in these related processes. Membrane ruffling requires the constant recycling of membranes to endocytic structures, which is ARF6 dependent [85]. PLD2 likely participates in the membrane recycling process.

Yet another membrane trafficking event is the translocation of GLUT4 glucose transporter from an intracellular compartment to the plasma membrane which is stimulated by insulin. PLD1 has been found to co-localise with GLUT4-containing structures and may play some role in exocytosis of these vesicles. However, PLD activation has only been observed in cells which express high levels of insulin receptors, but not in 3T3-Li adipocytes, which are responsive to insulin with respect to GLUT4 translocation to membranes but not with respect to PLD activation [86]. Clearly, further studies are required before a clear picture emerges concerning insulin-stimulated GLUT4 insertion into the plasma membranes.

Aggregation of the high-affinity receptor for IgG, FcγRI, by immune complexes results in the internalisation of these complexes and their trafficking to lysosomes for degradation. Activation of PLD is necessary for the efficient trafficking of the internalised immune complexes to lysosomes using butanol as a suppressor of PA production [87, 88]. Aggregation of the receptor stimulates the association of PLD1 with ARF6 and PKCα. An interesting aspect of this study was the observation that PKC activity required PLD1 activation, as butanol or downregulation of PLD1 using antisense oligonucleotides were inhibitory. This implies that PKC is downstream of PLD [89].

Another trafficking event that requires PLD activity is in the liver. The major function of hepatic very low density lipoproteins (VLDLs) is to deliver triacylglycerol from the liver to peripheral tissues. The assembly of VLDLs containing apolipoprotein B is a complex process that occurs in the lumen of the secretory pathway. The process consists of two relatively well-identified steps. In the first step, two VLDL precursors are formed simultaneously and independently: an apolipoprotein B-containing VLDL precursor (a partially lipidated apolipoprotein B) and a VLDL-sized lipid droplet that lacks apolipoprotein B. These two components are probably made in separate membrane compartments. In the second step, these two precursors fuse to form a mature VLDL particle. The apolipoprotein B-containing VLDL precursor is formed during the translation, and concomitant translocation of the protein to the lumen of the endoplasmic reticulum. The VLDL precursor is completed shortly after the protein is fully synthesized. The process is dependent on the

microsomal triglyceride transfer protein. Although the mechanism by which the lipid droplets are formed is unknown, recent observations indicate that the process is dependent on microsomal triglyceride transfer proteins. The conversion of the apolipoprotein B-containing precursor to VLDL seems to be dependent on ARF1 and its activation of PLD. This step of VLDL assembly is inhibited by butanol as well as brefeldin A, which inhibits the formation of active ARF [90, 91].

PLD1 and ARF have together been implicated in the release of nascent secretory vesicles from the trans golgi network in growth hormone-secreting GH3 cells [92–94]. In these experiments, butanol was used at 1.5% compared to the usual requirement of 0.2–0.5% for maximal transphosphatidylation. This concentration of butanol was found to dismantle the Golgi apparatus. Whether this effect is due to disruption of PA formation has to be questioned.

Many of the functional responses described above where PLD activity has been implicated relate to membrane-trafficking events. Many of the membrane-trafficking events include the lysosomes, endosomes, plasma membranes, endoplasmic reticulum and Golgi, and PLD enzymes have been found in all of these locations (see table 2). The assembly of VLDL takes places at the endoplasmic reticulum and at least two studies have identified the endoplasmic reticulum as a site of PLD activity.

Functions of PA

What is the role of PLD-derived PA? Figure 3 illustrates some current ideas for which some evidence has been presented. The further metabolism of PA to LPA and DG is the least favoured explanation and very little data exist to support these possibilities. The two most favoured ideas are the potential of PA to function as a membrane-recruiting device for transiently targeting specific proteins to membranes. Some evidence to support this notion has been forthcoming. The first PA-binding protein was Raf-1 kinase which is translocated to membranes under conditions where PLD2 is activated [95, 96]. PA was not found to activate Raf-1 kinase but only acted as a mechanism for protein recruitment. A more recent study has purified PA-binding proteins from rat brain cytosol and identified 15 known and 5 novel proteins. These include proteins involved in intracellular traffic, ARF, coatomer complex, kinesin and N-ethylmaleimide-sensitive fusion protein, and also neurochondrin (thought to be involved in somatodendritic functions in neuronal cells), puromycin-sensitive aminopeptidase and Raf-1 [97]. These data are in accord with previous studies which suggested that PA mediated the recruitment of the coatomer complex during the formation of transport vesicles [98, 99]. However, the importance of PA in the budding of COP1-coated vesicles has been questioned by more recent analysis [69–71, 100, 101]. Major arguments against PA being essential in coatomer recruitment are the observation that recruitment of coatomer to Golgi membranes is not inhibited by ethanol up to 6% and, moreover, coatomer can directly interact with ARF to mediate its recruitment.

Finally, we have proposed that PA is a regulator of PIP_2 synthesis [2, 64]. This was based on the observation that PA could stimulate the activity of type I PIP 5-kinase [102]. Recent studies with this enzyme indicate that not only PA but also ARF can directly regulate the activity of this enzyme in vitro. Depending on the composition of the vesicles used, PA or ARF can independently regulate PIP 5-kinase activity. Under specific in vitro conditions, ARF can only stimulate PIP 5-kinase activity provided that PA is present as a co-activator [67, 72]. The possibility that PIP 5-kinase activity can be increased by ARF alone complicates the interpretation of some of the older studies where ARF was shown to increase PIP_2 synthesis in permeabilised cells [64]. This could have been either a direct effect of ARF on PIP 5-kinase or it could have been indirectly via PA-derived PLD. Finally, within different cellular compartments, different mechanisms could operate. Recent analysis indicates that at the Golgi membranes prepared from rat liver, PIP_2 synthesis is mediated by ARF1 via a direct activation of PI 4-kinaseβ and a type I PIP 5-kinase [67, 103]. A requirement for PLD was excluded by the demonstration that PIP_2 synthesis could not be blocked by 0.5% butanol, a concentration that gives maximal transphosphatidylation. In contrast, Golgi membranes prepared from GH3 cells were shown to make a modest amount of PIP_2 upon addition of cytosol, with no requirement for ARF. This was blocked by the addition of butanol, however [94]. The main difference between the two studies was the amount of butanol used: 0.5% in the studies with rat liver Golgi compared to 1.5% in the study with GH3 cells derived Golgi membranes. In a different study, which examined purified lysosomes, PIP_2 synthesis was also found to be dependent on PLD activity and was shown to be responsible for the initiation of clathrin coat assembly [68]. But again, inhibition was observed with 1.5% butanol although PLD activation was monitored at 0.5% butanol.

Why much higher concentrations of alcohol are required to inhibit PIP_2 synthesis compared to maximally divert PA to phosphatidylalcohol is not clear. In a recent study, we compared the concentration of alcohol required to inhibit receptor-mediated activation of PLD with GTPγS-mediated activation of PLD and found near maximal production of phosphatidylethanol with 1% ethanol [79]. Higher concentrations of alcohol did not produce a further increase in phosphatidylethanol. The reason for comparing a receptor-activated system versus stimulation with GTPγS is due to the nature of the degree of stimula-

tion. A much higher level of PLD activation is observed with GTPγS compared to an agonist. Since transphosphatidylation is always incomplete and is also accompanied with some PA production [104], with GTPγS as a stimulus, a higher level of PA will be formed compared to an agonist. We have noted that functional responses, e.g. secretion, can be blocked more completely with alcohols when stimulated with a receptor compared to the stimulation with GTPγS. This is precisely what one would expect. Enough PA would still be made in the presence of an alcohol when GTPγS is used as an activator. This does not entirely explain the discrepancies noted in the literature, however, since GTPγS was not used for activation in either of the studies discussed here [68, 94]. Specific inhibitors of PLD are urgently required to resolve these discrepancies.

Conclusions

The demonstration that mammalian cells have PLD activity was first reported just over 20 years ago and shortly thereafter, PLD activation was identified as a receptor-activated step. We have travelled a long way since then and have now identified many of the key players. There are two bona fide PC-hydrolysing PLD enzymes in the human genome, both of which are regulated in a complex manner by small GTPases, PIP_2 and PKC. The product of PLD activity is PA and the most favoured scenario is that PA has two major functions in cells: one is to recruit proteins and the second is to regulate the activity of enzymes including protein and lipid kinases. The best data are available for activation of a lipid-metabolising enzyme, PIP 5-kinase to make PIP_2. Here we come full circle as it is clear that PIP_2 itself can regulate the activity of PLD. This brings to mind a previous conundrum that existed in the PLC field. All PLCs are dependent on Ca^{2+} for activity and increasing levels of Ca^{2+} enhance PLC activity. Nonetheless, the product of PLC activity is IP_3, whose downstream function is to mobilise Ca^{2+}. Thus for PLD to be active in the cell, the basal pool of PIP_2 would suffice. The activation would lead to a local increase in PIP_2 which could enhance the activation of PLD further. PIP_2 together with PA may be responsible for governing the specific reversible recruitment of proteins to execute many of the trafficking events discussed here and for modulation of the actin cytoskeleton. It is interesting to note that PIP_2 in the presence of PA is effective at regulating the activity of ARF-GAPs which would terminate ARF activity.

The critical factor will be the location of the PLD enzymes, as these areas will be sites of PA and PIP_2 production, and here much progress has been made concerning the location of the enzymes, using tagged proteins in transfected cells. Co-localisation of PLDs with PIP 5-kinases will provide further evidence for a relationship between these two enzymes. Evidence to that effect has been presented [72, 84]. This review has focused on PLD1 and PLD2. However, the possibility that Hu-K4 also has PLD activity remains an open question and this possibility cannot be disregarded.

Acknowledgements. I thank the Wellcome Trust for their financial support for the research conducted in my laboratory. The ongoing research has provided many of the concepts and ideas discussed here. I thank Michelle Li for making the illustrations. The images shown in figure 4 are the work of Naimh O'Luanaigh, a Ph.D. student in my laboratory.

1 Cockcroft S. (1996) ARF-regulated phospholipase D: a potential role in membrane traffic. Chem. Phys. Lipids **80:** 59–80
2 Cockcroft S. (1997) Phospholipase D: regulation by GTPases and protein kinase C and physiological relevance. Prog. Lipid Res. **35:** 345–370
3 Exton J. H. (1997) New developments in phospholipase D. J. Biol. Chem. **272:** 15579–15582
4 Exton J. H. (1997) Phospholipase D: enzymology, mechanisms of regulation, and function. Physiol. Rev. **77:** 303–320
5 Liscovitch M., Czarny M., Fiucci G. and Tang X. (2000) Phospholipase D: molecular and cell biology of a novel gene family. Biochem. J. **345:** 401–415
6 Morris A. J., Engebrecht J. and Frohman M. A. (1996) Structure and regulation of phospholipase D. Trends Pharmacol. Sci. **17:** 182–185
7 Frohman M. A., Morris A. J. (1999) Phospholipase D structure and regulation. Chem. Phys. Lipids **98:** 127–140
8 Wakelam M. J., Hodgkin M. N., Martin A. and Saqib K. (1997) Phospholipase D. Semin. Cell Dev. Biol. **8:** 305–310
9 Daniel L. W. ed. (1999) Phospholipase D. Biochim. Biophys. Acta **1439:** 119–299
10 Wang X., Xu L. and Zheng L. (1994) Cloning and expression of phosphatidylcholine-hydrolyzing phospholipase D from *Ricinus communis* L. J. Biol. Chem. **269:** 20312–20317
11 Rose K., Rudge S. A., Frohman M. A., Morris A. J. and Engebrecht J. (1995) Phospholipase D signaling is essential for meiosis. Proc. Natl. Acad. Sci. USA **92:** 12151–12155
12 Hammond S. M., Altshuller Y. M., Sung T.-C., Rudge S. A., Rose K., Engebrecht J. et al. (1995) Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. J. Biol. Chem. **270:** 29640–29643
13 Hammond S. M., Jenco J. M., Nakashima S., Cadwallader K., Gu Q.-M., Cook S. et al. (1997) Characterization of two alternatively spliced forms of phospholipase D1: activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-α. J. Biol. Chem. **272:** 3860–3868
14 Colley W. C., Sung T.-C., Roll R., Jenco J., Hammond S. M., Altshuller Y. et al. (1997) Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Curr. Biol. **7:** 191–201
15 Okamura S. and Yamashita S. (1994) Purification and characterisation of phosphatidylcholine phospholipase D from pig lung. J. Biol. Chem. **269:** 31207–31213
16 Ponting C. P. and Kerr I. D. (1996) A novel family of phospholipase D homologues that includes phospholipid synthases and putative endonucleases: identification of duplicated repeats and potential active site residues. Protein Sci. **5:** 914–922

17 Hinnebusch J., Cherepanov P., Du Y., Rudolph A., Dixon J. D., Schwan T. et al. (2000) Murine toxin of *Yersinia pestis* shows phospholipase D activity but is not required for virulence in mice. Int. J. Med. Microbiol. **290:** 483–487
18 Pederson K. M., Finsen B., Celis J. E. and Jensen N. A. (1998) Expression of a novel murine phospholipase D homolog coincides with late neuronal development in the forebrain. J. Biol. Chem. **273:** 31494–31504
19 Sung T. C., Roper R. L., Zhang Y., Rudge S. A., Temel R., Hammond S. M. et al. (1997) Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. EMBO J. **16:** 4519–4530
20 Schultz J., Copley R. R., Doerks T., Ponting C. P. and Bork P. (2000) SMART: a web-based tool for the study of genetically mobile domains. Nucleic Acids Res. **28:** 231–234
21 Wishart M. J., Taylor G. S. and Dixon J. E. (2001) Phoxylipids. revealing PX domains as phosphoinositide binding modules. Cell **105:** 817–820
22 Park S.-K., Provost J., Bae C. D., Ho W.-T. and Exton J. (1997) Cloning and characterization of phospholipase D from rat brain. J. Biol. Chem. **272:** 29263–2921
23 Gottlin E. B., Rudolph A. E., Zhao Y., Matthews H. R. and Dixon J. E. (1998) Catalytic mechanism of the phospholipase D superfamily proceeds via a covalent phosphohistidine intermediate. Proc. Natl. Acad. Sci. USA **95:** 9202–9207
24 Rudolph A. E., Stuckey J. A., Zhao Y., Matthews H. R., Patton W. A., Moss J. et al. (1999) Expression, characterization, and mutagenesis of the *Yersinia pestis* murine toxin, a phospholipase D superfamily member. J. Biol. Chem. **274:** 11824–11831
25 Stuckey J. A. and Dixon J. E. (1999) Crystal structure of a phospholipase D family member. Nat. Struct. Biol **6:** 278–284
26 Leiros I., Secundo F., Zambonelli C., Servi S. and Hough E. (2000) The first crystal structure of a phospholipase D. Structure Fold. Des. **8:** 655–667
27 Xie Z., Ho W. T. and Exton J. H. (2000) Association of the N- and C-terminal domains of phospholipase D. J. Biol. Chem. **275:** 24962–24969
28 Ella K. M., Meier K. E., Kumar A., Zhang Y. and Meier G. P. (1997) Utilization of alcohols by plant and mammalian phospholipase D. Biochem. Mol. Biol. Int. **41:** 715–724
29 Devlin M. A., Das S., Singh I., Bourgoin S., Brindley D. N. and Ginsberg J. (2000) The characterization of phospholipase D in FRTL-5 thyroid cells. Mol. Cell. Endocrinol. **167:** 107–115
30 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
31 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
32 Saito S., Sakagami H. and Kondo H. (2000) Localization of mRNAs for phospholiapse D (PLD) type 1 and 2 in the brain of developing and mature rat. Dev. Brain Res. **120:** 41–47
33 Lee M. Y., Kim S. Y., Min D. S., Choi Y. S., Shin S. L., Chun M. H. et al. (2000) Upregulation of phospholipase D in astrocytes in response to transient forebrain ischemia. Glia **30:** 311–317
34 Griner R. D., Qin F., Jung E., Sue-Ling C. K., Crawford K. B., Mann-Blakeney R. et al. (1999) 1,25-dihydroxyvitamin D3 induces phospholipase D-1 expression in primary mouse epidermal keratinocytes. J. Biol. Chem. **274:** 4663–4670
35 Ohguchi K., Nakashima S. and Nozawa Y. (1999) Phospholipase D development during differentiation of human promyelocytic leukemic HL60 cells. Biochim. Biophys. Acta **1439:** 215–227
36 El Marjou M., Montalescot V., Buzyn A. and Geny B. (2000) Modifications in phospholipase D activity and isoform expression occur upon maturation and differentiation in vivo and in vitro in human myeloid cells. Leukemia **14:** 2118–2127
37 Uchida N., Okamura S. and Kuwano H. (1999) Phospholipase D activity in human gastric carcinoma. Anticancer Res. **19:** 671–675
38 Uchida N., Okamura S., Nagamachi Y. and Yamashita S. (1997) Increased phospholipase D activity in human breast cancer. J Cancer Res. Clin. Oncol. **123:** 280–285
39 Zhao Y., Ehara H., Akao Y., Shamoto M., Nakagawa Y., Banno Y. et al. (2000) Increased activity and intranuclear expression of phospholipase D2 in human renal cancer. Biochem. Biophys. Res. Commun. **278:** 140–143
40 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
41 Noh D. Y., Ahn S. J., Lee R. A., Park I. A., Kim J. H., Suh P. G. et al. (2000) Overexpression of phospholipase D1 in human breast cancer tissues. Cancer Lett. **161:** 207–214
42 Brown H. A., Gutowski S., Moomaw C. R., Slaughter C. and Sternweis P. C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell **75:** 1137–1144
43 Cockcroft S., Thomas G. M. H., Fensome A., Geny B., Cunningham E., Gout I. et al. (1994) Phospholipase D: a downstream effector of ARF in granulocytes. Science **263:** 523–526
44 Sung T.-C., Zhang Y., Morris A. J. and Frohman M. A. (1999) Structural analysis of human phospholipase D1. J. Biol. Chem. **274:** 3659–3666
45 Yamazaki M., Zhang Y., Watanabe H., Yokozeki T., Ohno S., Kaibuchi K. et al. (1999) Interaction of the small G protein RhoA with the C terminus of human phospholipase D1. J. Biol. Chem. **274:** 6035–6038
46 Cai S. and Exton J. H. (2001) Determination of interaction sites of phospholipase D1 for RhoA. Biochem. J. **355:** 779–785
47 Du G., Altshuller Y. M., Kim Y., Han J. M., Ryu S. H., Morris A. J. et al. (2000) Dual requirement for rho and protein kinase C in direct activation of phospholipase D1 through G protein-coupled receptor signaling. Mol. Biol. Cell **11:** 4359–4368
48 Zhang.Y, Altshuller Y. A., Hammond S. M., Hayes F. and Morris A. J. (1999) Loss of receptor regulation by a phospholipase D1 mutant unresponsive to protein kinase C. EMBO J. **18:** 6339–6348
49 Kim Y., Han J. M., Park J. B., Lee S. D., Oh Y. S., Chung C. et al. (1999) Phosphorylation and activation of phospholipase D1 by protein kinase C in vivo: determination of multiple phosphorylation sites. Biochemistry **38:** 10344–10351
50 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
51 Sung T.-C., Altshuller Y. M., Morris A. J. and Frohman M. A. (1999) Molecular analysis of mammalian phospholipase D2. J. Biol. Chem. **274:** 494–502
52 Siddiqi A. R., Srajer 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
53 Slaaby R., Du G., Altshuller Y. M., Frohman M. A. and Seedorf K. (2000) Insulin-induced phospholipase D1 and phospholipase D2 activity in human embryonic kidney-293 cells mediated by the phospholipase C gamma and protein kinase C alpha signalling cascade. Biochem. J **351:** 613–619
54 Kim J. H., Kim Y., Lee S. D., Lopez I., Arnold R. S., Lambeth J. D. et al. (1999) Selective activation of phospholipase D2 by unsaturated fatty acid. FEBS Lett. **454:** 42–46
55 Lee S. Y., Yeo E. J. and Choi M. U. (1998) Phospholipase D activity in L1210 cells: a model for oleate-activated phospholipase D in intact mammalian cells. Biochem. Biophys. Res. Commun. **244:** 825–831

56 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

57 Park J. B., Kim J. H., Kim Y., Ha S. H., Kim J. H., Yoo J. S. et al. (2000) Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by α-actinin in an ADP-ribosylation factor-reersible manner. J. Biol. Chem. **275:** 21295–21301

58 Hodgkin M. N., Masson M. R., Powner D., Saqib K. M., Ponting C. P. and Wakelam M. J. (2000) Phospholipase D regulation and localisation is dependent upon a phosphatidylinositol 4,5-biphosphate-specific PH domain. Curr. Biol. **10:** 43–46

59 Xie Z., Ho W. T. and Exton J. H. (2001) Requirements and effects of palmitoylation of rat pld1. J. Biol. Chem. **276:** 9383–9891

60 Sugars J. M., Cellek S., Manifava M., Coadwell J. and Ktistakis N. T. (1999) Fatty acylation of phospholipase D1 on cysteine residues 240 and 241 determines localization on intracellular membranes. J. Biol. Chem. **274:** 30023–30027

61 Sciorra V. A., Rudge S. A., Prestwich G. D., Frohman M. A., Engebrecht J. and Morris A. J. (1999) Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. EMBO J. **20:** 5911–5921

62 Borregaard N., Lollike K., Kjeldsen L., Sengelov H., Bastholm L., Nielsen M. H. et al. (1993) Human neutrophil granules and secretory vesicles. Eur. J. Haematol. **51:** 187–198

63 Morgan C. P., Sengelov H., Whatmore J., Borregaard N. and Cockcroft S. (1997) ARF-regulated phospholipase D activity localizes to secretory vesicles and mobilizes to the plasma membrane following fMetLeuPhe stimulation of human neutrophils. Biochem. J. **325:** 581–585

64 Fensome A., Cunningham E., Prosser S., Tan S. K., Swigart P., Thomas G. et al. (1998) ARF and PITP restore GTPγS-stimulated protein secretion from cytosol-depleted HL60 cells by promoting PIP_2 synthesis. J. Biol. Chem. **6:** 730–738

65 Whatmore J., Morgan C. P., Cunningham E., Collison K. S., Willison K. R. and Cockcroft S. (1996) ADP-ribosylation factor1-regulated phospholipase D is localized at the plasma membrane and intracellular organelles in HL60 cells. Biochem. J. **320:** 785–794

66 Ktistakis N. T., Brown A., Sternweis P. C. and Roth M. G. (1995) Phospholipase D is present on golgi-enriched membranes and its activation by ADP ribosylation factor is sensitive to brefeldin A. Proc. Natl. Acad. Sci. USA. **92:** 4952–4956

67 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 $PI(4,5)P_2$ synthesis in the Golgi compartment. J. Biol. Chem. **275:** 13962–13966

68 Arneson L., Kunz J., Anderson R. A. and Traub L. M. (1999) Coupled inositide phosphorylation and phospholipase D activation initiated clathrin-coat assembly on lysosomes. J. Biol. Chem. **274:** 17794–17805

69 Jones D. H., Morgan C. and Cockcroft S. (1999) Phospholipase D and membrane traffic: potential roles in regulated exocytosis, membrane delivery and vesicle budding. Biochim. Biophys. Acta **1439:** 229–244

70 Jones D. H., Bax B., Fensome A. and Cockcroft S. (1999) ADP ribosylation factor 1 mutants identify a phospholipase D effector region and reveal that phospholipase D participates in lysosomal secretion but is not sufficient for recruitment of coatomer I. Biochem. J. **341:** 185–192

71 Kuai J., Boman A. L., Arnold R. S., Zhu X. and Kahn R. A. (2000) Effects of activated ADP-ribosylation factors on Golgi morphology require neither activation of phospholipase D nor recruitment of coatomer. J Biol. Chem. **275:** 4022–4032

72 Honda A., Nogami M., Yokozeki T., Yamazaki M., Nakamura H., Watanabe H. et al. (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

73 Brown F. D., Thompson N., Saqib K. M., Clark J. M., Powner D., Thompson N. T. et al. (1998) Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane located on cellular stimulation. Curr. Biol. **8:** 835–838

74 Toda K., Nogami M., Murakami K., Kanaho Y. and Nakyama K. (1999) Colocalization of phospholipase D1 and GTP-binding defective mutant of ADP-ribosylation factor 6 to endosomes and lysosomes. FEBS Lett. **442:** 221–225

75 Pike L. J. and Casey L. (1996) Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains. J. Biol. Chem. **271:** 26453–26456

76 Hope H. R. and Pike L. J. (1996) Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains. Mol. Biol. Cell **7:** 843–851

77 Pike L. J.and Millerand J.M. (1998) Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. J. Biol. Chem. **273:** 22298–22304

78 Laine J., Bourgoin S., Bourassa J. and Morisset J. (2000) Subcellular distribution and characterization of rat pancreatic phospholipase D isoforms. Pancreas **20:** 323–336

79 Way G., O'Luanaigh N. and Cockcroft S. (2000) Activation of exocytosis by cross-linking of the IgE receptor is dependent on ARF-regulated phospholipase D in RBL-2H3 mast cells: evidence that the mechanism of activation is via regulation of PIP_2 synthesis. Biochem. J. **346:** 63–70

80 Galas M. C., Helms J. B., Vitale N., Thierse D., Aunis D. and Bader M.-F. (1997) Regulated exocytosis in chromaffin cells: a potential role for a secretory granule-associated ARF6 protein. J. Biol. Chem. **272:** 2788–2793

81 Caumont A. S., Vitale N., Gensse M., Galas M. C., Casanova J. E. and Bader M. F. (2000) Identification of a plasma membrane-associated guanine nucleotide exchange factor for ARF6 in chromaffin cells: possible role in the regulated exocytotic pathway. J. Biol. Chem. **275:** 15637–15644

82 Caumont A. S., Galas M. C., Vitale N., Aunis D. and Bader M. F. (1998) Regulated exocytosis in chromaffin cells: translocation of ARF6 stimulates a plasma membrane-associated phospholipase D. J. Biol. Chem. **273:** 1373–1379

83 Cross M. J., Roberts S., Ridley A. J., Hodgkin M. N., Stewart A., Claesson-Welsh L. et al. (1996) Stimulation of actin stress fibre formation mediated by activation of phospholipase D. Curr. Biol. **6:** 588–597

84 Divecha N., Roefs M., Halstead J. R., D'Andrea S., Fernandez-Borga M., Oomen L. et al. (2000) Interaction of the type Iα 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

85 Radhakrishna H., Al-Awar O., Khachikian Z. and Donaldson J. G. (1999) ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. J. Cell Sci. **112:** 855–866

86 Emoto M., Klarlund J. K., Waters S. B., Hu V., Buxton J. M., Chawla A. et al. (2000) A role for phospholipase D in GLUT4 glucose transporter translocation. J. Biol. Chem. **275:** 7144–7151

87 Melendez A., Floto R. A., Gillooly D. J., Harnett M. M. and Allen J. M. (1998) FcγRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. J. Biol. Chem. **273:** 9393–9402

88 Gillooly D. J., Melendez A. J., Hockaday A. R., Harnett M. M. and Allen J. M. (1999) Endocytosis and vesicular trafficking of immune complexes and activation of phospholipase D by the human high-affinity IgG receptor requires distinct phosphoinositide 3-kinase activities. Biochem. J **344:** 605–611

89 Melendez A., Harnett M. M. and Allen J. M. (2001) Crosstalk between ARF6 and protein kinase C alpha in FcγRI mediated activation of phospholipase D1. Curr. Biol. **11**: 869–874

90 Asp L., Claesson C., Boren J. and Olofsson S. O. (2000) ADP-ribosylation factor 1 and its activation of phospholipase D are important for the assembly of very low density lipoproteins. J. Biol. Chem. **275:** 26285–26292

91 Olofsson S. O., Asp L. and Boren J. (1999) The assembly and secretion of apolipoprotein B-containing lipoproteins. Curr. Opin. Lipidol. **10:** 341–346

92 Chen Y.-G. and Shields D. (1996) ADP-ribosylation factor-1 stimulates formation of nascent secretory vesicles from the *trans*-Golgi network of endocrine cells. J. Biol. Chem. **271:** 5297–5300

93 Chen Y. G., Siddhanta A., Austin C. D., Hammond S. M., Sung T. C., Frohman M. A. et al. (1997) Phospholipase D stimulates release of nascent secretory vesicles from the trans-Golgi network. J. Cell Biol. **138:** 495–504

94 Siddhanta A., Backer J. M. and Shields D. (2000) Inhibition of phosphatidic synthesis alters the structure of the Golgi apparatus and inhibits secretion in endocrine cells. J. Biol. Chem. **275:** 12023–12031

95 Ghosh S., Strum J. C., Sciorra V. A., Daniel L. and Bell R. M. (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. J. Biol. Chem. **271:** 8472–8480

96 Rizzo M. A., Shome K., Vasudevan C., Stolz D. B., Sung T.-C., Frohman M. A. et al. (1999) Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. J. Biol. Chem. **274:** 1131–1139

97 Manifava M., Thuring J. W. J. F., Lim Z.-Y., Packman L., Holmes A. B. and Ktistakis N. T. (2001) Differential binding of traffic-related proteins to phosphatidic acid- or phosphatidylinositol(4,5)-bisphosphate-coupled affinity reagents. J. Biol. Chem. **276:** 8987–8994

98 Ktistakis N. T., Brown H. A., Waters M. G., Sternweis P. C. and Roth M. G. (1996) Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. J. Cell Biol. **134:** 295–306

99 Kun B., Roth M. G. and Ktistakis N. T. (1997) Phosphatidic acid formation by phospholipase D is required for transport from the endoplasmic reticulum to the golgi complex. Curr. Biol. **7:** 301–307

100 Jones D., Bax B. and Cockcroft S. (1999) ADP-ribosylation factor GTPases in signal transduction and membrane traffic: independent functions? Biochem. Soc. Trans. **27:** 642–647

101 Stamnes M., Schiavo G., Stenbeck G., Sollner T. H. and Rothman J. E. (1998) ADP-ribosylating factor and phosphatidic acid levels in Golgi membranes during budding of coatomer-coated vesicles. Proc. Natl. Acad. Sci. USA **95:** 13676–13680

102 Jenkins G. H., Fisette P. L. and Anderson R. A. (1994) Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. J. Biol. Chem. **269:** 11547–11554

103 Godi A., Pertile P., Di Tullio G., Iurisco C., Luini A., Corda D. et al. (1999) ARF recruits PI 4-kinase type β and a PIP 5-kinase and stimulates PIP_2 synthesis at the Golgi. Nat. Cell Biol. **1:** 280–287

104 Geny B. and Cockcroft S. (1992) Synergistic activation of phospholipase D by a protein kinase C- and a G-protein-mediated pathway in streptolysin O-permeabilized HL60 cells. Biochem. J. **284:** 531–538

105 Fallman M., Gullberg M., Hellberg C. and Andersson T. (1992) Complement receptor-mediated phagocytosis is associated with accumulation of phosphatidylcholine-derived diglyceride in human neutrophils: involvement of phospholipase D and direct evidence for a positive feedback signal of protein kinase C. J. Biol. Chem. **267:** 2656–2663

106 Fensome A., Whatmore J., Morgan C. P., Jones D. and Cockcroft S. (1996) ADP-ribosylation factor and Rho proteins mediate fMLP-dependent activation of phospholipase D in human neutrophils. Current Biology **273:** 13157–13164

107 Marcil J., Harbour D., Houle M. G., Naccache P. H. and Bourgoin S. (1999) Monosodium urate-crystal-stimulated phospholipase D in human neutrophils. Biochem. J. **337:** 185–192

108 Meacci E., Vasta V., Moorman J. P., Bobak D. A., Bruni P., Moss J. et al. (1999) Effect of Rho and ADP-ribosylation factor GTPases on phospholipase D activity in intact human adenocarcinoma A549 cells. J. Biol. Chem. **274:** 18605–18612

109 Shome K., Nie Y. and Romero G. (1998) ADP-ribosylation factor proteins mediate agonist-induced activation of phospholipase D. J. Biol. Chem. **273:** 30836–30841

110 Shome K., Rizzo M. A., Vasudevan C., Andresen B. and Romero G. (2000) The activation of phospholipase D by endothelin-1, angiotensin II, and platelet-derived growth factor in vascular smooth muscle A10 cells is mediated by small G proteins of the ADP-ribosylation factor family. Endocrinology **141:** 2200–2208

111 Lee S. D., Lee B. D., Kim Y., Suh P. G. and Ryu S. H. (2000) Bradykinin activates phospholipase D2 via protein kinase C-delta in PC12 cells. Neurosci. Lett. **294:** 130–132

112 Rumenapp U., Geiszt M., Wahn F., Schmidt M. and Jakobs K. H. (1995) Evidence for ADP-ribosylation factor-mediated activation of phospholipase D by M3 muscarinic acetylcholine receptor. Eur. J. Biochem. **234:** 240–244

113 Schurmann A., Schmidt M., Asmus M., Bayer S., Fliegert F., Koling S. et al. (1999) The ADP-ribosylation factor (ARF)-related GTPase ARF-related protein binds to the ARF-specific guanine nucleotide exchange factor cytohesin and inhibits the ARF-dependent activation of phospholipase D. J. Biol. Chem. **274:** 9744–9751

114 Rumenapp U., Asmus M., Schablowski H., Woznicki M., Han L., Jakobs K. H. et al. (2001) The M_3 muscarinic acetylcholine receptor expressed in HEK-293 cells signals to phospholipase D via G_{12} but not G_q type G proteins: RGS proteins as tools to dissect pertussis toxin-resistant G proteins in receptor-effector-coupling. J. Biol. Chem. **276:** 2474–2479

115 Hitomi T., Yanagi S., Inatome R. and Yamamura H. (1999) Cross-linking of the B cell receptor induces activation of phospholipase D through Syk, Btk and phospholipase C-γ2. FEBS Lett. **445:** 371–374

116 Hess J. A., Ji Q. S., Carpenter G. and Exton J. H. (1998) Analysis of platelet-derived growth factor-induced phospholipase D activation in mouse embryo fibroblasts lacking phospholipase C-γ1. J. Biol. Chem. **273:** 20517–20524

117 Kotter K., Jin S., Eichel-Streiber C., Park J. B., Ryu S. H. and Klein J. (2000) Activation of astroglial phospholipase D activity by phorbol ester involves ARF and Rho proteins. Biochim. Biophys. Acta **1485:** 153–162

118 Kotter K. and Klein J. (1999) Adrenergic modulation of astroglial phospholipase D activity and cell proliferation. Brain Res. **830:** 138–145

119 Gelas P., Ribbes G., Record M., Terce F. and Chap H. (1989) Differential activation by fMet-Leu-Phe and phorbol ester of a plasma membrane phosphatidylcholine-specific phospholipase D in human neutrophil. FEBS Lett. **251:** 213–218

120 Edwards Y. S. and Murray A. W. (1995) Accumulation of phosphatidylalcohol in cultured cells: use of subcellular fractionation to investigate phospholipase D activity during signal transduction. Biochem. J. **308:** 473–480

121 Decker C., Miro Obradors M. J., Sillence D. J. and Allan D. (1996) Phorbol ester-sensitive phospholipase D is mainly localized in the endoplasmic reticulum of BHK cells. Biochem. J. **320:** 885–890

122 Brown F. D., Thompson N., Saqib K. M., Clark J. M., Powner D., Thompson N. T. et al. (1998) Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. Curr. Biol. **8:** 835–838
123 Kim Y., Kim J. E., Lee S. D., Lee T. G., Kim J. H., Park J. B. et al. (1999) Phospholipase D1 is located and activated by protein kinase C alpha in the plasma membrane in 3Y1 fibroblast cell. Biochim. Biophys. Acta **1436:** 319–330
124 Kim J. H., Han J. M., Lee S., Kim Y., Lee T. G., Park J. B. et al. (1999) Phospholipase D in caveolae: regulation by protein kinase Cα and caveolin-1. Biochemistry **38:** 3763–3769
125 Iyer S. S. and Kusner D. J. (1999) Association of phospholipase D activity with the detergent-insoluble cytoskeleton of U937 promonocytic leukocytes. J. Biol. Chem. **274:** 2350–2359
126 Hodgkin M. N., Clark J. M., Rose S., Saqib K. and Wakelam M. J. (1999) Characterization of the regulation of phospholipase D activity in the detergent-insoluble fraction of HL60 cells by protein kinase C and small G-proteins. Biochem. J. **339:** 87–93
127 Czarny M., Fiucci G., Lavie Y., Banno Y., Nozawa Y. and Liscovitch M. (2000) Phospholipase D2: functional interaction with caveolin in low-density membrane microdomains. FEBS Lett. **467:** 326–332
128 Czarny M., Lavie Y., Fiucci G. and Liscovitch M. (1999) Localization of phospholipase D in detergent-insoluble, caveolin-rich membrane domains: modulation by caveolin-1 expression and caveolin-182-101. J. Biol. Chem. **274:** 2717–2724