1. Introduction

1.1 Phospholipids in cellular signalling

Signalling through lipid metabolites has gained a lot of attention since the finding of MABEL R. and LOWELL E. HOKIN (1953) that [^{32}P]-orthophosphate is incorporated into phosphatidylinositol upon acetylcholine stimulation. This reaction step is now recognised as part of the phosphoinositide cascade. Its activation results in the release of D-*meso*-inositol-1,4,5-trisphosphate and *sn*-1,2-diacylglycerol (DAG) from phosphatidyl-inositol-4,5-bisphosphate (PIP_2) (BERRIDGE and IRVINE, 1984). These second messengers are products of a phospholipase C (PLC), other second messengers are generated by phospholipase A_2 (PLA_2) and phospholipase D (PLD) (Fig. 1).

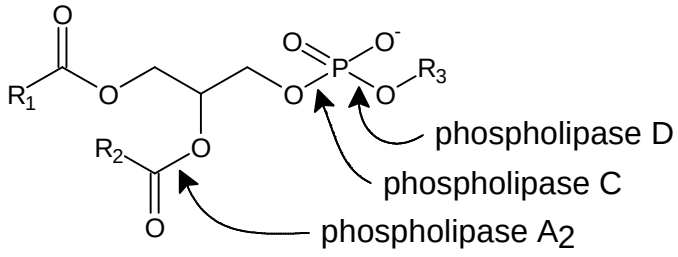

Figure 1.
Mechanism of action of phospholipases involved in signalling on phospholipids. R_1 is mostly a saturated, R_2 an unsaturated carbon chain and R_3 is a polar head group like choline, ethanolamine or inositol.

Activation of the phosphoinositide cascade occurs through many cell surface receptors and has been characterised in great detail. Its second messenger D-*meso*-inositol-1,4,5-trisphosphate releases Ca^{2+} from intracellular stores whereas *sn*-1,2-diacylglycerol activates protein kinase C (PKC). At present, three PIP_2-specific phospholipase C families are characterised, referred to as phospholipase Cβ, phospholipase Cγ and phospholipase Cδ (REBECCHI and PENTYALA, 2000). Each of these families comprises several members and is used in different signal transduction pathways. Receptors coupled to heterotrimeric G-proteins activate phospholipase Cβ through the latter while activation of phospholipase Cγ is mediated via tyrosine kinase receptors. As regulators of phospholipase Cδ the atypical G-protein G_h and the GTPase-activating protein p122 RhoGAP have been described.

Another phospholipase C, sphingomyelinase converts sphingomyelin to ceramide. Ceramide is the sphingolipid analogue of *sn*-1,2-diacylglycerol but provokes an apoptotic cellular response in contrast to the mitogenic action of *sn*-1,2-diacylglycerol.

Sphingomyelinases are activated, for example through receptors of the tumour necrosis factor receptor family. This activation occurs via protein-protein interactions at the receptor and involves adaptor proteins (ADAM-KLAGES *et al.*, 1998). The regeneration of sphingomyelin is coupled with the generation of *sn*-1,2-diacylglycerol from phosphatidylcholine (PC) via the phosphatidylcholine:ceramide phosphocholine transferase (LUBERTO and HANNUN, 1998). Therefore, this activity might represent a phosphatidylcholine-specific phospholipase C involved in signal transduction.

Phospholipase A_2 is specific for the *sn*-2 position of phosphatidylcholine, phosphatidylethanolamine (PE) and phosphatidic acid (PA) to yield the corresponding *lyso*-phospholipid and fatty acids. Interestingly, the major fatty acid produced is arachidonic acid which is a precursor for eicosanoids (EXTON, 1994). Both, extracellular and intracellular forms of phospholipase A_2 exist of which only the latter is believed to be involved in cell signalling. Cytosolic phospholipase A_2 is regulated by Ca^{2+} and by phosphorylation through mitogen activated protein kinases (LESLIE, 1997).

Several phospholipase D isoforms with various substrate specificities have been identified recently. Phosphatidylcholine-specific phospholipase D (EC 3.1.4.4) has gained a lot of interest because its activity is under the control of proteins involved in important cellular processes like mitogenesis, cytoskeletal organisation and vesicle trafficking (LISCOVITCH *et al.*, 2000). How phospholipase D activity is regulated and which role it serves in physiological processes is still not completely resolved. Nevertheless, its importance in cellular function is underlined by the fact that yeast harbouring a mutant phospholipase D1 are defective in the completion of the meiotic division and subsequent gamete differentiation (HONIGBERG *et al.*, 1992). The hydrolysis of phosphatidylcholine results in generation of choline and phosphatidic acid. The latter is believed to be an effector in several physiological processes, including secretion and cell proliferation. An important feature of phosphatidylcholine-specific phospholipase D is the transphosphatidylation reaction. In the presence of a primary alcohol, this reaction yields phosphatidylalcohol (YANG *et al.*, 1967). This non-physiologic phospholipid can be used to specifically measure phospholipase D activity. Phosphatidylcholine-specific phospholipase D activity was initially discovered in plants but is also present in bacteria, yeast and mammalian cells. Three phosphatidylcholine-specific phospholipase D activities have been described for mammalian cells.

SAITO and KANFER (1973) described the first phospholipase D in mammalian cells. They found a phosphatidylcholine-specific activity that is dependent on unsaturated fatty acids

(UFA) such as oleic acid or arachidonic acid in millimolar concentrations and is therefore named UFA-PLD. The purification of porcine lung UFA-PLD yielded a protein band of $M_r = 190 \cdot 10^3$ but it has not been cloned yet (OKAMURA and YAMASHITA, 1994). This activity has primarily been detected in the endoplasmic reticulum and nucleus but also in the plasma membrane.

The first human phospholipase D to be cloned was phospholipase D1 (PLD1; HAMMOND *et al.*, 1995). Later, it was shown to be expressed in two splicing variants, PLD1a and the shorter PLD1b (HAMMOND *et al.*, 1997). PLD1a comprises 1,074 amino acids with a calculated molecular mass of $M_r = 124 \cdot 10^3$ and PLD1b comprises 1,036 amino acids with a calculated molecular mass of $M_r = 120 \cdot 10^3$. Its activity is dependent on PIP_2 and strongly activated by protein kinase C and monomeric G-proteins such as ADP-ribosylation factors and Ras homology (Rho) family proteins. Unsaturated fatty acids are potent inhibitors of its activity. ADP-ribosylation factor-dependent phospholipase D activities have been described for the endoplasmic reticulum, the Golgi apparatus, secretory and endosomal vesicles and the plasma membrane.

Phospholipase D2 (PLD2) shares more than 50 % amino acid homology with PLD1 (COLLEY *et al.*, 1997). The human protein comprises 933 amino acids with a calculated molecular mass of $M_r = 106 \cdot 10^3$. Overexpressed, the enzyme exhibits high activity in the presence of PIP_2 and can be further activated by micromolar concentrations of unsaturated fatty acids. Activation by Ca^{2+} and ADP-ribosylation factor (ARF) has been reported. Endogenous PLD2 is localised to the intracellular side of the plasma membrane and its activity can be inhibited by synucleins and phosphorylation.

1.2 Mechanisms of regulation of phosphatidylcholine-specific phospholipase D

The first direct evidence for receptor-linked activation of phosphatidylcholine-specific phospholipase D in intact cells was provided with the chemotactic peptide *N*-formylmethionyl-leucylphenylalanine in granulocytic-differentiated HL60 cells (PAI *et al.*, 1988). Since then, the list of mammalian cells in which receptor-induced phospholipase D activity was observed expanded rapidly. Activation of phospholipase D can be established via receptors which couple to heterotrimeric G-proteins or receptors possessing intrinsic tyrosine kinase activity (EXTON, 1997). Protein kinase C is often required for receptor-mediated phospholipase D activation and perturbation of an increase of cytosolic Ca^{2+} can disturb hormone-induced phospholipase D activation in some systems. More recent data imply a role

for monomeric G-proteins of the Ras superfamily, especially the Rho family and ADP-ribosylation factors. The role of phosphorylation-dependent mechanisms, particularly the phosphorylation of phospholipase D itself, is still unclear. These signalling pathways are summarised in figure 2. A further regulatory mechanism observed is the regulation of phospholipase D mRNA expression.

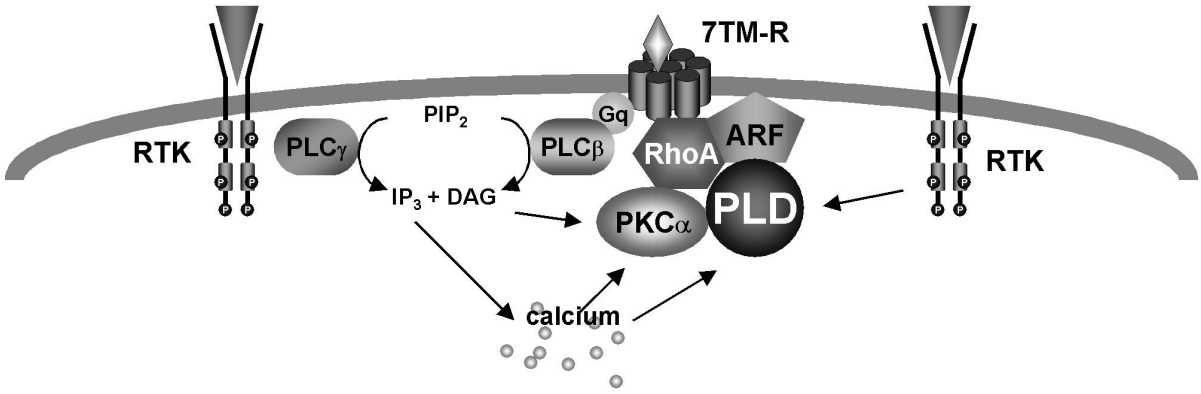

Figure 2.
Proposed signalling pathways for extracellular ligands to stimulate phospholipase D activation. For details see text. 7TM-R, G-protein coupled receptor; ARF, ADP-ribosylation factor; DAG, *sn*-1,2-diacylglycerol; IP_3, D-*meso*-inositol-1,4,5-trisphosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; RTK, receptor tyrosine kinase.

Protein kinase C

Phorbol-12-myristate-13-acetate (PMA) stimulated choline release from HeLa cells was the first observation indicating a protein kinase C-dependent phospholipase D activity (MUFSON *et al.*, 1981). Confirmation that this choline release is a product of a phospholipase D was achieved five years later by CABOT *et al.* (1988). Thereafter, the list of different cells in which a phorbol ester-sensitive phospholipase D activation was observed expanded rapidly. The role of protein kinase C in receptor-mediated phospholipase D activation was therefore extensively studied.

Prolonged phorbol ester treatment is a powerful tool to downregulate diacylglycerol-sensitive protein kinase C isotypes. MARTIN *et al.* (1989) reported that long-term phorbol ester treatment prevented bradykinin-induced phospholipase D activation but not bradykinin-induced phosphoinositide hydrolysis and prostacyclin synthesis in bovine endothelial cells. Subsequently, the pivotal role of protein kinase C in phospholipase D activation was demonstrated in many other cell types using a wide variety of agonists (EXTON, 1999). However, it must be recognised that in some situations, decreased protein kinase C activity leads to partial or no inhibition of the stimulation of phospholipase D. The use of protein kinase C inhibitors

revealed a further feature of protein kinase C-dependent phospholipase D activation. While some inhibitors block activation by interfering with the catalytic domain, others interact with the substrate binding domain (HOFMANN, 1997). The latter, represented by chelerythrine, effectively blocked hormone-induced phospholipase D activation whereas the former like staurosporine, H-7 and Ro-31-8220 under the same conditions did not (ESKILDSEN-HELMOND *et al.*, 1997; SINGH *et al.*, 1998). A clue to these findings was given by CONRICODE *et al.* (1992), who showed that phorbol ester induced choline production in Chinese hamster lung fibroblasts depends on the presence of either a cytosolic fraction or partially purified protein kinase C but did not require ATP. This led to the conclusion that a non-phosphorylation mechanism is involved and that formation of a complex between protein kinase C and phospholipase D is sufficient for the latter to become activated.

The protein kinase C family comprises twelve isoforms and can be divided into three classes (JAKEN, 1996). To address the question which protein kinase C isotype activates phospholipase D, CONRICODE *et al.* (1994) added different protein kinase C isoforms to membranes of CCL39 fibroblasts. Phorbol ester-dependent phospholipase D activation occurred only upon addition of PKCα or PKCβ but not PKCγ, PKCδ, PKCε or PKCζ. Identical results were obtained using membranes from HL60 cells (OHGUCHI *et al.*, 1996). Furthermore, studies on effects of proteolysis on the ability of PKCα to stimulate phospholipase D demonstrated that the stimulatory activity resides in the regulatory domain of the kinase (SINGER *et al.*, 1996). In agreement with these studies, recombinant human PLD1 responds directly to PKCα and PKCβ and is co-immunoprecipitated in a phorbol ester-dependent manner (HAMMOND *et al.*, 1997; LEE *et al.*, 1997). PLD2 was also shown to be activated by PKCα although no activation using recombinant enzymes has been observed (SIDDIQI *et al.*, 2000; COLLEY *et al.*, 1997).

The conventional isoforms of protein kinase C play a dominant role in agonist-induced phospholipase D activation. Evidence shows that protein kinase C physically interacts with PLD1 albeit that this interaction does not necessarily lead to phosphorylation of the enzyme. The role of direct phosphorylation is still a matter of debate and will be discussed later (see chapter 1.2.6). The activity of the third phosphatidylcholine-specific phospholipase D, UFA-PLD, has not yet been connected with protein kinase C.

Calcium

Similar to phorbol esters, an increase in cytosolic Ca^{2+} readily increases phospholipase D activity in many cell types. In addition, many studies have shown that depletion of cellular Ca^{2+} results in inhibition of the activation of phospholipase D by various agonists.

Initially, it was proposed that Ca^{2+} switch on the classical protein kinase C isoforms to activate phospholipase D but a number of studies argue for a second pathway. Notably, treatment with the calmodulin antagonist W-7 effectively inhibited phospholipase D activation via prostaglandin D2 in osteoblast-like cells (IMAMURA *et al.*, 1995). Moreover, the calcium/calmodulin-dependent kinase II inhibitor KN-62 was shown to inhibit Ca^{2+}-induced phospholipase D activation in synaptosomes (SARRI *et al.*, 1998). In reconstitution systems phospholipase D can be stimulated by calmodulin (TAKAHASHI *et al.*, 1996). Additionally, recombinant PLD1 and PLD2 were shown to be responsive to Ca^{2+} and calmodulin *in vitro* (SIDDIQI *et al.*, 2000).

All three phosphatidylcholine-specific phospholipase D isotypes require Ca^{2+} for full activity *in vitro*. Besides the most dominant effect of Ca^{2+}, the activation of protein kinase C, a further pathway acting via calmodulin and calcium/calmodulin kinase II might be responsible for subsequent phospholipase D activation.

Monomeric G-proteins

Guanosine-5'-*O*-(3-thiotriphosphate) (GTPγS), a non-discriminatory activator of GTP-binding proteins, very potently stimulates phospholipase D activity in many different permeabilised and cell-free systems. Although there are some reports on involvement of heterotrimeric G-proteins in phospholipase D activation, the vast majority deals with monomeric G-proteins from the Ras superfamily. In particular, ADP-ribosylation factors and members of the Rho family were found to be involved in signal transduction cascades that activate phospholipase D. In complex with GTP these proteins transduce signals to effector proteins whereas bound GDP inactivates them. The cycling between these two states is controlled by guanine nucleotide exchange factors (GEF), which catalyse the exchange of GDP for GTP and GTPase activating proteins (GAP) which promote the intrinsic GTP-hydrolysing activity. Additionally, guanine nucleotide dissociation inhibitors (GDI) maintain the G-protein in its GDP-bound, inactive state.

ADP-ribosylation factor

GENY *et al.* (1993) reported the requirement of a cytosolic factor of $M_r = 16 \cdot 10^3$ for GTPγS stimulated, membrane bound phospholipase D. This factor was subsequently identified to be ADP-ribosylation factor 1 (ARF1; BROWN *et al.*, 1993). The family of ADP-ribosylation factors is implicated in the regulation of membrane trafficking in yeast and mammalian cells (KAHN *et al.*, 1996). At present, this family consists of the six members ARF1 through ARF6 with an amino acid sequence identity of 65 % - 95 % (ROTH, 1999).

Evidence for a role of ADP-ribosylation factors in receptor-mediated phospholipase D activation was achieved predominantly by the use of brefeldin A. This drug acts by inhibiting some but not all GEFs of ADP-ribosylation factors (JACKSON and CASANOVA, 2000). In human embryonic kidney cells stably expressing the human M3 muscarinic acetylcholine receptor, brefeldin A inhibited the stimulation of phospholipase D by carbachol in intact cells (RÜMENAPP *et al.*, 1995). Protein kinase C-dependent phospholipase D activation was not affected using brefeldin A. Intriguingly, *N*-formylmethionyl-leucylphenylalanine stimulated phospholipase D activity was unaffected by brefeldin A in HL60 cells. Overexpression of dominant negative ARF1 and ARF6 in HIRcB fibroblasts abolished platelet-derived growth factor induced phospholipase D activation and partially inhibited phorbol ester induced phospholipase D activity (SHOME *et al.*, 1998).

ADP-ribosylation factor-dependent phospholipase D activation is established through direct interaction since addition of purified recombinant ARF1 to PLD1 resulted in a GTPγS-dependent activation of the enzyme with myristoylation of ARF1 being a prerequisite (HAMMOND *et al.*, 1997). Furthermore, co-immunoprecipitation of ARF1 with recombinant PLD1 underscored this finding (KIM *et al.*, 1998). Chimeras between ARF1 and the poor phospholipase D activator ARF2 restricted the interaction site to amino acids 35 - 94 of ARF1 (LIANG *et al.*, 1997). Cloning and characterisation of PLD2 revealed also a direct interaction of these proteins although the extent of activation is much lower than with PLD1 (LOPEZ *et al.*, 1998). However, mutational removal of the amino terminal region of PLD2, comprising amino acids 1 - 308, resulted in a protein with much lower basal activity that was stimulated up to 13-fold by ADP-ribosylation factors (SUNG *et al.*, 1999a). It is therefore possible that PLD2 constitutes at least in part of the ADP-ribosylation factor stimulated phospholipase D activity observed in mammalian cells. The full length protein may acquire its responsiveness

to ADP-ribosylation factors through an interaction with another protein or by post-translational modification.

ADP-ribosylation factors are monomeric G-proteins involved in membrane trafficking and vesicle transport. PLD1 and PLD2 have been shown to be responsive to ADP-ribosylation factors. The suggested involvement of phospholipase D in these cellular processes will be discussed in chapter 1.4.3.

Rho protein family

Another GTPγS-dependent factor stimulating membrane-bound phospholipase D activity was found in neutrophil lysates by BOWMAN *et al.* (1993). This stimulation was prevented by addition of RhoGDI inhibiting the dissociation of GDP in complex with Rho proteins. The Rho family consists of ten members and is divided into Rho-, Rac- and Cdc42-like groups (BISHOP and HALL, 2000). Rho proteins are activated by a variety of growth factors, cytokines and adhesion molecules and regulate a wide range of biological processes, including reorganisation of the actin cytoskeleton, transcriptional regulation and vesicle trafficking (VAN AELST and D'SOUZA-SHOREY, 1997; HALL, 1998).

Rho proteins are tightly regulated by GEFs, GAPs and GDIs, and responses to extracellular signalling could occur through the modulation of the activities of any of the three. However, how these modulators are activated in signal transduction pathways is still poorly defined. The heterotrimeric G-proteins $G_{\alpha 12}$ and $G_{\alpha 13}$ can stimulate p115 RhoGEF and may therefore act as intermediary in hormonal regulation of Rho proteins (HART *et al.*, 1998; KOZASA *et al.*, 1998). Conversely, it has been demonstrated that monomeric G-proteins can directly interact with G-protein coupled receptors (MITCHELL *et al.*, 1998). Many studies used *Clostridium botulinum* C3 exoenzyme (C3 toxin) which ADP-ribosylates RhoA rendering it inactive, as tool to investigate its role in phospholipase D activation. In rat brain homogenates, nuclei from MDCK cells and permeabilised human embryonic kidney cells C3 toxin treatment completely abolished GTPγS induced phospholipase D activation (BALBOA and INSEL, 1995; KURIBARA *et al.*, 1995; SCHMIDT *et al.*, 1996). However, the use of C3 toxin *in vivo* has been questioned since RÜMENAPP *et al.* (1998) reported that PIP_2 levels were significantly lowered in human embryonic kidney cells employing this toxin. Moreover, addition of exogenous PIP_2 to C3 toxin treated human embryonic kidney cells fully restored GTPγS-induced phospholipase D activity (SCHMIDT *et al.*, 1997). As PIP_2 is a prerequisite of PLD1 and PLD2 (see chapter 1.3.7), lowering the levels of PIP_2 affects their activity.

To address the question which members of the Rho family can stimulate phospholipase D activity rat liver plasma membranes and homogenates of human neutrophils were treated with RhoGDI to deplete Rho proteins. Addition of recombinant RhoA fully reconstituted GTPγS-induced phospholipase D activation whereas recombinant Rac1 only had a partial effect (MALCOLM *et al.*, 1994). In line with these observations, cloning of PLD1 showed that RhoA and RhoB are strong activators of the enzyme while Rac1, Rac2 or Cdc42Hs were less effective (HAMMOND *et al.*, 1997; BAE *et al.*, 1998). PLD2 was not activated by RhoA *in vitro* (COLLEY *et al.*, 1997). Additionally, geranylgeranylation of RhoA greatly increases its potency to activate PLD1. Direct interaction of the two proteins was demonstrated by yeast two-hybrid analysis and co-immunoprecipitation (YAMAZAKI *et al.*, 1999). However, in a plasma membrane fraction of human neutrophils phospholipase D activity was not restored by addition of RhoA alone but required the presence of a thus far unidentified factor of $M_r = 50 \cdot 10^3$ (KWAK *et al.*, 1995).

A synergistic effect of phorbol ester and GTPγS on phospholipase D activity in permeabilised cells was demonstrated by several studies (GENY and COCKCROFT, 1992; DUBYAK and KERTESY, 1997). This is in agreement with the observation that recombinant PLD1 is maximally stimulated upon the combined action of PKCα, RhoA and ARF1 (HAMMOND *et al.*, 1997). However, *in vivo* the situation seems to be different since in some cell types phospholipase D is stimulated in either an ADP-ribosylation factor- or Rho-dependent manner.

Several members of the Ras superfamily including ADP-ribosylation factors and RhoA can modulate the activation state of phospholipase D. Especially PLD1 is activated by PKCα, ARF1 and RhoA in a synergistic manner. However, whether cell surface receptors use one or more of these monomeric G-proteins to promote phospholipase D activity is not resolved so far.

Tyrosine phosphorylation

Many of the agonists that stimulate phospholipase D act via receptors that have intrinsic tyrosine kinase activity or activate soluble tyrosine kinases. Both, studies with protein tyrosine kinase inhibitors and protein tyrosine phosphatase inhibitors provided evidence for regulation of phospholipase D by tyrosine phosphorylation.

N-formylmethionyl-leucylphenylalanine and platelet derived growth factor stimulated phospholipase D activity was inhibited by tyrosine kinase inhibitors and the protein tyrosine phosphatase inhibitor pervanadate induced an accumulation of tyrosine phosphorylated

proteins and stimulated phospholipase D activity in HL60 cells and neutrophils (BOURGOIN and GRINSTEIN, 1992; UINGS *et al.*, 1992). Using anti-phosphotyrosine antibodies phospholipase D activity was immunoprecipitated from *N*-formylmethionyl-leucylphenylalanine treated but not from untreated human neutrophils (GOMEZ-CAMBRONERO, 1995). This suggests that phospholipase D or an associated protein is tyrosine phosphorylated. More recently, MARCIL *et al.* (1997) showed that immunoprecipitated PLD1 from pervanadate treated HL60 cells is tyrosine phosphorylated.

Murine PLD2 expressed in human embryonic kidney cells is constitutively associated with the epidermal growth factor receptor and is activated by agonist ligation (SLAABY *et al.*, 1998). Interestingly, tyrosine-11 becomes phosphorylated upon stimulation but, however, mutation of this tyrosine residue to phenylalanine does not alter the magnitude of the epidermal growth factor stimulation. This phosphorylation is restricted to mouse and rat since human PLD2 does not exhibit a tyrosine residue at this position.

Additionally, there is evidence for upstream regulation of phospholipase D by tyrosine phosphorylation. For instance, GTPase activating proteins such as the Rho-specific p190 RhoGAP are targets of protein tyrosine kinases. Phosphorylation by v-Src related kinases induces its association with p120 RasGAP- and p62 RasGAP-associated protein (ZRIHAN-LICHT *et al.*, 2000). Furthermore, inhibition of GTP- and epinephrine-induced phospholipase D activity by anti-Src and anti-p120 RasGAP antibodies has been reported (JINSI-PARIMOO and DETH, 1997).

Phospholipase D activity is regulated by tyrosine phosphorylation and the enzyme itself can be tyrosine phosphorylated. At present, it is unclear whether this phosphorylation influences the activity of the enzyme or if it is important for interaction with src homology 2 (SH2) domain containing proteins.

Serine/threonine phosphorylation

In contrast to the studies discussed in chapter 1.2.1, stimulation of phospholipase D is not always independent of ATP. Moreover, recent studies suggest the phosphorylation of PLD1 and PLD2 on serine end threonine residues to be a further regulatory mechanism.

For instance, brain protein kinase C or conventional protein kinase C isoforms activate phospholipase D in membranes from neutrophils, but the effect requires ATP or ATPγS and is abolished by staurosporine (LOPEZ *et al.*, 1995). Induction of phospholipase D by PKCα and PKCβ in HL60 cell membranes is also dependent on millimolar concentrations of

magnesium-ATP (OHGUCHI *et al.*, 1996). However, the requirement for ATP for induction of phospholipase D activity is mostly thought to account for synthesis of PIP_2. Conversely, in reconstitution systems ATP was found to significantly reduce protein kinase C stimulated phospholipase D activity (HAMMOND *et al.*, 1997; LEE *et al.*, 1997). In agreement, phosphorylation of PLD1 using PKCα *in vitro* resulted in a decrease of activity (MIN *et al.*, 1998). Recently, several phosphorylated serine and threonine residues on PLD1 were identified (KIM *et al.*, 1999b). Mutation of selected residues into alanine resulted in reduced phorbol ester induced phospholipase D activity suggesting a positive regulation by phosphorylation. Treatment with the protein serine/threonine phosphatase inhibitor okadaic acid was shown to increase spontaneous phosphorylation of overexpressed PLD2 in HeLa cells (WATANABE and KANAHO, 2000). This was accompanied by decreased activity and dephosphorylation of immunoprecipitated PLD2 using the catalytic subunit of protein phosphatase $1\gamma_1$ stimulated its activity.

Regulation of phospholipase D by serine/threonine phosphorylation is still a matter of debate. The amino acid sequences of PLD1 and PLD2 exhibit a multitude of phosphorylation consensus sites not only for protein kinase C. This suggests a complex regulation of phospholipase D by multisite phosphorylation (COHEN, 2000).

Phosphatidylinositol-4,5-bisphosphate

In reconstitution systems the activity of PLD1 and PLD2 is marginal in the absence of phosphatidylinositol-4,5-bisphosphate (PIP_2) or phosphatidylinositol-3,4,5-trisphosphate (PIP_3). An active role of these phosphoinositides in regulation of phospholipase D *in vivo* is therefore likely but has not been proven in intact cells.

Increases in PIP_2 levels are possibly mediated by activation of phosphatidylinositol-4-phosphate 5-kinase via Rho proteins or tyrosine kinases (OUDE WEERNINK *et al.*, 2000; CASTELLINO and CHAO, 1999). In agreement with a role for Rho proteins in the cellular regulation of PIP_2, pretreatment of human embryonic kidney cells with C3 toxin and *Clostridium difficile* toxin B, which inactivates Rho proteins by glucosylation, reduces PIP_2 levels and phospholipase D activity (SCHMIDT *et al.*, 1997). Basal and GTPγS-stimulated phospholipase D activity was fully restored by addition of PIP_2. Moreover, the ADP-ribosylation factor GTPase activating proteins ASAP1 and other ARF-GAPs are activated by PIP_2, suggesting a second indirect effect of PIP_2 on phospholipase D activity (KAM *et al.*, 2000).

Many agonists such as insulin or platelet derived growth factor elevate PIP_3 levels while PIP_2 is often transiently decreased by agonist induced PIP_2-specific phospholipase C (FRY, 1994). Inhibitors of phosphatidylinositol 3-kinase such as wortmannin and LY294002 abolish agonist-induced phospholipase D activation (KOZAWA *et al.*, 1997). This inhibition does not affect phorbol ester or calcium ionophore induced phospholipase D activation (NAKAMURA *et al.*, 1997).

PIP_2 and PIP_3 directly activate PLD1 and PLD2 and it is therefore possible that phosphoinositide generation may represent a mechanism of agonist regulation of these enzymes. Moreover, phosphoinositides are also involved in upstream regulators of phospholipase D such as effector proteins of monomeric G-proteins and thus act synergistic on the enzyme.

Unsaturated fatty acids

Unsaturated fatty acids are potent inhibitors of PLD1 whereas PLD2 was shown to be activated by micromolar concentrations and inhibited by millimolar concentrations (HAMMOND *et al.*, 1995; KIM *et al.*, 1999a). In contrast, the unsaturated fatty acid stimulated phosphatidylcholine-dependent phospholipase D (UFA-PLD) reaches its full activity at millimolar concentrations (CHALIFOUR and KANFER, 1982; OKAMURA and YAMASHITA, 1994). This isoform was demonstrated to be the major form detected *in vitro* in lysates of Jurkat T cells (KASAI *et al.*, 1998). Induction of apoptosis dramatically elevated phosphatidylbutanol formation in the presence of 1-butanol in intact cells, as well as *in vitro* phospholipase D activity. This was accompanied by raised levels of non-esterified fatty acids and loss of responsiveness to oleate of the enzyme *in vitro*. These data suggest a model in which apoptosis provokes an increase in cellular non-esterified fatty acids, most probably via a cellular phospholipase A_2, which than activate UFA-PLD. Evidence for an *in vivo* regulation of PLD1 or PLD2 by unsaturated fatty acids has not been provided so far.

Inhibitory proteins

Phospholipase D activity can be inhibited by several proteins *in vitro* but their physiological role in regulation of the enzyme is unclear. Two of them, synaptojanin and fodrin most probably act indirectly on phospholipase D activity via sequestering PIP_2. Synaptojanin exhibits phosphoinositide 5-phosphatase activity whereas the non-erythroid form of spectrin fodrin inhibits phosphoinositide biosynthesis (CHUNG *et al.*, 1997; LUKOWSKI *et al.*, 1996; LUKOWSKI *et al.*, 1998). The synapse-specific clathrin assembly protein AP180 apparently

inhibits PLD1 by binding and association (LEE *et al.*, 1997). Similarly, the amphiphysins I and II inhibit PLD1 and PLD2 (LEE *et al.*, 2000). Regulation by inhibition was requested for PLD2 because it shows high basal activity when expressed in insect cells (COLLEY *et al.*, 1997). A heat stable protein factor in brain extracts was found to effectively and selectively inhibit PLD2 (JENCO *et al.*, 1998). The purified inhibitory activity was identified as a mixture of α- and β-synucleins which are involved in neurodegenerative diseases (CLAYTON and GEORGE, 1998). Inhibition cannot be overcome by phosphoinositides, is unaffected by protein activators of PLD1 and not competitive with substrate. α-Synuclein was found to be a negative regulator of dopamine neurotransmission suggesting that PLD2 is involved in synaptic vesicle fusion (ABELIOVICH *et al.*, 2000).

Expression

Besides regulation of enzyme activity several studies show alterations in mRNA and protein expression.

Induction of differentiation of C6 glioma cells by dibutyryl cyclic AMP results in a transient elevation of PLD1 expression and concurrently, a decrease in expression of PLD1b and PLD2 (YOSHIMURA *et al.*, 1996). Conversely, granulocytic differentiation of HL60 cells using dibutyryl cyclic AMP or all-trans retinoic acid induces expression of both PLD1 isoforms but also of PLD2 (NAKASHIMA *et al.*, 1998). The same response was seen after treatment of HL60 cells with dimethylsulfoxide (HOULE and BOURGOIN, 1999). In PC12 cells mRNA levels of PLD1a and PLD1b but not PLD2 were elevated after treatment with nerve growth factor (HAYAKAWA *et al.*, 1999). Epidermal keratinocytes differentiated with 1α,25-dihydroxyvitamin D_3 exhibited increased expression of PLD1 whereas PLD2 levels were unaltered (GRINER *et al.*, 1999). In rat brain, PLD1 expression is elevated *in vivo* during days 5 - 15 of postnatal development (ZHAO *et al.*, 1998). This time coincides with the major period of synaptogenesis and myelination. Ceramide induced apoptosis and a decrease of phospholipase D in HaCaT keratinocytes and FRTL-5 thyroid cells (IWASAKI-BESSHO *et al.*, 1998; PARK *et al.*, 1999). In agreement, ceramide-induced apoptosis is accompanied by a decrease in PLD1a and PLD1b mRNA whereas PLD2 levels were only slightly altered rat C6 glial cells (YOSHIMURA *et al.*, 1997).

Altered mRNA or protein levels of PLD1 have been found especially in differentiating cells. In contrast, PLD2 levels are unchanged in most cases. The promoter regions of PLD1

and PLD2 have not been cloned so far. However, the data support a role for PLD1 in regulation of differentiated cell function in diverse cell types.

1.3 Biochemistry of phosphatidylcholine-specific phospholipase D

1.3.1 Catalysis

Phosphatidylcholine-specific phospholipase D shows high preference for a choline head group, but the nature of the acyl- or alkyl groups in position 1 and 2 also influences their susceptibility to hydrolysis by these enzymes. The recent cloning and crystallisation of phospholipase D allowed further insights into the catalytic mechanism involved.

Phorbol ester activated phospholipase D predominantly catalysed the degradation of the ether- but not the ester-linked molecular species of phosphatidylcholine (DANIEL *et al.*, 1993; HUANG *et al.*, 1995). In contrast, GTPγS stimulated predominantly the hydrolysis of ester-linked molecular species. Moreover, phorbol ester and v-Src induced phospholipase D activity could be discriminated using differential radiolabelling of the phospholipids (SONG and FOSTER, 1993; JIANG *et al.*, 1994). Phorbol ester induced phospholipase D activity was detected irrespective of the label used. Conversely, no v-Src stimulated phospholipase D activity was observed upon prelabelling with either arachidonic acid or 1-*O*-alkyl-*sn*-glyceryl-3-phosphorylcholine. The stimulatory effect of the tyrosine kinase became apparent when the phospholipids were labelled with either myristic acid or palmitate. At present, the physiologic implication for the different pathways to either ether-linked or ester-linked species degradation is unclear.

The most unique feature of phosphatidylcholine-specific phospholipase D is the transphosphatidylation reaction and it has proven to be a powerful tool to study the properties of this enzyme. In the presence of small primary alcohols like methanol, ethanol, 1-propanol, 1-butanol and glycerol, the primary hydroxyl-group is preferred as nucleophil in the hydrolysis respective alcoholysis of the phosphate diester (Fig. 3). Primary alcohols are excellent substrates for phospholipase D and are preferred over water by at least a factor of 260 (CHALIFA-CASPI *et al.*, 1998). The resulting phosphatidylalcohol can be used to specifically determine phospholipase D activity. Cloning of phosphatidylcholine-specific phospholipase D revealed a conserved motif comprising the three amino acids histidine, lysine and aspartic acid (HKD). This motif is suggested to be responsible for the catalytic activity of the enzyme since it is also found in other phosphodiesterases. Most interestingly, this motif is also present in bacterial

cardiolipin synthase and phosphatidylserine synthase. Both of these enzymes catalyse phospholipid synthesis reactions in that an alcohol is conjugated to a phospholipid. These enzymes and phospholipase D isoforms contain two of these motifs per molecule. As depicted in figure 3, this reaction involves a phosphatidyl-enzyme intermediate, most probably with the histidine of the catalytic histidine-lysine-aspartate triad (GOTTLIN *et al.*, 1998). When Nuc 8, an endonuclease from *Salmonella typhimurium* that comprises only one such motif was crystallised with tungstate, it was found dimerised with the tungstate hydrogen bonded by the amino acids of both motifs (STUCKEY and DIXON, 1999). Recently, the crystal structure of a phospholipase D from *Streptomyces* sp. strain PMF was reported and in agreement found to bear a tungstate hydrogen bonded as with Nuc 8 (LEIROS *et al.*, 2000). In the presence of phosphate instead of tungstate, the phosphate was bound in the same way and suggests that this represents the orientation of the phosphate moiety of the phosphatidylcholine substrate. It is proposed that one histidine functions as the attacking nucleophile while the second protonates the oxygen of the leaving group.

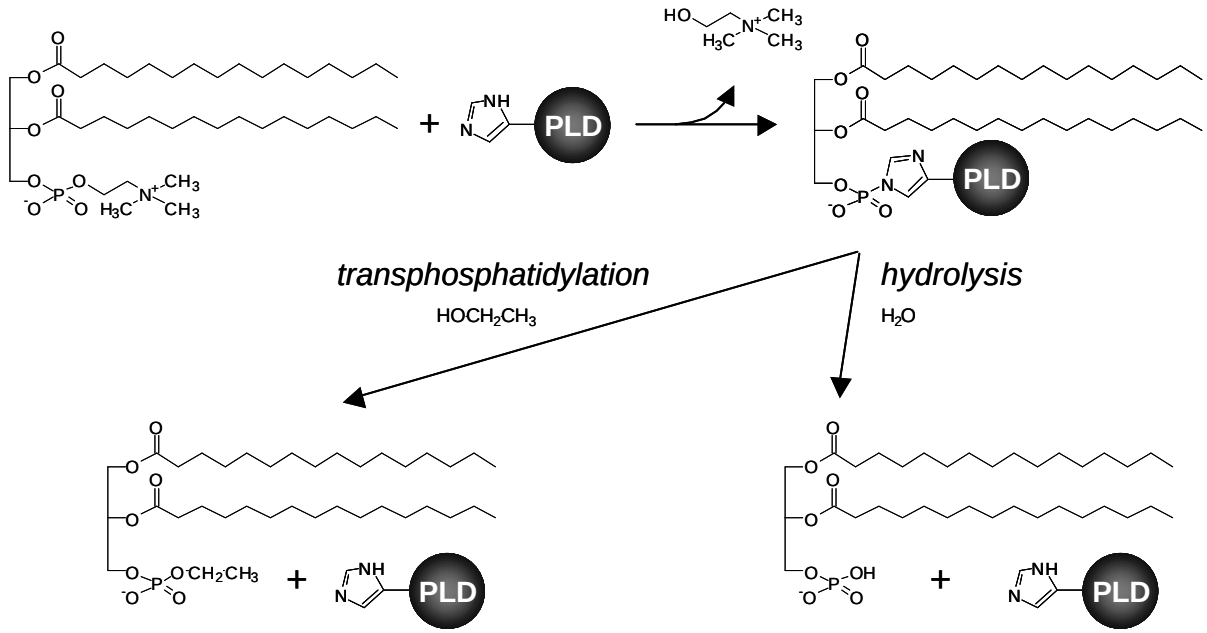

Figure 3.
Mechanism of the catalytic reactions performed by phosphatidylcholine-specific phospholipase D. Phospholipase D (PLD) attacks the phosphate of phosphatidylcholine with a histidine of the catalytic triad. The intermediary phosphatidyl-enzyme complex is then hydrolysed by water yielding phosphatidic acid or alcoholysed by a primary alcohol such as ethanol generating the respective phosphatidylalcohol.

1.3.2 Structure

Two mammalian phosphatidylcholine-specific phospholipase D isoforms, PLD1 and PLD2 have been cloned to date.

Cloning was achieved based on four conserved motifs of cloned phosphatidylcholine-specific phospholipase D isoforms from the bacterium *Corynebacterium pseudotuberculosis*, the yeast *Saccharomyces cerevisia* and the plant *Ricinus communis* from a HeLa library (HAMMOND *et al.*, 1995). The first conserved region (CRI) comprises amino acids 362 - 421 of human PLD1a, the second (CRII) amino acids 463 - 487, the third (CRIII) amino acids 743 - 786 and the fourth (CRIV) amino acids 894 - 919. The conserved regions CRII and CRIV contain the catalytic motif $HxKx_4D$ (see chapter 1.3.1). Whereas a doublet of this core-motif is also found in cardiolipin and serine synthases, careful sequence comparison reveals the phospholipase D-specific motif $HxKx_4Dx_nHxKx_4Dx_6GSxN$. The last three conserved amino acids are also involved in coordination of the catalytic site but the position of other essential residues is less strictly conserved. No clear function could be assigned to the conserved region CRI but CRIII was suggested to be involved in binding of the choline head group (SUNG *et al.*, 1997). Another critical region for catalytic function of mammalian phospholipase D appears to be the carboxy terminal 42 amino acids. Several groups reported upon generation of tagged mutants, that solely amino terminal tagged proteins retain full activity and that removal of the carboxy terminal amino acids abolishes enzyme activity (SUNG *et al.*, 1999b; LIU *et al.*, 2001).

The requirement for PIP_2 by PLD1 and PLD2 led to the proposal that membrane binding is achieved through PIP_2 most probably via a pleckstrin homology (PH) domain. A weakly conserved PH domain comprising amino acids 220 - 329 of PLD1a was reported by STEED *et al.* (1998) and mutations within this domain abolish membrane localisation (HODGKIN *et al.*, 2000). Recently, however, SUGARS *et al.* (1999) showed that the two cysteine residues cysteine 240 and cysteine 241 within this domain are fatty acylated and membrane association is achieved through these fatty acids. PIP_2-binding could be designated to a conserved stretch of predominantly basic and hydrophobic residues in mouse PLD2 (SCIORRA *et al.*, 1999). Mutation of two arginine residues in this region to glycine, completely abolished PIP_2 binding to phospholipase D, demonstrating that this region is the PIP_2 interaction site. Moreover, membrane association of this mutant was not altered. This region is also found in human PLD1a comprising amino acids 691 - 712.

Another motif, the phox consensus sequence (PX) has been assigned to mammalian PLD1, PLD2 and yeast phospholipase D1 through its significant conservation (SUNG *et al.*, 1999a). PX domains mediate a wide variety of protein-protein interactions including kinase and src homology 3 (SH3) domain binding (PONTING; 1996). This domain comprising amino acids 99 - 213 of PLD1 is critical for phospholipase D proteins but the factors that are interacting are unknown. However, PKCα does not functionally associate via the PX or PH domain but binds another region of PLD1 that is located amino terminal from these domains between amino acids 50 and 115 (PARK *et al.*, 1998; ZHANG *et al.*, 1999). This region contains an auto-inhibitory domain which is suggested to be removed by protein kinase C interaction. Protein-protein interaction is nonetheless achieved through additional regions. The interaction regions for RhoA and ADP-ribosylation factors are located in the carboxy terminus of PLD1. Whereas the region for RhoA association is flanking the conserved motif CRIV the ADP-ribosylation factor interaction site has not been clearly allocated (YAMAZAKI *et al.*, 1999; PARK *et al.*, 1998; SUNG *et al.*, 1999b).

Amino acid sequence analysis reveals a plethora of possible phosphorylation sites for PLD1 and PLD2. Recently, KIM *et al.* (1999b) demonstrated several phosphorylated peptides from PLD1 after cotransfection with PKCα. They identified serine 2, threonine 147 which lies within the PX domain and serine 561 which lies within the loop-region as phorbol ester-dependent phosphorylation sites. No serine/threonine phosphorylation sites have been assigned to phospholipase D2. Tyrosine phosphorylation of PLD1 was demonstrated by MARCIL *et al.* (1997) but the modified residues were not defined. Consensus sites for tyrosine phosphorylation suggest tyrosine 295 that lies within the PH domain and tyrosine 815 as possible targets. Although murine PLD2 was found to be tyrosine phosphorylated on tyrosine 11, this residue is not available in the human protein (SLAABY *et al.*, 1998). A second consensus site contains tyrosine 470 which is present in all mammalian PLD2 proteins but is not phosphorylated upon epidermal growth factor stimulation.

The intron-exon structure of human PLD1 has not been resolved to date but preliminary genomic sequence data suggests 28 exons (accession numbers AC008134 and AC019265; fig. 4). PLD1 exists in two spliced forms, designated PLD1a and PLD1b where one exon, exon 17 in figure 4, is excluded. This exon lies within a region named the loop-region that comprises exons 16 and 17. The loop-region encodes a 119 amino acid stretch that is exclusively present in mammalian PLD1 and possibly in *Caenorhabditis elegans* and *Drosophila melanogaster* phospholipase D (FROHMAN *et al.*, 1999). PLD2 lacks this region but shares high homology

throughout the rest of the amino acid sequence. It is tempting to speculate that the loop region and its alternative splicing is responsible for subcellular localisation or effector interaction of PLD1. This would provide an explanation for the incongruous results of the influences of effector proteins on phospholipase D activation *in vivo*. However, alternative splicing does not significantly affect PLD1 activity or regulation (HAMMOND *et al.*, 1997; KATAYAMA *et al.*, 1998). Moreover, mutational deletion of the loop-region from PLD1 or insertion into PLD2 hardly affects the activity or regulation of the enzyme (SUNG *et al.*, 1999ab).

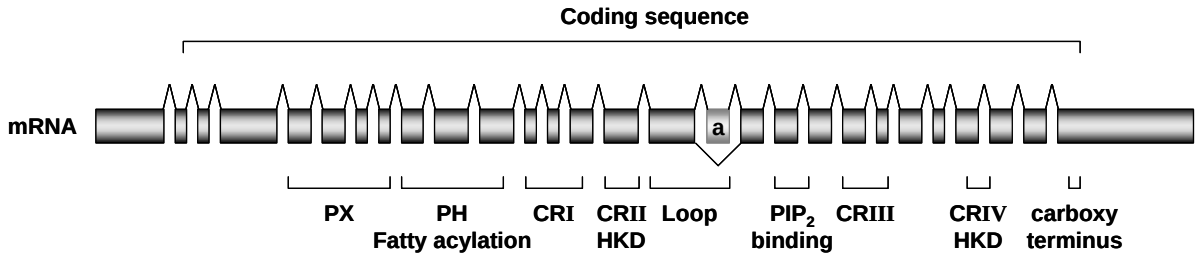

Figure 4.
Preliminary intron-exon structure of human phospholipase D1. Shaded blocks represent exons, the thin kinked lines indicate the positions occupied by the introns before they are spliced out. The lighted block marked with 'a' is the alternatively spliced exon. Inclusion of the exon generates the splicing variant phospholipase D1a, exclusion the isoform phospholipase D1b. Annotated domains are depicted with their position below. PX, phox consensus sequence; PH, pleckstrin homology; CRI-IV, conserved phospholipase D region I-IV; HKD, catalytic triad motif.

UFA-PLD has not been cloned so far but the fact that it catalyses the transphosphatidylation reaction suggests that it also contains the catalytic motif and their high conservation implies all four conserved regions to be present.

1.4 Cellular functions of phosphatidylcholine-specific phospholipase D

Phospholipase D hydrolyses phosphatidylcholine to yield phosphatidic acid and choline. Phosphatidic acid can be further metabolised to diacylglycerol by phosphatidic acid phosphohydrolase. Whereas diacylglycerol production via the phosphoinositide cycle is fast and transient, often a second, sustained diacylglycerol elevation is observed and respective protein kinase C activation. For this reason, a phosphatidylcholine cycle was proposed where phosphatidylcholine is hydrolysed and the generated phosphatidic acid subsequently hydrolysed to diacylglycerol. Diacylglycerol is then condensed with CDP-choline to recover phosphatidylcholine. The nature of a phosphatidylcholine-specific phospholipase C that directly generates diacylglycerol from phosphatidylcholine is still under debate (see chapter 1.1). Furthermore, phosphatidic acid is a metabolite in lipid biosynthesis. Condensation with CTP yields *sn*-3-CDP-1,2-diacylglycerol which is the precursor of phosphatidylinositol and

phosphatidylserine. Additionally, there is evidence that phosphatidic acid serves as a second messenger involved in processes such as mitogenesis, inflammation, tissue damage, respiratory burst, cell signalling, vesicle trafficking and cytoskeletal rearrangements. The direct stimulation of phospholipase D activity by ADP-ribosylation factors stresses the proposal of an involvement in vesicle trafficking of this enzyme.

1.4.1 Phosphatidic acid-derived diacylglycerol

In many systems hormone-induced production of diacylglycerol is biphasic by nature (HUANG and CABOT, 1990; HODGKIN *et al.*, 1998). A small transient increase is followed by a larger sustained increase. Polyunsaturated diacylglycerols predominate during the initial phase of stimulation and are thought to be derived from PIP_2 by the action of phospholipase C (PESSIN and RABEN, 1989). During the sustained phase, the concentration of mono-unsaturated and saturated diacylglycerols rises. Phosphatidylcholine-specific phospholipase D has some preference for mono-unsaturated and saturated phosphatidylcholine molecular species (HEUNG and POSTLE, 1995). Therefore, it is proposed that phospholipase D contributes to the production of diacylglycerol by promoting the formation of phosphatidic acid, which is subsequently converted into diacylglycerol by phosphatidic acid phosphohydrolase. Since phosphatidylalcohols are not substrate for phosphatidic acid phosphohydrolase, the transphosphatidylation reaction was exploited to demonstrate that the second diacylglycerol peak results from phospholipase D (BILLAH *et al.*, 1989). Additionally, the phosphatidic acid phosphohydrolase inhibitor propanolol likewise abolished diacylglycerol production.

The primary target of diacylglycerol is activation of protein kinase C. Whereas the PIP_2-derived polyunsaturated molecular species readily activates the conventional protein kinase C isoforms, a role for mono-unsaturated and saturated species in protein kinase C activation is not well established (WAKELAM, 1998). In gonadotropin-releasing hormone-stimulated pituitary cells, a prolonged protein kinase C stimulation was associated with increased expression of the transcription factor c-fos (CESNJAJ *et al.*, 1995). In agreement with a role of phospholipase D, c-fos expression was inhibited by ethanol and inhibition of phosphatidic acid phosphohydrolase activity by propanolol. Similarly, in hepatocyte growth factor stimulated rat hepatocytes, induction of c-jun and c-fos expression was suppressed by 1-butanol and propanolol (ADACHI *et al.*, 1996). However, in platelets protein kinase C activation occurs during the first rather than the second phase of diacylglycerol formation (BALDASSARE *et al.*, 1992). In addition, *lyso*-phosphatidic acid activated phospholipase D independently of phospholipase C

and without activation of protein kinase C in porcine aortic endothelial cells (PETTITT *et al.*, 1997). Moreover, a large increase in diacylglycerol changes the physical state of the membrane and may act as a fusogen in vesiculation (see chapter 1.4.3).

1.4.2 Protein targets of phosphatidic acid

Extracellular signal evoked phosphatidic acid generation is observed in many different cells suggesting that it serves as a second messenger. *In vitro* studies revealed several targets of phosphatidic acid but *in situ* evidence is lacking. PLCγ has been described to be activated by phosphatidic acid (JONES and CARPENTER, 1993). The induced phosphoinositide cascade leads to stimulation of Ca^{2+}-dependent protein kinase C isotypes which may be responsible for mitogenic responses to phosphatidic acid formation. Besides this activation of classical isoforms, PKCη and PKCζ can be stimulated directly with phosphatidic acid (LIMATOLA *et al.*, 1994). The serine/threonine protein kinase Raf-1, a component of the mitogen activated protein kinase cascade has been shown to interact directly with phosphatidic acid (GHOSH *et al.*, 1996; RIZZO *et al.*, 2000). Several phosphatidylinositol-4-phosphate 5-kinase isoforms are stimulated by phosphatidic acid providing a possible feed forward loop through phospholipase D activation via PIP_2 formation (ISHIHARA *et al.*, 1996; ISHIHARA *et al.*, 1998). Other examples are a protein kinase of $M_r = 125 \cdot 10^3$ that phosphorylates NADPH oxidase (WAITE *et al.*, 1997), the epidermal growth factor-associated protein tyrosine phosphatase 1c (TOMIC *et al.*, 1995) and cAMP-specific phosphodiesterase PDE4D3 where a phosphatidic acid binding site was identified (GRANGE *et al.*, 2000). Recently, MANIFAVA *et al.* (2001) purified phosphatidic acid binding proteins using a phosphatidic acid coupled matrix. The coatomer protein β-cop, ADP-ribosylation factor, NSF and kinesin, all proteins involved in intracellular trafficking, are described among others.

1.4.3 Vesicle transport

The observation that ADP-ribosylation factors are efficacious activators of phospholipase D provoked interest in studies on the role of phospholipase D in the regulation of vesicle transport and protein trafficking (JONES *et al.*, 1999). Secretion of β-glucuronidase was markedly inhibited by 1-butanol in HL60 cells, demonstrating a role of phospholipase D generated phosphatidic acid (STUTCHFIELD and COCKCROFT, 1993). Matrix metalloproteinases play an important role in degrading the basement membrane and thereby facilitating invasion and metastasis of cancer cells. Laminin induces release of matrix metalloproteinase 2 and this

release is inhibited by primary but not secondary alcohols (REICH *et al.*, 1995). Furthermore, laminin stimulates phospholipase D activity and exogenously added phosphatidic acid stimulated matrix metalloproteinase 2 secretion. Similarly, in human fibrosarcoma cells matrix metalloproteinase 9 secretion was stimulated by a short chain phosphatidic acid analogue and inhibited by 1-propanol (WILLIGER *et al.*, 1999). Studies on subcellular localisation of ADP-ribosylation factor stimulated phospholipase D showed co-localisation with sites of vesicle trafficking. The bulk of phospholipase D activity in Chinese hamster ovary cells was detected in Golgi membranes while a smaller amount was present in the endoplasmic reticulum (KTISTAKIS *et al.*, 1995). In accordance, primary alcohols were shown to inhibit both protein transport from the endoplasmic reticulum to the Golgi complex and release of secretory vesicles from the *trans*-Golgi network (CHEN *et al.*, 1997; BI *et al.*, 1997). Initiation of coat assembly on Golgi membranes was demonstrated to occur independently of ADP-ribosylation factors in cell lines exhibiting high constitutive phospholipase D activity (KTISTAKIS *et al.*, 1996; BI *et al.*, 1997). The formation of coated vesicles was sensitive to ethanol at concentrations that inhibit the phospholipase D catalysed phosphatidic acid production. Exogenous bacterial phospholipase D was able to induce the binding of coatomers to Golgi membranes. This led to the idea that phospholipase D catalysed production of phosphatidic acid is a key event in the formation of coatomer-coated vesicles. In addition, assembly of assembly protein 2 (AP2) containing clathrin coats on rat liver lysosomal membranes appears to depend on a PLD1-like activity (ARNESON *et al.*, 1999; WEST *et al.*, 1997). Moreover, ARF1, reconstituted purified coatomer proteins and chemically defined synthetic liposomes containing phosphatidic acid can form coated vesicles *in vitro* in the absence of phospholipase D (SPANG *et al.*, 1998). This is underscored by the finding that the coatomer protein β-cop and ADP-ribosylation factor directly bind to phosphatidic acid (MANIFAVA *et al.*, 2001). However, activation of phospholipase D may not be an essential part of the basic ADP-ribosylation factor-mediated vesicle budding machinery in the Golgi of eukaryotic cells, because in yeast such a functionality does not exist (RUDGE and ENGEBRECHT, 1999). Thus, a role for ARF1-dependent PLD1 in budding of coatomer-coated Golgi vesicles remains controversial.

1.4.4 Stress fibre formation

Differentiation of IIC9 fibroblasts from a semi-round to an elongated form can be induced by α-thrombin (HA and EXTON, 1993). This differentiation is accompanied by an increase in stress fibres and a similar increase in stress fibres was observed following incubation of these

cells with exogenous phospholipase D or phosphatidic acid. In porcine aortic cells, *lyso*-phosphatidic acid-induced stress fibre formation was inhibited by 1-butanol and mimicked by exogenous phosphatidic acid (CROSS *et al.*, 1996). The formation of stress fibres in response to exogenous phosphatidic acid was inhibited by C3 toxin, suggesting a role of RhoA downstream rather than upstream of phospholipase D. Spreading and proliferation of murine mammary adenocarcinoma cells was reduced by 1-butanol and stimulated by phosphatidic acid (AGUIRRE GHISO *et al.*, 1997). The Rho protein isotypes RhoA and RhoC are localised to the submembraneous actin network and RhoB is associated with multivesicular bodies (ROBERTSON *et al.*, 1995). Therefore, RhoA and RhoC are thought to be involved in cell migration and cadherin-mediated cell-cell adhesion whereas RhoB likely participates in endocytic receptor trafficking (GAMPEL *et al.*, 1999). Rho proteins are overexpressed in colon, breast and lung tumours (FRITZ *et al.*, 1999). Overexpression of RhoC correlates with progression of ductal adenocarcinoma of the pancreas (SUWA *et al.*, 1998) and the inflammatory breast cancer phenotype (VAN GOLEN *et al.*, 1999). Moreover, mammary epithelial cells transfected with RhoC showed an increase in actin stress fibre and focal adhesion contact formation (VAN GOLEN *et al.*, 2000). These cells exhibited enhanced motility and were invasive. In addition, *in vivo* selection of a highly metastatic melanoma cell line revealed elevated expression of RhoC among others (CLARK *et al.*, 2000). Transfection with RhoC of the low metastatic original cell line produced a metastatic phenotype. Moreover, enhanced phospholipase D expression was described in colon and breast tumours (YOSHIDA *et al.*, 1998; NOH *et al.*, 2000). Several effectors of Rho proteins respond to phosphatidic acid but PLD1 is also responsive to Rho proteins. Therefore, the role of phospholipase D in tumourigenesis is unclear.

These data suggest phospholipase D to be implicated in mitogenic as well as in differentiation processes. Enhanced phospholipase D activity can generate the mitogen diacylglycerol whereas the precursor phosphatidic acid increases vesicle transport and Rho protein-related cytoskeletal reorganisation. Consequently, dysregulation of phospholipase D might contribute to carcinogenesis.