



Universitat Autònoma de Barcelona

**Studies on the molecular mechanisms of cell proliferation: phosphatidylcholine-derived lipids and lithium modulation of the MEK/ERK pathway.**

Memoria presentada per Raül Pardo Pardo, llicenciat en Bioquímica, per tal d'optar al grau de Doctor en Ciències per la Universitat Autònoma de Barcelona.

Aquesta tesi ha estat realitzada sota la direcció dels doctors Fernando Picatoste Ramón, Enrique Claro Izaguirre i Elisabeth Sarri Plans al departament de Bioquímica i Biologia Molecular de la UAB i al departament de Fisiologia de l'University College de Londres.

Raül Pardo Pardo

Bellaterra, Març del 2003.

**Esforzarse y luchar contra algo que se resiste es  
una necesidad esencial de la naturaleza humana.**

**Superar obstáculos es un placer.**

Ángela Vallvey

**El que te quiere nunca muere, no,...jamás!.**

De la canción 537 C.U.B.A.

ORISHAS. A lo cubano 1999, Chrysalis

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**Thesis contents**


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**Part I: Role of phospholipase D in cellular proliferation and membrane ruffling**


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# **PART I:**

**Role of phospholipase D in cellular proliferation and  
membrane ruffling**

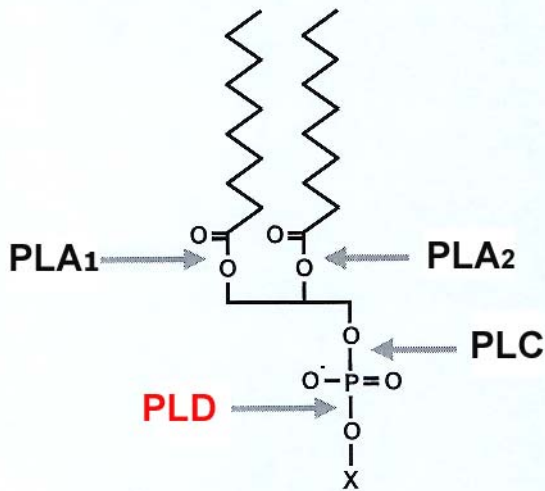
## **INTRODUCTION**

# Introduction

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## 1. General introduction to lipid signaling.

The evolution of multicellular organisms is tightly dependent on the ability of each cell to communicate with other cells within the organism and with the environment. In this scenario extracellular signals and its correspondent receptors play a key role, as these are the molecules that transmit information from outside to the inside of the cell. Late in the 1950s initial studies on the actions of acetylcholine on pancreatic acini suggested the possibility of lipidic constituents of cell membranes as messenger molecules able to transmit signals upon receptor stimulation [1]. Nowadays, many lipidic molecules, among them phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>), diacylglycerol (DAG), phosphatidic acid (PtdOH), lysophosphatidic acid (lysoPtdOH), arachidonic acid, ceramide and sphingosine 1-phosphate ([2]), have been found to regulate a wide variety of complex cellular processes that include proliferation, differentiation, senescence and cell death. These lipids are generated by means of phosphatases, kinases and phospholipases acting on major lipid constituents of cellular membranes, its catalytic activity being under elaborate control by cell membrane receptors.



**Figure 1. Cleavage site of different phospholipases on a glycerophospholipid molecule.**

**Phospholipases** are enzymes that hydrolyse phospholipids. They are localised either intracellularly or extracellularly. Most of them are constituents of gut secretions and collaborate in digestion of food, some are present in venoms from a wide variety of animal species and a few, which are localised intracellularly, participate in the degradation of phospholipids in the lysosomes. A set of these intracellularly expressed

phospholipases have been widely studied because of their ability to generate signaling molecules required in wide variety of cellular processes. Depending on the specific bond that is targeted, they have been classified as phospholipases A1, A2, C or D (see Fig.1).



**Phospholipase A1** (PLA<sub>1</sub>) enzymes with specific selectivity for phosphatidylserine or PtdOH are known to be present in mammalian cells. PtdSer-PLA1 produces 2-acyl lysoPtdSer, which is a lipid mediator for mast cells and neurones [3]. This lipid stimulates mast cell degranulation and is also reported to induce neurite outgrowth. A PtdOH-PLA1 has been recently cloned and has been involved in the production of 2-acyl lysoPtdOH, a lipid that might elicit cellular responses through stimulation of Edg7 receptors [4].

**Phospholipase A2** (PLA<sub>2</sub>) enzymes have attracted considerable interest as a pharmacological target in view of its role in lipid signaling and its involvement in a variety of inflammatory conditions. To date, at least 19 distinct enzymes have been found in mammals. PLA<sub>2</sub> enzymes hydrolyse the sn-2 ester bond of cellular phospholipids, producing a free fatty acid and a lysophospholipid, both of which are lipid signaling molecules [5]. The free fatty acid produced is frequently arachidonic acid, the precursor of the eicosanoid family of potent inflammatory mediators that includes prostaglandins, thromboxanes, leukotrienes and lipoxins. From a functional point of view, PLA<sub>2</sub>s from mammalian sources have been classified in secreted (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII), Ca<sup>2+</sup>-dependent cytosolic (groups IVA, IVB and IVC), and Ca<sup>2+</sup>-independent cytosolic PLA<sub>2</sub>s (groups VIA-1, VIA-2 and VIB). Of these, only a few have been studied in sufficient detail to elucidate their role in arachidonic acid release and eicosanoid production in mammalian cells.

**Phospholipase C** (PLC) hydrolyses phospholipids at the proximal bond of the glycerol backbone. It has been widely studied for its implication in signal transduction [6]. It catalyses the hydrolysis of PtdIns(4,5)P<sub>2</sub> to inositol (1,4,5)-trisphosphate and DAG in response to the activation of more than 100 different cell surface receptors. The former releases Ca<sup>2+</sup> from intracellular stores and the latter activates various isoforms of protein kinase C (PKC). To date, eleven different PLC enzymes have been identified and classified according to sequence homology: PLCβ<sub>1-4</sub>, PLCγ<sub>1,2</sub>, PLCδ<sub>1-4</sub> and PLCε [7]. They are localised in the cytosol and they are also found associated to membranes. Probably they are not integral membrane proteins since sequence analysis revealed no signs of hydrophobic transmembranal domains. All PLC isoforms contain X and Y domains, which form the catalytic core, as well as various combinations of regulatory domains that are common to

many other signaling proteins. These domains serve to tether the PLC enzymes to the vicinity of their substrate or activators through protein-protein or protein-lipid interactions. The presence of these regulatory domains in PLCs renders them susceptible to different modes of activation [6]. PLC $\beta$  isoforms are activated by heterotrimeric Gq proteins, via  $\alpha$  subunit or  $\beta\gamma$  dimers targeting its C2 and plekstrin homology (PH) domains. The mechanism of PLC $\gamma$  activation is different, being dependent on Src homology 2 (SH2) motifs that permit its association with phosphotyrosine residues of receptor tyrosine kinases. PLC $\delta$  isoforms contain a Ca<sup>2+</sup> binding C2 domain and a PH-PtdIns(4,5)P<sub>2</sub> binding domain that are involved in its mechanism of activation. The last isoform to be identified, PLC $\epsilon$ , has a Ras binding domain that permits its activation upon nucleotide exchange on Ras GTPase.

The existence of a **phospholipase D** (PLD) activity was first described in plants, and PLD was the first identified PtdCho-hydrolysing enzyme to be purified and successfully cloned [8]. PLD catalyses the hydrolysis of the major membrane phospholipid, PtdCho, to produce PtdOH and choline. The PtdOH can then be converted to DAG by the ubiquitous enzyme PtdOH phosphohydrolase, or be deacylated by PLA2 or PLA1 to produce lysoPtdOH. Thus, PtdCho turnover can result in the generation of PtdOH, DAG and lysoPtdOH as potential second messengers. Compared to transient PtdIns-derived DAG, accumulation of PtdCho-derived DAG is more persistent in time and accounts for most of the DAG mass formation [9]. For that reason it was suggested that PtdCho-derived DAG could elicit long term PKC activation. However, recent evidences suggest that this is unlikely, as both DAG pools are different molecular entities with different properties towards PKC activation. Unlike phosphatidylinositol-derived DAG, those generated from PtdCho degradation are reported to contain both acyl and alkyl linkages. Alkyl-DAG does not activate either classical or new PKCs, suggesting a different role for its stimulated formation [9].

Studies reporting the existence of PLD activity in mammalian cells date from early 80s [10] and prior attempts at purifying the enzyme failed due to lack of knowledge about their regulators and relative low abundance. The cloning of the first plant PLD and the subsequent realisation that the yeast sporulation gene SPO14 had sequence homology to

plant PLD led to the identification of yeast SPO14 as the yeast PLD [11]. Later, counterparts were found to be present in higher organisms and mammalian cells [12-15]. To date, a great variety of signal molecules acting through specific receptors are reported to modulate PLD activity in different tissues and cell types: neurotransmitters (acetylcholine, glutamate, histamine, bradykinin, noradrenaline), immunoreceptors (Fc $\gamma$ R, Fc $\epsilon$ RI), hormones (vasopresin, gonadotrophin releasing hormone), components of extracellular matrix (collagen, laminin, fibronectin), growth factors (EGF, PDGF), cytokines (TNF $\alpha$ ), oxidants (H<sub>2</sub>O<sub>2</sub>) and lipid molecules (lysoPtdOH)(reviewed in [16-18]). Because a good part of the work presented in this thesis has been focused on this specific phospholipase, it will be analysed further in detail in the next section.

## 1.1 Phospholipase D

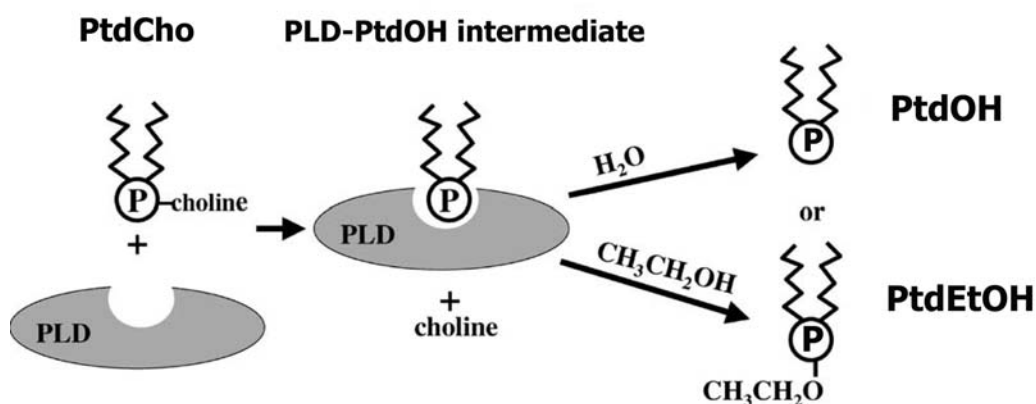
### 1.1.1 Reactions catalysed by PLD

The catalysis of PtdCho by PLD involves two steps as described in Fig. 2 [19]. In the first step, a covalent phosphatidyl-enzyme intermediate is formed and choline is released. In a second step the phosphatidyl group is transferred to water, yielding PtdOH. A hallmark of PLDs is the ability to use primary alcohols as nucleophiles instead of water (transphosphatidylation reaction) leading to the production of phosphatidylalcohols instead of PtdOH [20]. Transphosphatidylation is specific for primary alcohols, which are preferred over water by at least 1000 fold. If used in the millimolar range (50-100mM), primary alcohols can prevent up to 70% the formation of PtdOH by PLD. Therefore, they can be used *in vivo* as inhibitors of PtdOH formation in response to physiological activators of PLD. The affinity of the enzyme towards alcohols depends on the length of the chain, being 1-butanol the one with lower K<sub>m</sub>. For branched alcohols, activity increases with distance from the alcohol group to the branch point. Thus, iso-butanol is a transphosphatidylation substrate but secondary and tertiary butanol are not.

Due to the the lack of more specific inhibitors of PLD activity, **the transphosphatidylation reaction has been successfully exploited to unmask the function of PLD-derived PtdOH in different cellular processes** [21, 22]. This use relies on the assumption that either PtdOH or its derivate products (lysoPtdOH and DAG) are the active second messengers. Secondary alcohols are generally used as internal controls for the non-

specific effects of such pleiotropic compounds, since they are bad PLD substrates. Additionally, because of the relative stability of phosphatidylalcohols (in contrast to PtdOH, which is rapidly metabolised), **transphosphatidylation is often the method of choice for the determination of PLD activity.**

To date, the transphosphatidylation reaction is considered a unique property of enzymes of the PLD superfamily.



**Figure 2. PLD catalysed hydrolysis and transphosphatidylation reactions.** The first part of the reaction involves the formation of a PtdOH-PLD intermediate. Either water or a primary alcohol can act as a nucleophile in the second step of the reaction. In the presence of a primary alcohol (i.e. ethanol or butan-1-ol) the reaction product is a phosphatidylalcohol. In the absence of alcohols, the hydrolysis reaction yields PtdOH. This lipid is considered the molecule responsible for PLD-mediated cellular functions. No functions have been attributed yet to phosphatidylalcohols.

### 1.1.2 Structure of PLD1 and PLD2

The limited but significant similarity shared by plant, yeast and mammalian PLDs comprise a gene family. These PLD genes all belong to an extended gene superfamily that includes poxvirus envelope proteins, bacterial endonucleases, K4 protein, *Yersinia pestis* murine toxin and, interestingly, the bacterial phosphatidyltransferases cardiolipine synthase and phosphatidylserine synthase [23]. These phosphotransferases catalyse reactions where an alcohol (phosphatidylglycerol and serine, respectively) performs a nucleophilic attack over a phosphodiester bond (phosphatidylglycerol or CDP-DAG respectively). Taking into account that most PLDs retain the ability to perform transphosphatidylation (use alcohols instead of water in an hydrolysis reaction), it is suggested that PLD derived from

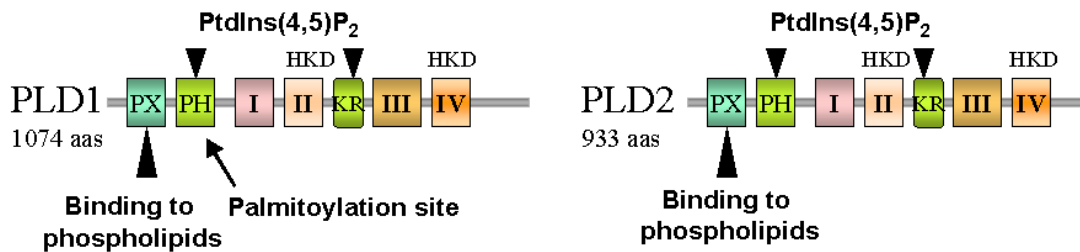
an ancient superfamily of alcohol phosphatidyl transferases [23]. It should be emphasized that while some PLD superfamily members use exclusively the transphosphatidylation reaction, for PLD enzymes this reaction only takes place in the presence of short chain primary alcohols.

Two mammalian PLDs, PLD1 and PLD2, have been cloned to date. Mammalian PLD1 was cloned from a HeLa cell cDNA library and was found to encode a 1074 amino acid protein [12]. PLD2 was cloned from a rat brain cDNA library using highly degenerate PCR primers corresponding to conserved regions among various eukaryote PLDs, and it was found to be a 933 amino acid protein sharing some 55% sequence identity with PLD1 and with different regulatory properties [13]. Two splice forms of each isozyme have been described, PLD1a, PLD1b, PLD2a and PLD2b [24, 25]. Sequence analysis studies on members of the PLD superfamily revealed four motifs of conserved sequence, I-IV and no obvious transmembrane(s) domain(s) (see Fig. 3). Yet, PLDs must interact with a phospholipid substrate in cell membranes and thus must either be constitutively associated with, or transiently recruited to the membrane through a membrane-interaction module. At least three these lipid-interacting modules are reported to be present in mammalian PLD isoforms. All isoforms show the existence of a putative Phox homology domain (PX) followed by a Plekstrin Homology (PH) domain which both bind polyphosphoinositides. A region between motifs II and III, rich in basic and aromatic aminoacids, is well conserved between PLD1 and PLD2 and is also reported as a site of interaction with phosphoinositides [26]. PLD1 is found to be postranslationally modified by palmitoylation in cysteine residues 240 and 241, which lie within the putative PH domain, and this is reported to influence the localisation and association of PLD1 to membranes [27] (see fig. 3).

All known forms of PLDs show the existence of four domains of conserved sequence (I-IV). Conserved Region II (also known as HKD motif) is defined by the presence of the sequence  $HxK(x)_4D(x)_6GSxN$ , where x denotes any aminoacid. This domain is found duplicate and is thought to be involved in catalysis. The crystal structure of a bacterial PLD confirms that HKD motifs bind to a phosphodiester bond [28]. Also from the structure of a PLD superfamily member, the involvement of HKD motifs in catalysis was suggested: the two HKD motifs would form a single active site, a histidine from one motif would serve as

a nucleophile in the reaction forming a phosphoenzyme intermediate, while a histidine from the other motif would be a general acid that functions in the hydrolysis of the phosphodiester bond [29].

Analysis of proteins containing HKD motifs in *homo sapiens* identified three proteins: PLD1, PLD2 and K4. The K4 gene is also found in *Caenorhabditis elegans*, *Dictyostelium discoideum* and mouse. Interestingly, mouse K4 gene (also called SAM-9) is expressed in mature neurons of the forebrain and its expression appears to be turned on at late stages of neuritogenesis [30]. However, the enzymatic reaction catalysed by K4 remains to be characterised.



**Figure 3. Domain structure of PLD1 and PLD2.** Regions of conserved sequence are shown. PX, phox homology domain; PH, plekstrin homology domain; motifs I,II,III and IV, regions of sequence conserved among all PLD isozymes from different species. Motifs II and IV are also named HKD motifs and contain residues essential for catalysis. Phosphoinositide interacting regions and the palmitoylation site on PLD1 are marked by arrows.

### 1.1.3 Expression and subcellular distribution

Homologues of PLD1 and PLD2 are found throughout the animal kingdom. Measurements of biochemical activity indicate that PLD is expressed in most cells, although expression of a specific isozyme varies widely within tissues and cell lines. Antibodies to PLD are available, but detection of endogenous protein is often a problem due to a combination of low levels of expression and also low affinity of the antibody for the antigen. Therefore, the majority of the data regarding PLD expression are derived from analysis of mRNA levels [31-33]. Most cells seem to express both isoforms of PLD with few exceptions [31]. Interestingly, no PLD expression has been detected in mature lymphocytes and we have been unable to detect stimulated PLD activity in cerebral cortical

neurons in primary culture [34]. In that sense the glia seems to account for most of the PLD activity found in central nervous system. In situ hybridization histochemistry in the brain of developing and mature rats revealed that PLD1 mRNA expression was mainly found in presumptive oligodendrocytes, while PLD2 mRNA expression was detected in presumptive astrocytes [32]. Furthermore, PLD1 was found to be upregulated in astrocytes in response to transient ischemia [35]. Considering that the mammalian brain is one of the organs with the highest PLD specific activity, the finding that neurons are not the major PLD-expressing cell types is rather unexpected.

Many reports reveal that expression of mRNA for both PLD1 and PLD2 can be regulated at the transcriptional level by growth and differentiation factors in primary and cultured cell lines. For example, in primary mouse keratinocytes, 1,25-dihydroxyvitamin D3 induces PLD1 expression during differentiation [36]. In human HL60 cells, differentiation to neutrophil-like phenotype leads to an increase in both PLD1 and PLD2 expression [37, 38]. PLD activity has also been shown to be significantly elevated in human cancers suggesting that PLD might be implicated in tumorigenesis [39-41].

Since localisation is key to understand function, several studies have focussed on the precise subcellular localisation of PLD enzymes. Overexpression of tagged forms of PLD have been used to try to establish its precise location. Green Fluorescent Protein-tagged PLD1 (GFP-PLD1), when expressed in a mast cell line (RBL-2H3), was localised to a lysosomal/endosomal compartment [42, 43], and some studies have reported recruitment to plasma membrane upon stimulation [44]. In PC12 cells, Hela cells and rat embryo fibroblasts, GFP- or HA-tagged PLD2 was localised to the plasma membrane [45, 46], whilst in HT29-c119A epithelial cells it was localised at the Golgi compartment [47]. In PAE cells, GFP-PLD2 was localised in a submembraneous vesicular compartment [48]. Thus, the localisation of PLD isoenzymes seems to be dependent on cell type. It is noteworthy to mention that the data based on overexpression of tagged-PLDs could be misleading, since there is no guarantee that the overexpressed tagged enzyme localises to the same compartment as the endogenous protein. A few studies based on whole cell fractionation on continuous linear sucrose gradients have addressed the subcellular localisation of endogenous PLDs. In these works, PLD activity was found to colocalise

with specific markers of the plasma membrane, endoplasmatic reticulum and the Golgi compartment [49].

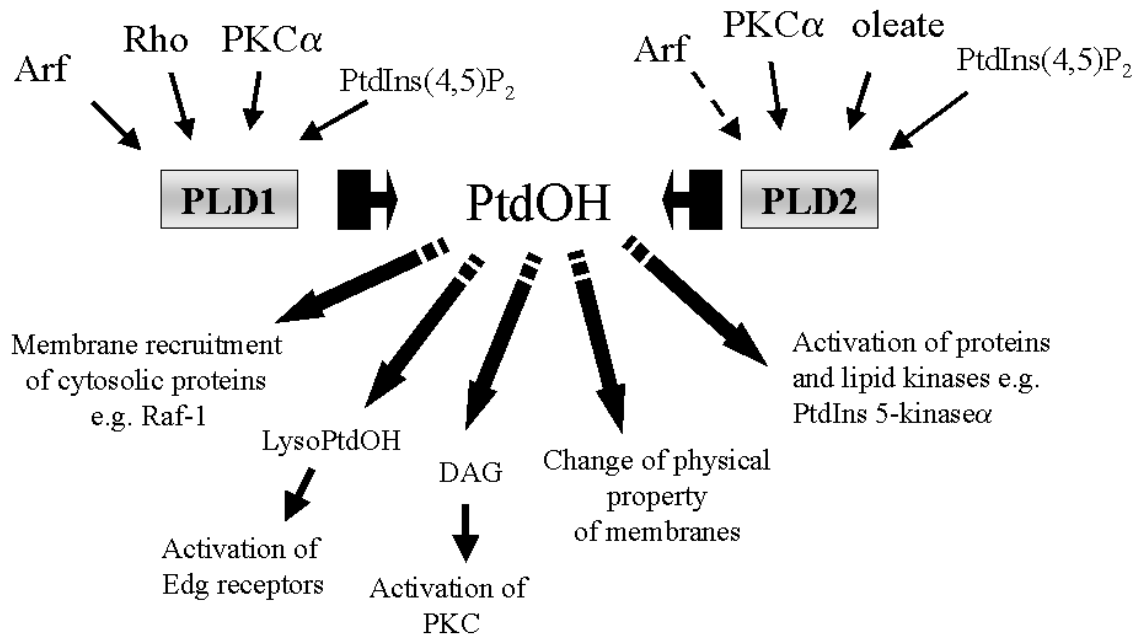
PLD activity is found to be enriched in low-density detergent-insoluble membrane microdomains that contain the caveolae marker proteins caveolin-1 and caveolin-2. Caveolae are plasma membrane ‘flasked shaped’ invaginations, 50-100 nm in diameter, that are seen in cells from epithelial and mesenchymal origin. PLD2 and also PLD1 were found in caveolin-rich membranes from different cell types including human keratinocytes, human breast cancer cells, Cos-7 and promonocytic U937 cells [16].

In table 1 (page 19) the reader will find a list summarising the different cellular compartments and organelles where either PLD isoforms or PLD activity have been found.

#### 1.1.4 Regulation of PLD1 and PLD2

There is substantial evidence that receptor-mediated regulation of PLD is complex. A very large number of agonists increase the activity of PLD in many cell types acting through G-protein-coupled receptors, receptors for growth factors and immuno-receptors (see table 2). Many of these receptors also activate PLC activity, which in turn activates PKC. This kinase is considered a major PLD activator for many different cell types. PLD activation is also in part mediated by small G proteins of ARF and Rho families. A great bulk of data also point towards dependence on phosphoinositides. PtdIns(4,5)P<sub>2</sub> is now established as a cofactor required for activity. All these effectors interact directly with PLD enzymes, can act alone to stimulate PLD, but in physiological conditions act in combination to elicit a synergistic activation [50, 51]. Although PLD1 and PLD2 share the common PLD catalytic domain and are dependent on phosphoinositides for activity, the regulation of these proteins is very different. PLD1 has low basal activity that can be stimulated by ARF and Rho GTPases and certain PKC isoforms [16-18]. In comparison to PLD1, recombinant PLD2 exhibits high basal activity when expressed in mammalian cells and when assayed *in vitro* and is only mildly activated by ARF proteins [15] (see fig.4).





**Figure 4. Intracellular regulators of PLD1 and PLD2.** Two families of small GTPases (ARF and Rho) and PKC isozymes are involved in the activation of PLDs by extracellular signals. PtdIns(4,5)P<sub>2</sub> is required as a cofactor for the PLD reaction to take place. Some studies suggested that oleate and other unsaturated fatty acids might be specific activators of PLD2. Some potential functions of PtdOH and its metabolites (lysoPtdOH and DAG) are also indicated.

#### 1.1.4.1 Regulation by protein kinase C.

Protein kinase C (PKC) is an extensive group of serine/threonine kinases. As much as eleven different isoforms have been identified and classified according to sequence homology in three families: classical (cPKC:  $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$ ), new (nPKC:  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$  and  $\mu$ ) and atypical (aPKC:  $\zeta$  and  $\lambda$ ). Classical and new PKCs contain a Cys-rich conserved region (C1) responsible for DAG binding. Phorbol esters, DAG analogues that irreversibly activate PKCs, also bind to this specific region. Classical PKCs also contain a conserved region (C2 domain) that binds Ca<sup>2+</sup> and phospholipids and which is responsible for PKC translocation to membranes [52]. As mentioned before, a very large number of agonists increase the activity of PLD and many of these receptors activate also PLC, leading to increases in cytosolic Ca<sup>2+</sup> and DAG, which in turn activate PKC isoforms. The phorbol ester PMA is a potent activator of PLD activity in most cell types implying somehow that PKC activation may be upstream to PLD activation. A characteristic of PKC is that its prolonged stimulation results in desensitization by a mechanism that involves proteolytic

cleavage of the enzyme [52]. Many studies report that down-regulation of PKC by prolonged phorbol ester treatment impairs receptor-mediated PLD activation [53, 54].

The current mechanism mediating PKC activation of PLD isoforms remains without consensus. While some studies have shown that PKC can directly activate PLD by means of protein-protein interaction [51], others suggested that a phosphorylation-dependent mechanism was required [54].

When overexpressed in Cos7 or HEK-293 cell lines, activity of both PLD1 and PLD2 is greatly stimulated by phorbol ester treatment [15]. Furthermore, purified PKC $\alpha$ , - $\beta$ I and - $\beta$ II all stimulate PLD1 activity *in vitro* in a manner that is independent of, but synergistic with, ARF and Rho GTPases [51, 54].

*In vivo*, PLD1 is phosphorylated by PKC $\alpha$  at serine 2, threonine 147 (located in the PX domain) and serine 561 (loop region) and mutation of any of these residues leads to significant reductions in phorbol ester-induced PLD1 activity [55]. *In vitro*, activation of PLD1 by PKC is independent of the catalytic activity, since it does not require ATP [56], and the purified regulatory domain of PKC $\alpha$  either isolated from tissues, prepared *in vitro* by proteolysis of the holoenzyme, or expressed recombinantly, is an effective activator of PLD [57]. *In vivo*, however, the PKC regulation of PLD might involve a phosphorylation-dependent step, since selective PKC inhibitors (bisindolemaleimides, chelerytrine) frequently attenuate phorbol ester and receptor-stimulated PLD activity [53]. In that sense, PLD2 shows no regulation by PKC *in vitro*, but when overexpressed in Cos-7 or RBL-2H3 cells, it is significantly stimulated by phorbol esters (personal observations).

Some studies have reported an *in vitro* direct protein-protein association of PKC $\alpha$  with PLD1 and PLD2 [57, 58]. From mutational studies of PLD1, the site of interaction with PKC $\alpha$  lies in the N-terminal 340 amino acids of the protein [59]. Deletion of this region produces a catalitically active PLD1 that retains wild-type capacity for activation by ARF and Rho, but is completely unresponsive to PKC $\alpha$ .

#### 1.1.4.2 Regulation by small GTPases

The activation of PLDs is complex and PKC alone does not account for all the story. A number of reports described PLD activities in mammalian tissues and cell lines that were stimulated by guanine nucleotides. Researchers began to search for GTP-binding protein

regulators of PLD activity and, on the basis of reconstitution experiments, the cytosolic factors responsible for this activation were identified as the small G proteins of the ARF and Rho families [60, 61].

**ADP-ribosylation factors (ARFs)** were first described by their ability to activate the cholera-toxin-mediated ADP-ribosylation of the  $\alpha$  subunit of the heterotrimeric G-protein  $G_s$ . They have since been shown to play a role in the regulation of vesicular trafficking events. The ARF mediated GTP-dependent assembly of soluble coat complexes onto Golgi and endosomal membranes is considered critical for both maintenance of organelle structure and membrane traffic [62]. To date, six different mammalian ARF proteins have been identified, with homologues also described in plants, insects, and budding and fission yeasts. The fact of an ARF homologue being found in the simplest eukaryote, the protist *Giardia lamblia*, together with the high level of conservation across phylogenetic lines, suggests that ARF may have arisen early in evolution when intracellular compartments appeared [63]. As early cited, PLD1 basal activity is low and can be strongly stimulated by any ARF member *in vitro*, while recombinantly expressed PLD2 shows high basal activity that can be stimulated by ARF proteins [60, 61, 64]. Interestingly, stimulation of PLD2 by ARFs is more pronounced when its high basal activity is reduced through removal of the N-terminal 308 aminoacids [65]. The individual ARF proteins do not appear to differ significantly in their relative capacities to activate PLD1 or PLD2, but there are differences in their subcellular localisation. ARF6 is the only ARF member that is localised in the plasma membrane upon stimulation [66], while expression of ARF1 to 5 seem to be restricted to intracellular compartments. Activation of ARF6 through nucleotide exchange catalysed by specific proteins called ARF-GEFs (ARF Guanyl-nucleotide Exchange Factors) triggers ARF6 accumulation at the plasma membrane. Then, active ARF6 initiates cortical actin changes that result in formation of actin-containing surface protrusions and accelerated membrane recycling [67]. ARNO (ARF nucleotide binding site opener) is a well studied ARF-GEF that is presumed to catalyse nucleotide exchange of ARF6 at the plasma membrane. It consists of an N-terminal coiled-coil region, a PH domain presumably involved in membrane tethering, and a Sec7 domain which is responsible for the exchange factor activity. Sec7 domains of several ARF-GEFs are targeted by the fungal toxin brefeldin A. The addition of this drug to cell cultures prevents agonist-stimulated PLD

activation, albeit at much higher concentrations than those required for disruption of the Golgi apparatus [68-70]. A role of PLD mediating ARF6 actions at the plasma membrane has been suggested and studies on subcellular localisation of PLDs suggests PLD2 might be the isoform involved [15].

**Rho family GTPases** are involved in cell motility and cytoskeletal rearrangements. Rho family members were first reported to stimulate PLD activities in neutrophils, liver and HL60 cells [71, 72]. The cloning, expression and purification of PLD1 revealed that Rho family GTPases ( Rho, Rac and Cdc42) are all activators of PLD. The stimulatory effects of these GTP-binding proteins on PLD activity are GTP-dependent and require modification of the proteins by prenylation. A C-terminal RhoA-interacting region has been described for PLD1 [73]. The use of clostridial toxins also contributed to the study of Rho proteins and its role in PLD activation. C3 exoenzyme from *Clostridium botulinum* inactivates Rho proteins by ADP-ribosylation while *Clostridium difficile* toxins A and B do it by monoglycosylation [74, 75]. Treatment of cells with clostridial toxins results in inhibition of PLD activation elicited by a wide range of agonists and also *in vitro* activation of PLD by hydrolysis resistant GTP analogs (i.e. GTP $\gamma$ S) [74-76]. Furthermore, the inhibition of Rho proteins by Rho-GDI in membranes from HL60 cells abolished the GTP $\gamma$ S stimulated activation of PLD [68] while constitutive active forms of Rho (V14RhoA) were found to enhance PLD activity in PLD1 transfected Cos-7 cells [77].

The relative contribution of PKC, ARF and Rho proteins to PLD activation is cell-type dependent and is reported to be synergistic [ 50, 51]. In physiological conditions, they may act in combination to elicit a full PLD activation. For cultured astrocytes, glutamate-mediated PLD activation is reported to be PKC and ARF dependent, but Rho independent [53]. For RBL-2H3 mast cells, antigen-mediated IgE cross-linking activation of PLD is mainly ARF dependent [78].

#### 1.1.4.3 Direct and indirect regulation of PLD by phosphoinositides

The molecular cloning and subsequent expression and purification of PLD1 and PLD2 clearly established that, when measured *in vitro* with substrate-containing liposomes,

activity of both enzymes was stimulated by **PtdIns(4,5)P<sub>2</sub>**. Activity of the yeast PLD Spo14 and three plant PLDs are also reported to depend on PtdIns(4,5)P<sub>2</sub> for activity [16]. Binding of PLD2 to sucrose-loaded liposomes is highly dependent on PtdIns(4,5)P<sub>2</sub>, and this enzyme can be labelled with a photoreactive derivative of PtdIns(4,5)P<sub>2</sub>, indicating that the protein contains selective binding sites for this lipid. Along the PLD sequence, two sites of interaction with PtdIns(4,5)P<sub>2</sub> have been reported, a PH domain and a conserved region of basic aminoacids located between motifs II and III denoted as KR motif [24, 26]. The *in vitro* stimulating effects of phosphoinositides on PLD activity may arise in part from a tethering effect, by anchoring the enzyme on a substrate-containing surface and thus increasing its catalytic efficiency. In one study, point mutations within the PLD1-PH domain were reported to inhibit PLD1b enzyme activity, and deletion of the domain inhibited enzyme activity and disrupted normal PLD1 localisation [79]. In other studies however, removal of PH domain from PLD1 and PLD2 produced catalytically active proteins that were stimulated by phosphoinositides in an identical manner to the wild-type enzymes [26, 80]. Along the PLD sequence, another phosphoinositide binding domain has been reported: the KR motif. Its mutation results in the loss of PtdIns(4,5)P<sub>2</sub>-dependent activation, although the enzyme localises normally [26].

Some studies reported inhibition of PLD by proteins that hydrolyse PtdIns(4,5)P<sub>2</sub>. Synaptojanin and fodrin are two examples of proteins with polyphosphoinositide 5-phosphatase activity that inhibited PLD [81]. It is worth mentioning that since PtdIns(4,5)P<sub>2</sub> is substrate for PLC, the reported inhibition of PLD could just be a PLC-mediated phenomenon.

In addition to the direct effects of phosphoinositides on PLD activity, several of the identified protein activators of PLDs are themselves regulated by these lipids. These indirect roles of phosphoinositides on PLD regulation may be as important as the direct interaction with the enzyme. In particular, phosphoinositides clearly control ARF activation through guanine nucleotide exchangers (GEFs). All ARF-GEFs identified to date possess a Sec7 domain, a module of approximately 200 amino acids that is sufficient to catalyse exchange of GDP for GTP on ARF *in vitro*. In addition to this catalytic module, three identified mammalian ARF-GEFs families (ARNO/cyohesin/GRP) also contain a phosphoinositide-interacting PH domain that mediates its targeting to membranes (for a

good review on ARF regulators see [82]). The mechanism of ARF activation by ARNO has been studied in some detail. This widely expressed ARF-GEF has a PH domain that binds to PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> with high specificity. ARNO-mediated ARF activation is clearly phosphoinositide-dependent: a rise in PtdIns(4,5)P<sub>2</sub> or PtdIns(3,4,5)P<sub>2</sub> levels mediates recruitment of ARNO to membrane surfaces, and there interacts with membrane-bound ARF and triggers the nucleotide exchange. In that sense, some studies have reported that agonist-elicited ARNO translocation to the plasma membrane required phosphoinositide-3 kinase (PI3K) activation [83].

**Table 1.** Localisation of phospholipase D activities or isozymes in specific subcellular compartments and organelles (modified from Liscovitch et al, 1999 [16]).

Cell compartment or organelle	PLD activity or isozyme	References
Plasma membrane	Oleate-activated PLD	[120]
	GTPγS-stimulated PLD	[121]
	ARF/RhoA-stimulated PLD	[49]
	PLD2	[15]
Secretory granules	ARF-dependent PLD	[122]
	PLD1a and PLD1b	[44]
Golgi apparatus	ARF-stimulated PLD	[102],[103]
Endoplasmatic reticulum	PMA-stimulated PLD (in vivo)	[123]
Nucleus	ARF/RhoA-stimulated PLD	[124],[125]
	Oleate-activated PLD	[125],[126]
Mitochondria	Ca <sup>2+</sup> -dependent PE-PLD	[127]
Caveolae	PIP <sub>2</sub> -stimulated PLD	[128],[16]
Cytoskeleton	ARF/RhoA-stimulated PLD	[129]
Cytosol	PI/PE-preferring PLD	[130]
	ARF/RhoA-dependent PLD	[131],[132]

**Table 2.** Examples of PLD activation in primary tissues and cultured cell lines mediated by ARF, Rho and PKC(modified from Cockcroft S.(2001), [18])

Cell type	Agonist	Comments	References
Neutrophils	FMLP; monosodium urate crystals	dependent on ARF,PKC $\alpha$ and Rho	[110],[111]
Human airway epithelial adenocarcinoma A549 cells	bradykinin	involves RhoA	[71]
Fibroblasts	PDGF	dependent on ARF proteins but not Rho	[71]
Vascular smooth muscle cells	angiotensin II,endothelin-1,PDGF	stimulation is ARF-dependent and is via PLD2	[112]
PC12 cells	bradykinin	activates PLD2 via PKC $\delta$	[113]
HEK 293 cells expressing the M3 muscarinic receptor	carbachol	mediated by ARF and Rho	[114]
RBL-2H3 mast cells	antigen cross-linking of IgE	dependent on ARF proteins	[78]
Avian DT40B cells	antigen B cell receptor	Syk,Btk and PLC $\gamma$ 2 dependent	[115]
FRTL-5 thyroid cells	TSH	activation of PLD1 is dependent on ARF and Rho	[116]
Mouse embryo fibroblasts	PDGF	deletion of PLC $\gamma$ 1 inhibits activation of PLD	[117]
Rat-1 fibroblasts	EGF	dependent on Rho	[74]
Astrocytes	PMA, adrenalin	requires ARF, Rho and PKC for full activation	[118],[119]
Astrocytes	glutamate	PKC-dependent and Rho independent mechanism	[53]
Astrocytes	Endothelin-1	PKC-dependent	[34]

### 1.1.5 Cellular functions of PLD

The majority of the studies implicating PLD in a particular cellular event relied on the use of alcohols. As a control for the effects of such a pleiotropic compound, secondary and tertiary alcohols have often been employed. This strategy has to face the fact that because of water is present at high enough concentrations, transphosphatidylation reaction is never complete and some PtdOH will always be formed in presence of alcohols [20].

#### 1.1.5.1 Cell proliferation

The factors that promote cellular growth act through specific receptors on the plasma membrane that communicate with the nucleus, in a process that ends up with the duplication of the DNA content and the formation of two daughter cells. Growth factors can be classified according to the type of receptors that they activate. Firstly, there are growth factors, such as platelet-derived growth factor (PDGF) or epidermal growth factor (EGF), that act on receptors with a single transmembrane domain possessing intrinsic tyrosine kinase activity on the cytoplasmatic side. Secondly, there are growth factors, such as endothelin-1 (ET-1) or angiotensin II, that act on cell surface receptors with seven transmembrane domains that are coupled by G proteins to their primary effector mechanisms. **Table 2** compiles an extense list of examples where activation of PLD by growth factors has been reported. In addition, phorbol esters, which are among the most potent PLD activators for a wide variety of cell types, also cause hyperproliferation. These and later observations pointed towards PLD being part of the complex machinery that mediates proliferative signaling. Some further evidences are the following:

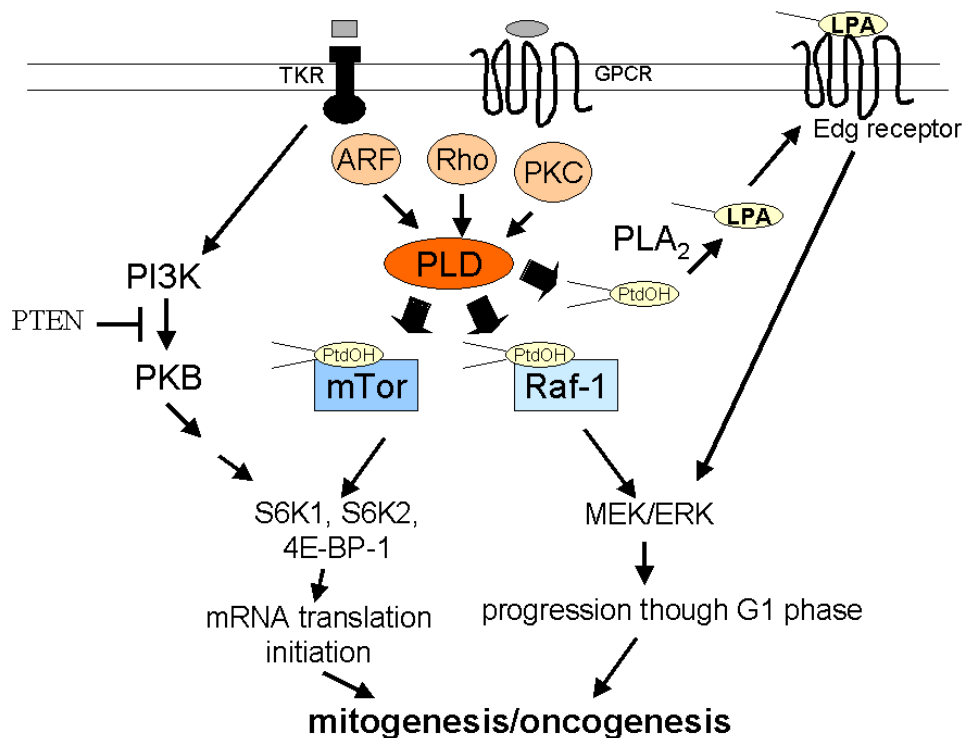
- a) Exogenously added PLD has mitogenic activity in various cell types including astrocytes [84-86, 22].
- b) Increased basal PLD activity has been reported for different carcinomas [38-41].
- c) Overexpression of PLD isozymes induces neoplastic transformation of GP+envAM 12 fibroblasts and generate tumours when injected into immunodepressed BALB/C mice [87]. The levels of cyclin D3 protein, known as an activator of G1 to S cell cycle phase transition, was found aberrantly high in cells overexpressing PLD1 and PLD2 compared to control cells.



- d) PLD activity is elevated in cells transformed by several oncogenes including v-Ras, v-Src, v-Raf and v-Fps, implying chronic stimulation of the PtdCho turnover in these transformed cells [84, 88-92].
- e) Primary alcohols can inhibit serum or growth factor-elicited cell proliferation [22, 93].
- f) PLD-derived products like lysophosphatidic acid (LPA), generated by deacylation of PtdOH by PLA<sub>2</sub>, can promote cell growth acting through an extensive family of specific G protein coupled receptors (Edg receptors) [94]. Some ovarian cancers have been found to secrete LPA, which would collaborate to the growth of the tumour by acting in a paracrine/autocrine manner [95, 96]. LPA is present in serum at micromolar concentrations (1-20µM), where it is mainly produced by stimulated platelets. LPA is thought to be a main contributor to the mitogenic effect of serum [97].

Several studies have identified Raf-1 as a downstream target of PLD activation [98, 99]. Raf-1 is a member of a serine/threonine protein kinase family (Raf-1, A-Raf and B-Raf) that plays a central role in the transmission of mitogenic signals from cell surface receptors to the nucleus via the mitogen activated protein kinase (MAPK) pathway. Gosh *et al.* developed an in vitro assay to determine the lipid binding of Raf proteins and demonstrated that a C-terminal domain of Raf-1 interacted strongly with PtdOH [98]. The interaction appeared to be quite specific for PtdOH, since other lipids tested did not bind under high-stringency conditions. Stimulation of cells with a phorbol ester caused a translocation of Raf-1 to the plasma membrane, and this translocation was inhibited by ethanol. The effect of ethanol was somehow selective on Raf, since it did not affect PKC $\alpha$  translocation. The same authors suggest that PLD-derived PtdOH facilitates the translocation of Raf-1 to the plasma membrane. They propose that activation of both Ras and PLD would create the environment to firmly anchor Raf-1 to the membrane. Moreover, PtdOH was found to be required for Raf-1 translocation in response to a physiological stimulus (insulin) [99]. Interestingly, many signals that activate the MAPK cascade through activation of Ras also activate PLD. The identification of PtdOH as participant in Raf-1 activation places PLD in a position to exert influence in the transduction of proliferative signals. It also points to a new mechanism that helps to explain the proliferative actions of phorbol esters.

Another link between PtdOH and cell proliferation comes from recent observations of PtdOH-mediated mitogenic activation of the mammalian target of rapamycin (mTor) [100, 101]. mTor governs cell growth and proliferation by mediating the mitogen and nutrient-dependent signal transduction that regulates mRNA translation initiation. A domain in mTor is reported to bind specifically to PtdOH-containing vesicles, and this interaction was required for the activation of downstream effectors as ribosomal subunit 6 kinase-1 and -2 (S6K1, S6K2). Furthermore, butanol efficiently prevented S6K1 and S6K2 activations.



**Figure 5. Phosphatidic acid (PtdOH) on mitogenic signaling.** mTor and Raf-1 require binding to PtdOH to activate downstream effectors, which ultimately lead to cell proliferation. The best well-known function of mTor is the regulation of translation initiation, a process that is mediated by ribosomal subunit S6 kinases 1 and 2 (S6K1, S6K2) and also eukaryotic initiation factor 4E-binding protein-1(4E-BP-1). Full activation of these factors require from the activation of PtdIns 3-kinase (PI3K) and protein kinase B (PKB). PTEN is a oncogenic phosphatase that dephosphorylates PtdIns-3P to yield PtdIns. Raf-1 is a Ser/Thr kinase that couples growth factor receptor stimulation to the activation of the ERK signaling pathway. ERKs trigger the phosphorylation and hence activation of AP1 and ETS transcription factors, turning on the expression of genes like cyclin D, which is required for progression through G1 phase of the cell cycle. PtdOH can be deacylated by a phospholipase A<sub>2</sub> (PLA<sub>2</sub>) to yield lysoPtdOH (LPA). This lipid messenger can stimulate in an autocrine/paracrine manner Edg receptors located on the plasma membrane and activate ERK signaling pathway. TRK, tyrosine kinase receptor; GPCR, G protein-coupled receptor.

### 1.1.5.2 Membrane trafficking.

PLD activity has been implicated in various trafficking events that include formation of COP1-coated Golgi vesicles [102], release of AP-1-coated vesicles from the Trans-Golgi Network (TGN) [103], recruitment of AP-2 in endosomes [104], and budding of endocytic vesicles in the plasma membrane [99] (see fig.6). In many cases, the involvement of PLD in these processes remain poorly defined and the implication of PLD is suggested mostly by virtue of it being an ARF-regulated enzyme. Evidence in support of a role of PLD is again based on the use of alcohols and the localisation of the PLD in those membrane compartments (see table 1). It is hypothesized that different ARF proteins would mediate PLD activation in those compartments, and the resultant production of PtdOH would be required for the recruitment of soluble coat-protein complexes [16]. PtdOH may also activate PtdIns(4,5)P<sub>2</sub> synthesis by activating type I phosphatidylinositol-4 phosphate 5-kinase (PIP5K) [105]. This lipid would allow the recruitment of proteins containing PH domains and facilitate the assembly of the protein complex required for coating. In some experiments, in particular those regarding the involvement of PLD in the release of nascent secretory vesicles from TGN, the implication of PLD is contentious, since butanol was used at high concentrations (1.5%) compared to the usual requirements of 0.2-0.5%. Therefore, whether the observed effects are due to disruption of PLD-derived PtdOH formation has to be questioned.

PLD has also been implicated in exocytosis. Early studies in mast cells identified a requirement for Ca<sup>2+</sup> and G-proteins for this process, and it was concluded that activation of G proteins was sufficient to drive the fusion machinery in mast cells. Later studies in permeabilised cells identified the key role of ARF proteins in neutrophil and mast cell exocytosis [78, 106]. These studies made use of Streptolysin O (SLO), a 69 KDa pore-forming bacterial toxin employed for controlled permeabilisation of cell membranes. When intact cells are treated with SLO, the toxin binds to the plasma membrane via interaction with cholesterol and oligomerises generating 30-50 nm diameter pores, which allow free passage of cytosolic proteins. Cytosol-depleted mast cells do not secrete when they are challenged with GTPγS or antigen, but secretion is restored simply by readdition of exogenous ARF proteins [78, 106]. More importantly, reconstitution of secretion by ARF

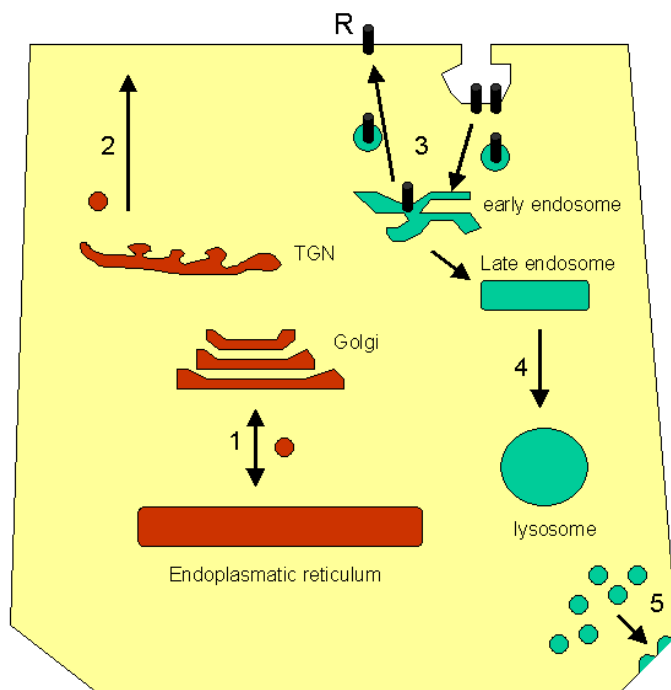
was inhibited by primary alcohols, indicating that production of PtdOH was vital for the process [78].

Another trafficking event that might require PLD activity is receptor-mediated endocytosis. This process involves physical removal of receptors upon its activation in endocytic structures, and serves as a negative feedback that prevents excessive input signaling. A recent study reports that endocytosis of the EGF receptor was accelerated by overexpression of PLD1 and was retarded by overexpression of catalytically inactive mutants of either PLD1 or PLD2 [21].

Assembly of very low density lipoproteins (VLDL) in the liver is also reported to depend on ARF and PLD. In particular, the conversion of the apolipoprotein-containing precursor to VLDL is inhibited by butanol as well as brefeldin A [107].

PLD has been found to play a role in controlling changes in the actin cytoskeleton. The assembly of actin structures in mammalian cells is regulated by members of the Rho family of small GTPases and also ARF members. In particular, RhoA has been implicated in the formation of stress fibers, Cdc42 in filopodia and Rac1 in the formation of membrane ruffles. PLD activation seems to be required for actin stress fiber formation, as it is inhibited by 1-butanol or overexpression of a catalytically inactive form of PLD1 [108]. **Membrane ruffling** is a very dynamic process that requires from an intense cortical actin rearrangement [45]. The small GTPases Rac1 and ARF6 are involved in these cytoskeletal changes [45, 67]. It is generally assumed that these proteins, which are cytosolic in the inactive GDP-bound form, become associated with the plasma membrane upon nucleotide exchange and then drive the cortical actin rearrangements. Cells expressing wild-type ARF6 or Rac1 form actin-containing surface protrusions and membrane ruffles upon stimulation with the G-protein activator aluminium fluoride, while overexpression of dominant negative forms (GTP-binding defective) inhibited the aluminium fluoride-induced ruffling [109]. When membrane ruffling is monitored in living cells, membrane trafficking events can also be observed, in particular, the constant recycling of plasma membrane to endocytic structures (which then may fuse again with the plasma membrane). It has been suggested that this membrane trafficking might be required for the cortical actin rearrangements [109]. The operation of an ARF6 cycle may account for these traffic events.

In some cell types (i.e. mast cells and related cell lines) ruffling accompanies the exocytosis of secretory vesicles. However, cell types that lack secretory vesicles (i.e. HeLa cells, fibroblasts) also ruffle upon stimulation of surface receptors, implying that secretion and ruffling are independent phenomena.



**Figure 6. Trafficking pathways in which involvement of PLD is inferred. (1)** Vesicular flux between endoplasmic reticulum and the Golgi. **(2)** Vesicular flux from the Trans-Golgi network (TGN) to the plasma membrane. **(3)** Receptor-mediated endocytosis (e.g. internalisation of EGF receptor) **(4)** Internalisation of membrane components to lysosomes (e.g. immune complexes). **(5)** Exocytosis of secretory granules (in neutrophils and mast cells secretory granules are modified lysosomes which can be exocytosed upon stimulation). R stands for receptor.

## **OBJECTIVES**

## 1.

In the introduction we presented the lipid-modifying enzyme PLD as a possible element of the cellular machinery which transduce mitogenic signals to the nucleus. Glial proliferation is a naturally occurring phenomenon in response to any damage or disturbance to the central nervous system. It is of tremendous medical interest, since it affects neuronal repair. Astrocytes are glial cells that express a wide variety of receptors which are coupled to PLD activation and exhibit a robust proliferative response following mitogen exposure. **The first objective was to explore the possible role of PLD on the proliferation of astrocytes in primary culture.** To do so, cultures were stimulated by growth factors and other mitogens and also challenged with non-mitogenic agonists. The aim of this strategy was to determine if correlation between PLD activation and mitogenesis did occur. Primary alcohols, which reduce PLD-derived PtdOH formation, were tested for their ability to prevent cell proliferation by determining [<sup>3</sup>H]thymidine incorporation into DNA. The possible cytotoxic effects associated with the use of alcohols were monitored by viable dye staining and membrane integrity markers (lactate dehydrogenase leakage).

## 2.

In the introduction PLD was also presented as an enzyme which has been involved in vesicular trafficking, receptor-mediated endocytosis and exocytosis events. All these processes involve the fusion of vesicles from different membrane compartments. Also, small GTPases of the Rho family were introduced as activators of PLD that control very dynamic processes such as the remodeling of the actin cytoskeleton, which determine cell shape and motility. In particular, RhoA has been implicated in the formation of stress fibers, Cdc42 in the formation of filopodial extensions and Rac1 in the formation of lamellipodia. We wanted to explore the possible implication of PLD in another cellular event that requires not only the remodelling of the actin cytoskeleton but also an intense membrane recycling: the membrane ruffling. **The second objective was the study of the possible implication of PLD in the membrane ruffling elicited by the antigen-mediated crosslinking of IgE receptors in a mast cell line (RBL cells).** The study was addressed by using alcohols to inhibit PLD-mediated PtdOH formation and by the transient transfection of tagged-PLD isoforms. In addition to PtdOH, we were also interested in the antigen-

stimulated formation of other bioactive lipids, such as PtdIns(4,5)P<sub>2</sub>, which might also be involved in membrane ruffling.

### 3.

The strategies employed when studying the involvement of PLD in cellular responses often make use of alcohols to inhibit PLD-catalysed PtdOH formation and the overexpression of wild-type or catalytically inactive forms. If alcohols are used, there is no way of implicating a specific PLD isoform in a particular cellular function, since both PLD1 and PLD2 are able to catalyse transphosphatidylation reaction and most cells seem to express both isoforms. The transfection of cell lines with tagged PLDs to study their function and subcellular distribution always create the doubt whether the transfected proteins localise to the same cell compartments as the endogenous forms. **In the third of the papers presented we examined the specificity of oleic acid as an activator of PLD2 and whether it could be used to study the subcellular localisation of endogenous PLD2.** Also, potential uses of oleic acid in the study of PLD2-mediated cellular processes, such as membrane ruffling, were explored.



## **RESULTS**

**Phospholipase D activation is not a general requirement for  
agonist-elicited proliferation in cultured astrocytes.**

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## SUMMARY

Phospholipid hydrolysis by phospholipase D (PLD) is believed to play an important role in cell signaling in many tissues including glial cells. Activation of PLD by mitogenic stimuli has been suggested to be involved in the onset of cell proliferation in other cell systems. In this work, we have explored the involvement of PLD in the proliferation of cultured rat brain astrocytes induced by the activation of G-protein-coupled receptors and tyrosine-kinase receptors of a variety of neurotransmitters, neuropeptides and growth factors. PLD activity was determined by measuring the formation of [ $^{32}\text{P}$ ]phosphatidylbutanol in [ $^{32}\text{P}$ ]P<sub>i</sub>-labelled cells stimulated in the presence of butanol, and cell proliferation was quantified by determining [ $^3\text{H}$ ]thymidine incorporation into DNA. We first observed that the ability of the different stimuli to activate cell proliferation was not matched by their efficacy as PLD stimulators. Furthermore, the presence of various short-chain alcohols during stimulation, which reduce the PLD-catalysed formation of phosphatidate, did not modify the incorporation of [ $^3\text{H}$ ]thymidine in cells stimulated by mitogens. Taken together, these results indicate that although several mitogenic stimuli are able to activate PLD, this response is not generally required in the signaling pathway leading to cell proliferation in astrocytes, suggesting that PLD-mediated phospholipid hydrolysis may be required for other physiological events in these cells.

Abbreviations: EGF, epithelial growth factor; ET-1, endothelin 1; FCS, fetal calf serum; Glu, glutamate; HPTLC, high performance thin layer chromatography; KHB Krebs-Henseleit buffer; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NA, noradrenaline; PDGF, platelet-derived growth factor; PMA, phorbol-12-myristate-13-acetate; PtdBut, phosphatidylbutanol; PtdOH, phosphatidic acid.

## INTRODUCTION

A wide variety of extracellular stimuli activate phospholipid hydrolysis by phospholipase D (PLD), leading to the generation of phosphatidic acid (PtdOH), which may then act as a second messenger itself or be a source for other messengers molecules, such as diacylglycerol and lysophosphatidic acid [1,2]. In the presence of short chain primary alcohols, PLD catalyses the transphosphatidylation reaction generating the corresponding phosphatidylalcohol [3]. This reaction is widely used to measure PLD activity, as well as to inhibit the signaling pathway subsequent to PLD activation in living

cells, since it switches the PLD-derived PtdOH formation to phosphatidylalcohol. As an effector system, PLD activation has been proposed to be involved in several cellular processes, including vesicular transport [4], secretion [5], and mitogenesis [6,7], although its downstream targets and actual role in cell signaling is not clearly established.

The involvement of PLD in mitogenic responses has been controversial. Supporting evidence includes the ability of many mitogens to stimulate PLD, the absence of PLD activity in non-dividing cells [6], the enhanced PLD activity shown by diverse tumour cells [8,9], the ability of PtdOH to bind and translocate Raf-1 to the plasma membrane [7], and the observation that prolonged incubation with alcohols to reduce the PLD-catalysed formation of PtdOH can inhibit, in some cases, the DNA synthesis elicited by PLD-activating mitogens [10-14]. However, there is evidence against a requirement for PLD activation in cell proliferation, since mitogenesis can occur in the absence of PLD activation or, conversely, signals that induce PLD activation do not necessarily lead to cell division [6,14,16]. In mammalian astrocytes, PLD can be activated by various agonists of G protein-coupled receptors, as well as by mitogenic agents [11,18-21]. As in other cells, the role of PLD in astrocyte proliferation is also controversial. Some results suggest that extracellular signals could trigger proliferation through PLD activation. These include the increase of [<sup>3</sup>H]thymidine incorporation into DNA upon exogenous addition of PtdOH [22] or bacterial PLD [13], and the inhibition of serum and platelet derived growth factor (PDGF)-elicited proliferation by long term exposure to primary alcohols [13,23]. In contrast, activation of PLD by noradrenaline (NA) is not followed by astrocyte proliferation [14]. Of note, the long term treatments with alcohols commonly used to explore the PLD requirement in mitogenesis may have non-specific cytotoxic effects [12], which could obscure their potential effect on PLD-mediated responses.

In this work, we have addressed the involvement of PLD in the proliferation of rat cultured astrocytes. We studied the effects on both PLD and proliferation elicited by a broad spectrum of agents, including G protein-coupled receptor agonists (endothelin-1 (ET-1), glutamate (Glu) and noradrenaline (NA)), growth factors (EGF and PDGF), fetal calf serum (FCS), and the protein kinase C stimulating agent phorbol-12-myristate-13-acetate (PMA). We found a lack of correlation between the extent of PLD activation and [<sup>3</sup>H]thymidine incorporation into DNA elicited by these agents. In addition, the mitogenic responses were insensitive to the reduction of PtdOH formation by primary alcohols,

performed under non-cytotoxic conditions. These results suggest that PLD activation is not a general requisite for the stimulation of astrocyte proliferation.

## MATERIALS AND METHODS

### *Materials*

Endothelin-1 was purchased from Alexis. Basal Eagle's Medium (BEM), FCS, penicillin, streptomycin and glutamine were obtained from Gibco. Phorbol-12-myristate-13-acetate, noradrenaline, glutamate, EGF, PDGF, trypsin, soybean trypsin inhibitor, DNase I and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Silica gel-60 HPTLC plates with concentrating zones were from Merck. [ $^{32}$ P]Orthophosphoric acid (carrier free) and [methyl- $^3$ H]thymidine were purchased from Amersham. Other chemicals used were of analytical grade.

### *Cell culture*

Primary cultures of cerebral cortical astrocytes were prepared from newborn (<24 h) Sprague-Dawley rats as previously described [20]. Cells were plated into 24-well plates ( $0.5 \times 10^6$  viable cells/well) in BEM supplemented with 10% FCS, 33 mM glucose, 2 mM glutamine, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. After 24 h, the medium was changed to remove non-adhered cells and then changed again after 3 days. Cells were used for experiments after 6 days in vitro, when cell cultures were still confluent. At this time, immunocytochemical staining for glial fibrillary acidic protein revealed that cultures consisted of 90-95% astrocytes.

### *Determination of PLD activity*

PLD was assayed by measuring the formation of [ $^{32}$ P]phosphatidylbutanol ([ $^{32}$ P]PtdBut) from prelabelled phospholipids by the PLD-catalysed transphosphatidyltransfer reaction in the presence of butanol, essentially as described by *Servitja et al.* [20]. Phospholipids were labelled by incubating astrocytes with [ $^{32}$ P]orthophosphoric acid ([ $^{32}$ P]P<sub>i</sub>, 1.5  $\mu$ Ci/well) in 300  $\mu$ l of Krebs-Henseleit buffer (KHB) without added P<sub>i</sub> (in mM: NaCl 116.0, KCl 4.7, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, CaCl<sub>2</sub> 1.3, and glucose 11.0) for 4 hours at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air. After the labelling period, cells were rinsed twice with KHB to remove non-incorporated [ $^{32}$ P]P<sub>i</sub> and cells were incubated for 10 min with agonists in the presence of butanol (final concentration 50 mM or 0.46%). The reaction was stopped by adding 800  $\mu$ l of ice cold methanol/HCl (98:2, vol/vol) and cells were scrapped and transferred to test tubes. Two phases were generated by adding 900  $\mu$ l of chloroform and 750  $\mu$ l of water. After a 5-min centrifugation at 2,000 xg, the upper phases were aspirated, and the lower (organic) phases containing  $^{32}$ P-lipids were washed with 1.55 ml methanol/water (1:1, vol/vol). Organic phases were centrifuged under vacuum to evaporate the solvent. Lipids were resuspended in 10  $\mu$ l chloroform/methanol (4:1, vol/vol) and spotted onto silica gel-60 HPTLC plates with concentrating zones that were developed with chloroform/methanol/acetic acid (65:15:2, by vol) to separate PtdBut (R<sub>f</sub> 0.45) from the rest of phospholipids (R<sub>f</sub> 0.05-0.30). Occasionally, the upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/H<sub>2</sub>O (65:10:15:50, by vol) was used as solvent system to separate PtdOH (R<sub>f</sub> 0.25) and PtdBut (R<sub>f</sub> 0.35) from the rest of phospholipids (R<sub>f</sub> 0-0.15). [ $^{32}$ P]PtdBut, [ $^{32}$ P]PtdOH, and  $^{32}$ P-phospholipids were quantified in a GS-525 Molecular Imager System (Bio-Rad). To correct for sample size and interexperimental variations of [ $^{32}$ P]P<sub>i</sub> labelling, accumulations of [ $^{32}$ P]PtdBut and [ $^{32}$ P]PtdOH were expressed as the percentage of total

radioactivity incorporated into the lipids present in the organic phase. The radioactivity present at the PtdBut position in butanol-free controls was considered to be background radioactivity and was subtracted from the value determined for each butanol-containing sample.

#### *Determination of [<sup>3</sup>H]thymidine incorporation*

Incorporation of [<sup>3</sup>H]thymidine into DNA was determined as an assay of cell proliferation. After 6 days in vitro cells were switched to BEM without FCS for 24 hours. Agonists were then added either in the presence or absence of short chain alcohols, which, when present, were added 5 min before agonists. After 1 hour, the medium containing the agonists and/or alcohols was removed, substituted for BEM without FCS, and cells were maintained in this medium for an additional period of 28 hours. [Methyl-<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) was present in the culture medium during the last 8 hours of incubation. In some experiments, agonists and/or alcohols were maintained in the culture medium throughout the entire incubation period including the [<sup>3</sup>H]thymidine-labelling step (29 hours). After removing radioactive medium, cells were rinsed with ice-cold KHB buffer and treated with ice-cold HClO<sub>4</sub> (1 M) for 30 min. The acid-precipitated material was washed with ethanol and solubilized with 0.5 M NaOH. An aliquot was collected for the measurement of [<sup>3</sup>H]thymidine incorporation.

#### *Cell viability*

The effect of alcohols on cell viability was explored by measuring both the leakage of lactate dehydrogenase (LDH) and the ability of cells to reduce MTT to formazan. LDH was determined in 10  $\mu$ l aliquots of both medium and cell layers solubilized with 0.1% Triton X-100 following the kinetics of NADH oxidation in the presence of 1 mM pyruvate. Mitochondrial reduction of MTT (0.2 mg/well) was determined after solubilization and spectrophotometric quantification of the formed formazan using the difference of absorbances at 570 and 630 nm.

#### *Data analysis*

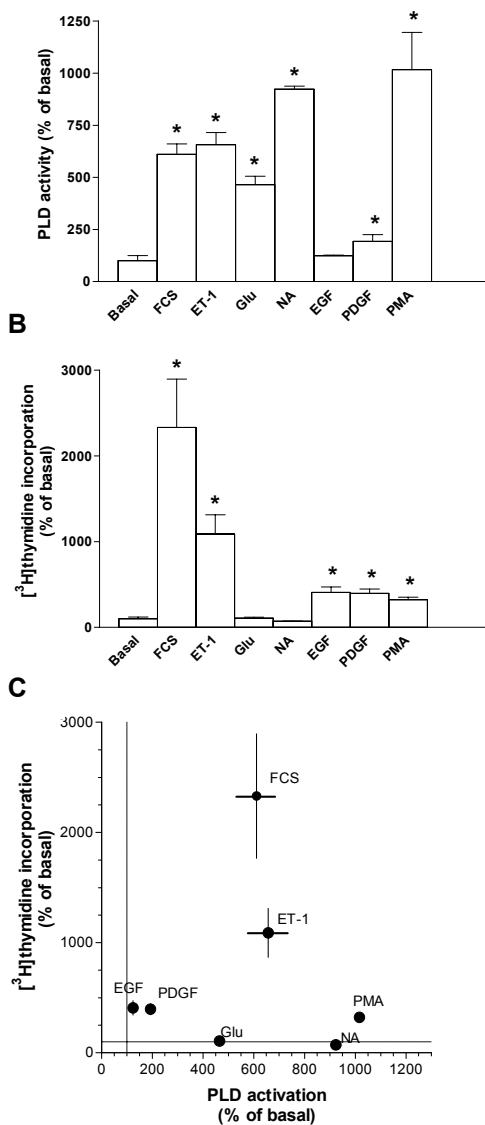
Statistical significance of differences between values was evaluated by performing analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons, using the SPSS statistical package. Significance was taken at  $P < 0.05$ .

## **RESULTS**

### *Lack of correlation between PLD activation and enhancement of DNA synthesis.*

As a first approach to explore the involvement of PLD activation in the regulation of cell proliferation by extracellular signals in cultured astrocytes, we compared the ability of a variety of stimuli to activate both PLD activity and DNA synthesis. These included the G protein-coupled receptor agonists ET-1 (25nM), glutamate (1 mM) and NA (10  $\mu$ M), the growth factors EGF and PDGF (both at 10 ng/ml), FCS (10%) and the protein kinase C activating phorbol ester PMA (100 nM). With the exception of EGF, all the agents tested stimulated PLD to a different extent after 10 min incubation (Fig.1A), a time at which the formation of [<sup>32</sup>P]PtdBut was still linear in all cases (not shown). However, ET-1, EGF, PDGF, FCS and PMA, but not glutamate and NA were able to enhance [<sup>3</sup>H]thymidine

incorporation into DNA (fig.1B). It is important to note that in these experiments cells were exposed to the stimuli for 1 hour and the radioactive labelling determined 28 hours later. Nevertheless, when cells were maintained in the presence of the above stimuli for the entire 29-hour incubation period, which is the most commonly reported procedure, the results were not significantly different from those shown in fig.1B, with the exception of stimulation by EGF, which elicited significantly higher  $[^3\text{H}]$ thymidine incorporation after 29 hours of incubation ( $407 \pm 65$  and  $788 \pm 39$  % of basal for 1hour and 29 hours of incubation, respectively;  $n = 3$ ;  $p < 0.01$ ).

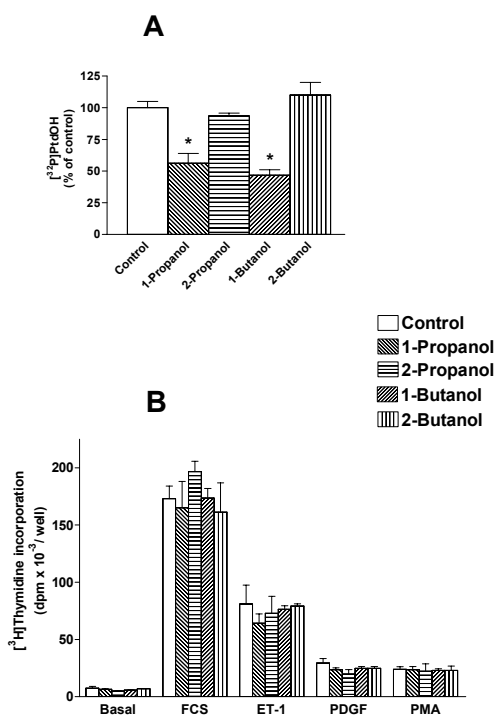


**Figure 1. A) PLD activation in cultured astrocytes.**  $[^{32}\text{P}]\text{P}_i$ -labelled astrocytes were incubated with agonists for 10 min in the presence of 50 mM butanol. PLD activity is expressed as counts on  $[^{32}\text{P}]\text{PtdBut} \times 100 / \text{counts on } ^{32}\text{P-lipids}$ , and are mean  $\pm$  SEM of three independent experiments. Basal :  $0.55 \pm 0.13$ . Concentrations used were: FCS, 10%; endothelin-1 (ET-1), 25nM; glutamate (Glu), 1mM; noradrenaline (NA), 10  $\mu\text{M}$ ; epidermal growth factor EGF, 10 ng/ml; platelet derived growth factor PDGF, 10 ng/ml; PMA, 100nM. **B).  $[^3\text{H}]$ thymidine incorporation into DNA.** Cells were incubated for 1h with the indicated stimuli. Medium was then removed and changed for fresh BEM. 20h later,  $1\mu\text{Ci/well}$   $[^3\text{H}]$ thymidine was added and maintained for an additional period of 8 hours. Values are expressed as percentage of basal ( $7427 \pm 1780$  dpm/well) and are mean  $\pm$  SEM of three independent experiments. Concentrations used are those shown in fig. 1A. **C) Lack of correlation between PLD activation and  $[^3\text{H}]$ thymidine incorporation into DNA.** The data is the same plotted in figures 1A and 1B. The dashed line indicates basal values.  $\star$  Significantly different from basal ( $P < 0.05$ ).

As shown in figure 1C, these results clearly demonstrate that the ability to activate PLD and that to induce cell proliferation are not correlated in astrocytes.

*Effects of short chain alcohols on [<sup>3</sup>H]thymidine incorporation.*

Short chain primary alcohols divert the PLD-catalysed generation of PtdOH to the formation of the corresponding phosphatidylalcohol, a characteristic which is not shared by secondary alcohols. Thus, comparing their respective effects on [<sup>3</sup>H]thymidine incorporation could help to elucidate whether PLD stimulation is required in the mitogenic pathway. In these experiments, we used FCS, ET-1, PDGF and PMA, since these agents were able to stimulate both PLD and cell proliferation. Astrocytes were treated with these mitogenic agents for 1 hour in the presence or absence of 1-propanol, 1-butanol and the correspondent secondary alcohols. As shown in fig. 2, none of the alcohols reduced [<sup>3</sup>H]thymidine incorporation in both control or stimulated cells (fig.2B), despite the fact that the primary alcohols reduced PMA (100 nM) stimulation of the PLD-catalysed formation of PtdOH by 45-60% (Fig.2A). When astrocytes were maintained in the presence of the stimuli and/or the alcohols throughout the entire 29-hour incubation, the incorporation of [<sup>3</sup>H]thymidine in control and stimulated cells was inhibited by 1-butanol and 2-butanol (both at 50 mM) by approximately 90% and 70%, respectively (not shown).



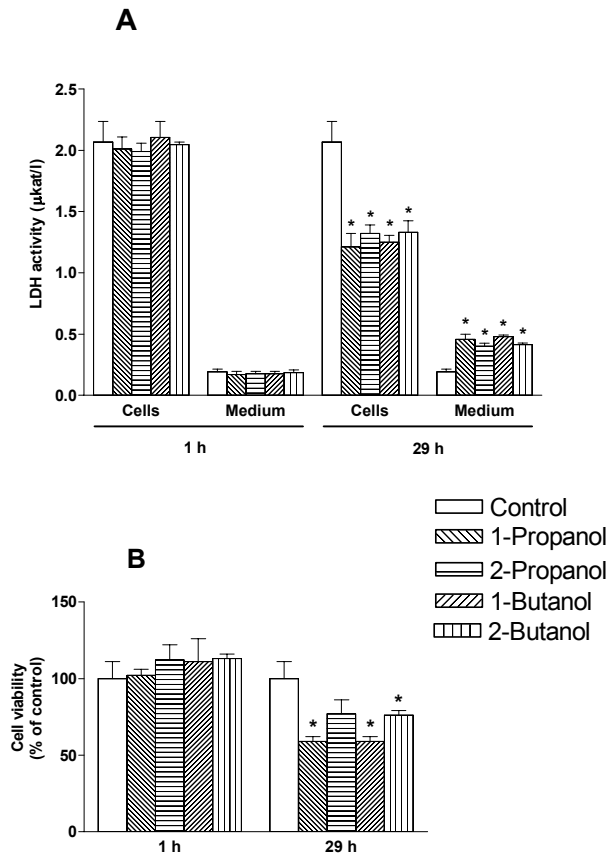
**Figure 2. Effect of alcohols on PtdOH formation and [<sup>3</sup>H]thymidine incorporation.** (A) Cells were incubated for 10 min with 100 nM PMA in the absence (control) or presence of either 1-propanol (100 mM), 2-propanol (100 mM), 1-butanol (50 mM) or 2-butanol (50 mM) and the formation of [<sup>32</sup>P]PtdOH was determined as described in Methods. Results are expressed as percentage of control (PMA: 3.41 ± 0.31 [<sup>32</sup>P]PtdOH x 100 / <sup>32</sup>P-lipids). (B) Cells were exposed for 1h to the indicated stimuli (FCS, 10%; ET-1, 25 nM; PDGF, 10 ng/ml; PMA, 100nM.) in the absence (control) or presence of the indicated concentrations of alcohols. Stimuli and alcohols were then removed and [<sup>3</sup>H]thymidine incorporation was determined 28 hours later. All data are mean ± SEM of three independent experiments. ★ Significantly different from control cells (P<0.05).



These results indicate that long term exposure of astrocytes to alcohols may have non specific cytotoxic effects, as reported for incubation with a relatively high (400 mM) ethanol concentration [9].

*Effects of short chain alcohols on cell viability.*

We next explored whether short term alcohol exposure, used in the experiments shown in fig. 3, as well as long term alcohol treatment could affect the cell viability in our system. For that purpose we monitored plasma membrane integrity by measuring LDH leakage and the mitochondrial ability to reduce MTT, which reflects cell viability. A 29-hour exposure to either primary or secondary alcohols enhanced the release of LDH to the culture medium. In contrast, the treatment with alcohols for 1 hour did not modify the cellular content of LDH (fig. 3A). Accordingly, MTT reduction was not altered after 1 hour incubation with alcohols, but was lowered after long term treatment with 1-propanol, 1-butanol and 2-butanol (fig. 3B). These results suggest that cell viability in our system is not compromised



**Figure 3. Effect of short-chain alcohols on cell viability.** Cells were incubated for either 1 h or 29 h with the indicated alcohols in BEM medium. In the first case, the medium was replaced for fresh BEM after 1 h and cells were maintained for an additional period of 28 h. Control cells were incubated for 1 or 29 h in the absence of alcohols. Cell viability was monitored by measuring leakage of cytosolic LDH (A), and MTT reduction (B). Values are given as percentage of controls (1h,  $0.322 \pm 0.044$ ; 29h,  $0.345 \pm 0.019$ ). All data are mean  $\pm$  SEM of three independent experiments. ★Significantly different from control cells ( $P < 0.05$ ).

when astrocytes are treated for 1 hour with alcohols, but is reduced after a 29-hour treatment.

## DISCUSSION

In the present work we provide evidence indicating that PLD activation is not a general requisite for the onset of a mitogenic response in cultured rat astrocytes. This conclusion rests on results obtained from two different experimental approaches. First, by comparing the ability of a wide range of stimuli to enhance PLD activity and DNA synthesis, we show a clear lack of correlation between both responses. With the exception of EGF, all stimuli activated PLD to a different extent, whereas only ET-1, EGF, PDGF, FCS and PMA, but not glutamate and NA, stimulated DNA synthesis. Moreover, for those stimuli that enhance PLD activity and DNA synthesis there was still no quantitative correlation between both responses. As a second approach, we studied the effects of short chain primary alcohols, which reduce the PLD-catalysed formation of PtdOH, on the stimulation of DNA synthesis by mitogens. Primary alcohols reduced the PMA-induced formation of PtdOH up to 60 %, whereas secondary alcohols had no effect. However, none of the alcohols had any effect on [<sup>3</sup>H]thymidine incorporation elicited by FCS, ET-1, PDGF or PMA, indicating that the natural product of PLD, PtdOH, may not be required in mitogenic signaling pathways. These results do not support a general requirement for PLD in mitogenic responses and are consistent with previous data showing cell proliferation without PLD activation, as described in fibroblasts stimulated with endothelin [24] or various growth factors [25]. Some studies also reported PLD activation not followed by cell proliferation, as for muscarinic agonist-stimulated fibroblasts [25], ATP-stimulated endothelial cells [26], or noradrenaline-stimulated astrocytes [14].

In our experiments, DNA synthesis was monitored after 1 hour stimulation by measuring the [<sup>3</sup>H]thymidine incorporated 28 hours later. With the exception of EGF, this protocol yielded similar results as those obtained when stimuli were maintained for the 29-hour period of incubation, which is the most common procedure. Therefore, our results indicate that 1 hour stimulation is sufficient to initiate the signaling events leading to astrocyte proliferation.

Although the length of incubation with the different stimuli does not seem to be crucial for the measurement of DNA synthesis by [ $^3\text{H}$ ]thymidine incorporation, it might be critical for cell cytotoxicity, since short-chain alcohols can interfere in multiple metabolic pathways [12]. In this work, we demonstrate that a long exposure to alcohols does affect cell viability in our system, measured as LDH leakage and a lowering of MTT reduction, which prevented us to draw any conclusion from the experiments where alcohols were present together with the stimuli for the 29-hour period. Thus the differences between the procedure we used to test the alcohol effects on stimulated DNA synthesis and the more generally used procedure, which maintains the presence of stimuli and alcohols for a long term incubation (29 hours), may account for the disagreement between the results shown in this work and those previously published that suggested an involvement of PLD activation in astrocyte mitogenesis [10, 13, 15].

As mentioned above, 1 hour incubation with the different mitogenic stimuli is sufficient to initiate astrocyte proliferation, and for all of them, except for EGF, to elicit a full response of [ $^3\text{H}$ ]thymidine incorporation. In this context, it is important to note that PLD activation for all the agents was measured after 10 minutes incubation (Fig.1A), and second, a 45-60% reduction of PtdOH derived from PLD activity by 1-propanol and 1-butanol is also detectable after 10 minutes (Fig. 2A). Therefore, if PLD activation was a signaling step leading to astrocyte proliferation, it is reasonable to expect these short-term (non-cytotoxic) incubations with primary alcohols to reduce the [ $^3\text{H}$ ]thymidine incorporation measured after a period of 28 hours without stimuli and alcohols.

On the other hand, we cannot rule out the possibility that for some of the mitogenic stimuli tested, amplification mechanisms between PLD activation and DNA synthesis may exist. In this case, the experimental approach we used, where primary alcohols reduce PtdOH production up to 60%, would not allow us to conclude that PLD is never involved in stimulated proliferation of cultured astrocytes. Taken together, the results shown in this work suggest that PLD activity is not a general requirement for stimulated proliferation in primary cultures of rat cortical astrocytes, and counsel caution in the use of alcohols to study the functions of PLD in order to avoid the non-specific toxic effects.

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