Phospholipase D in human blood platelets

Marta Vorland

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Abstract

We have studied phospholipase D (PLD) in human blood platelets. This enzyme hydrolyzes phosphatidylcholine to phosphatidic acid (PA) and choline, where PA is considered to be the main effector of PLDs function in cells. PA is reported to function as a second messenger, involved in membrane protein recruitment and membrane fusion processes, and PLD is proposed to play a role in signalling, intracellular transport and cytoskeletal rearrangements in cells. The role and regulation of PLD in platelets are largely unknown.

In this study we report that both isoforms of PLD, PLD1 and 2, are present in platelets. In resting platelets the two isozymes were localized all over the cells and upon addition of the platelet agonist thrombin they rapidly translocated to the membrane area. We showed that thrombin-induced PLD activity was enhanced by extracellular Ca^{2+} and autocrine stimulation, notably by ADP and binding of fibrinogen to its receptor. The thrombin-induced translocation was independent of Ca^{2+}, autocrine stimulation or PA from the PLD reaction, thus a primary thrombin effect.

We found that the platelet antagonist PGE_1 was able to induce a modest PLD activity at the same time as it inhibited PLD activation by thrombin. Further investigations using forskolin, inhibitors and specific activators of protein kinase A (PKA) and G, indicated that thrombin-induced PLD activity was inhibited by PKA. We observed that PLD1 and PLD2 had different regulation mechanisms in platelets as PKA/forskolin only inhibited PLD1 translocation by thrombin and also as phorbol 12–myristate 13-acetate (PMA), a direct activator of protein kinase C (PKC) only was able to induce PLD1 translocation. We wanted to study possible interactions between PLD and PKC isoenzymes by immunoprecipitation as previously demonstrated in C3H10T1/2 fibroblasts. We observed co-precipitation of both PLD1 and 2 with all PKC isoenzymes investigated in both stimulated and unstimulated platelets. PKCα showed a constitutive association with both PLD1 and 2 independent of the agents added (thrombin, PMA, forskolin or

PGE_1), while the association between PLDs and PKC βI, βII and δ varied with the different conditions. PLD1 and PLD2 associated differently with the PKC isoenzymes, again indicating different regulation mechanisms. We also report that PLD1 and 2 associated with PLCβII, which is believed to be upstream of PKC in the platelet activation pathway mediated by thrombin. Our findings that PLD1 and 2 associated with different PKC isoforms believed to be involved in distinct different mechanisms in platelets, indicate different roles for the PLD isozymes. PLD in platelets is thought so far to be implied in aggregation and secretion; we suggest in this work by correlation-studies, however, that PLD might be involved in lysosomal secretion and F-actin formation.

List of papers

This thesis is based upon the following papers:

Paper 1

Vorland M. Holmsen H. Phospholipase D in human platelets: Presence of isoenzymes and participation of autocrine stimulation during thrombin activation. Platelets 2008;19(3): 211-224.

Paper 2

Vorland M. Holmsen H. Phospholipase D activity in human platelets is inhibited by protein kinase A, involving inhibition of phospholipase D1 translocation. Platelets, in press, 2008.

Manuscript for Paper 3

Vorland M. Holmsen H. Phospholipase D in human platelets. Involvement of PKC isoenzymes and PLCβII. Submitted to Exp. Cell. Res. April 2008.

Paper 4

Vorland M. Thorsen V.A.T. Holmsen H. Phospholipase D in platelets and other cells. Submitted as invited review to Platelets. April 2008.

Related paper not included in the present thesis

Thorsen VA. Vorland M. Bjørndal B. Bruland O. Holmsen H. Lillehaug JR. Participation of phospholipase D and alpha/beta-protein kinase C in growth factor-induced signalling in C3H10T1/2 fibroblasts. Biochim Biophys Acta 2003;1632(1-3):62-71.

Abbreviation list

$\alpha_{IIb}\beta_3$	An integrin
12-HETE	12-Hydroxyeicosatetrenoate
14-3-3	A Binding protein
AC	Adenylyl cyclase
Akt	Protein kinase B
aPKC	Atypical protein kinase C
ARF	ADP- ribosylation factor
BAPTA	1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CP/CPK	Creatine phosphatase/creatine phosphatase kinase
cPKC	Conventional protein kinase C
DAG	Diacylglycerol
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellularly regulated kinase
FAK	Focal adhesion kinase (non receptor TK)
Fgr	A protein tyrosine kinase
Fyn	A protein tyrosine kinase
GEF	Guanine nucleotide exchange factor
GP	Glycoprotein
GPCR	G protein-coupled receptor
Grb	Growth factor-binding protein
$GTP_\gamma S$	Guanosine-5´-O(3-thio)-triphosphate
hPLD	Human phospholipase D
IAS	Inhibitor of autocrine stimulation
ITAM	Immunoreceptor tyrosine-based activation motif
Lyn	Protein tyrosine kinase
MEK	Mitogen activated protein kinase (MAPK)/ ERK kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
mTOR	Mammalian target of rapamycin
nPKC	Novel protein kinase C
PA	Phosphatidic Acid
PAK	Serine/threonine kinase p^{21}-activated kinase (Rac/Cdc42 effector)
PAR	Proteinase activated receptors
PC	Phosphatidylcholine
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PH	Pleckstrin homology domain
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PI4P 5K	Phosphatidylinositol 4-phosphate 5-kinase
PIP_2	Phosphatidylinositol 4,5- *bis*phosphate
PIP_3	Phosphatidylinositol 3,4,5 *tris*phosphate

PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PLD-PA	Phosphatidic acid derived from the phospholipase D catalyzed reaction
PMA	Phorbol 12–myristate 13-acetate
PPI	Polyphosphoinositides
PTB	Phosphotyrosine binding
PtdBut	Phosphatidylbutanol
PtdEth	Phosphatidylethanol
PTK	Protein tyrosine kinase
PX	Phox consensus sequence
PYK2 /RAFTK	Proline-rich tyrosine kinase/ related adhesion focal tyrosine kinase
Ral	Ras-related protein
Rap	Ribosomal acidic P proteins kinase
Raf	Serine/threonine kinase
Ras	Rat sarcoma virus
RGDS	The peptide Arg-Gly-Asp-Ser
Rho	Ras homology
ROCK	Rho kinase
rPLD	Rat phospholipase D.
SH	Src homology domain
Sos	Son of sevenless
Src	Non receptor protein tyrosine kinase
Syk	A tyrosine kinase
TXA_2	Thromboxane A_2
Vav	A Rho/Rac family guanine nucleotide exchange factor and adaptor protein
vWF	von Willebrand factor

Phospholipase D in platelets and other cells

M. VORLAND, V. A .T. THORSEN & H. HOLMSEN

Department of Biomedicine, University of Bergen, Bergen, Norway

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Corresponding author: Marta Vorland, Department of Biomedicine, Faculty of Medicine, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway,

Tel: +4755586444, Fax: +4755586360, e-mail: marta.vorland@biomed.uib.no

1. Introduction

1.1. Phospholipase D (PLD)

1.1.1. Catalysis and structure

PLD (EC 3.1.4.4) is a phosphodiesterase and was first demonstrated in mammalian tissues in 1975 [1]. The enzyme is stimulated by neurotransmitters, cytokines, hormones, growth factors and other extracellular signals [2, 3]. The major substrate for PLD is phosphatidyl choline (PC) which is hydrolyzed to phosphatidic acid (PA) and choline, but phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI) may also be substrates [4, 5]. A second substrate in the phosphatidate-producing PLD reaction is water (Figure 1). However, if the PLD reaction is carried out in the presence of a primary alcohol like 1-butanol or ethanol, the alcohol is the preferred substrate; giving phosphatidyl butanol (PtdBut) or phosphatidyl ethanol (PtdEth) as the products. This reaction is referred to as the PLD transphosphatidylation reaction and is regarded highly specific for PLD [6] (Figure 1).

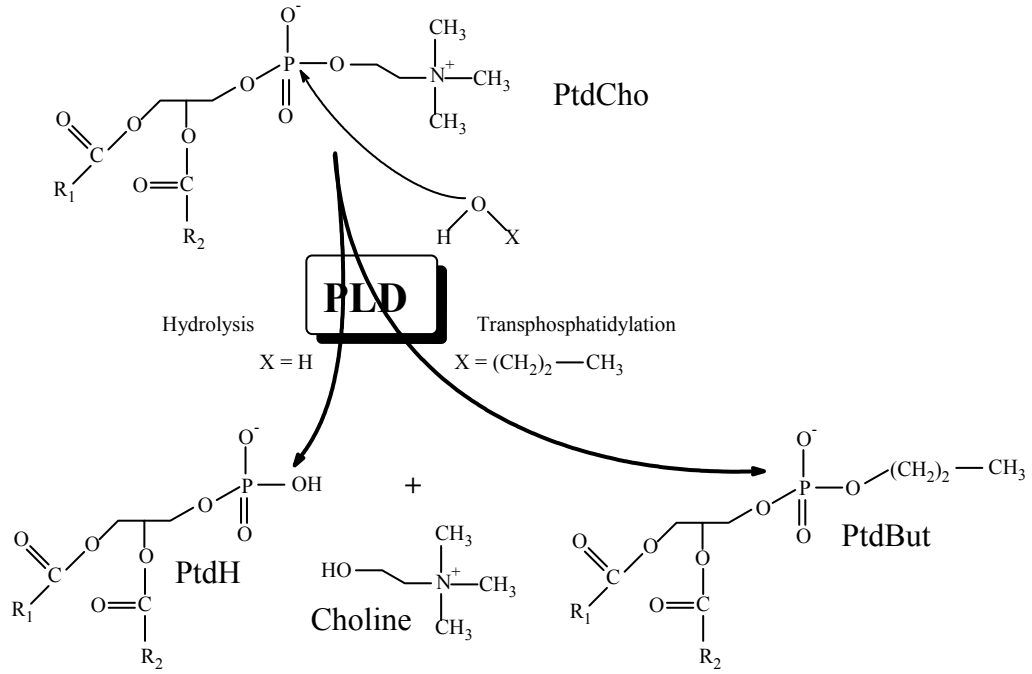

Figure 1. PLD-catalysed hydrolysis and transphosphatidylation of PtdCho. R_1 and R_2 are hydrocarbon chains of long fatty acids.

Two mammalian PLD isoforms exist, PLD1 (120 kDa) [7] and PLD2 (105 kDa) [8, 9], which have about 50 % sequence identity [8]. PLD1 exists in two splice variants (PLD1a and PLD1b) [10, 11], while PLD2 exists in four splice variants [12].

PLD is member of a PLD superfamily containing the highly conserved $HxKx_4$-Dx_6GSxN motif (HKD motif) [7, 13, 14] (Figure 2). Members of this family containing two copies of the HKD motif include mammalian and plant PLD, cardiolipin synthase and phosphatidylserine synthase, while a bacterial endonuclease (Nuc) and a helicase-like protein from *E.coli* contain a single copy of the HKD motif [13, 15].

Sung et al. [16] reported that the HKD motifs and a serine at the position 911 were critical for PLD activity, and suggested a two-step catalytic mechanism involving the two HKD motifs and a phosphoserine intermediate. Gottlin et al. [17] have presented evidence for a phosphohistidine intermediate in the phosphate (oxygen)-water exchange reaction catalyzed by the endonuclease Nuc. Both the crystal structure of Nuc [14] (dimer) and mutagenesis studies of *Yersinia pestis* murine toxin [15] indicate that two HKD motifs lie adjacent to one another, forming a single putative active site. It is also shown that the N- and C- terminal of rPLD1 can associate in vivo involving the conserved HKD motifs, and that the association is essential for catalytic activity [18], thus indicating that the two domains work together in forming an active site also in mammalian cells.

Human PLD1 (hPLD1) does not contain phospho tyrosine binding (PTB), Src homology (SH) 2 or SH3 domains and only a poorly defined pleckstrin homology (PH) domain in contrast to the various forms of phospholipase C (PLC) [7, 19].

Phox consensus sequences (PX motifs) is identified as a phosphatidylinositol binding domain [20]. Its function is critical for the mammalian PLD enzyme [21]. The PX motif of PLD1 have been reported to specifically interact with phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and is suggested to mediate signal transduction via ERK1/2 [22], while the PLD2 PX domain is reported to be involved in protein kinase C (PKC)ζ activation by PLD [23]. Recently, it was shown that PLD may function as a GTPase-activating protein (GAP) through its PX motif which activates dynamin and accelerates EGF-receptor endocytosis, identifying a novel role for PX motifs [24]. The PH domain binds to phosphatidylinositol (4,5)-bisphosphate (PIP_2) and is important for PLD regulation and localization [25], a phosphoinositide-binding motif that mediates activation of mammalian phospholipase D isoenzymes has also been identified [26], and both domains have been suggested to be involved in membrane targeting and catalysis by PLD [27].

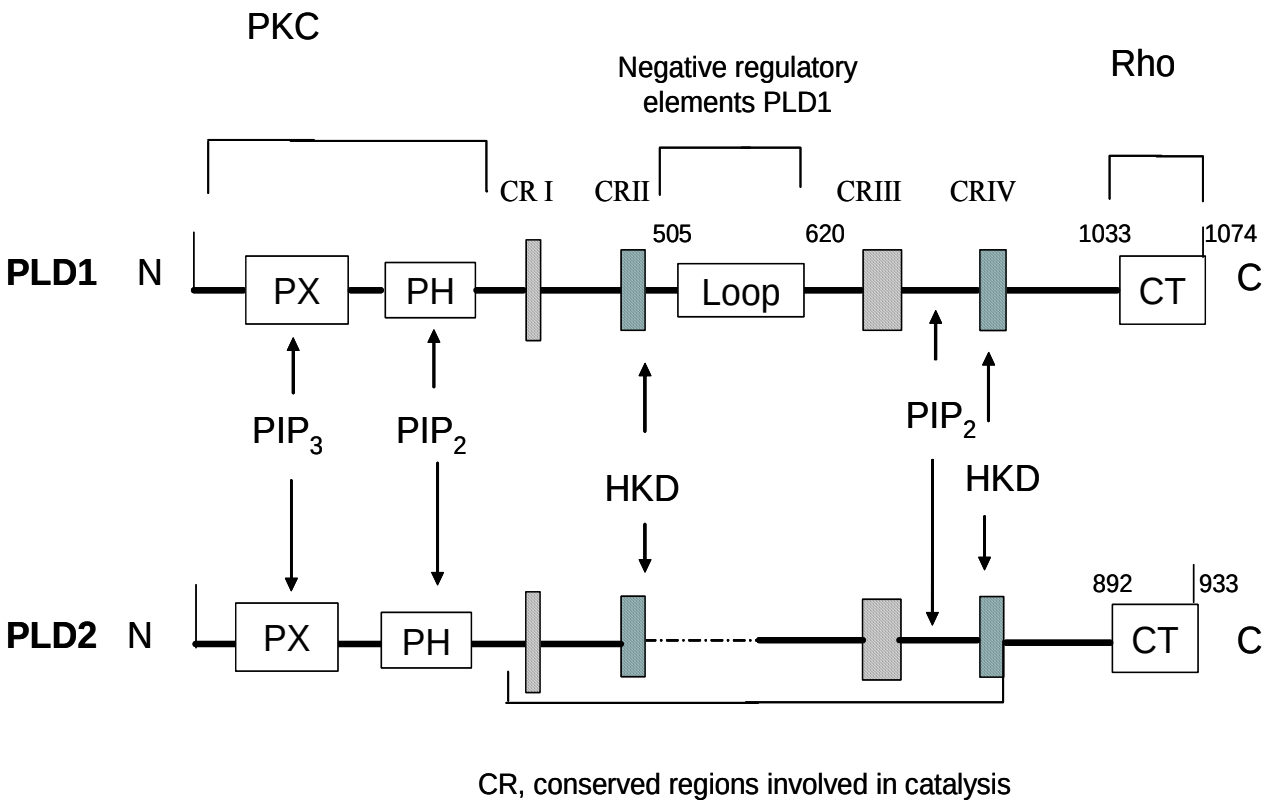

Figure 2. Domain organization of PLD. Roles of some PLD1 and PLD2 sequences. The loop sequence is unique to PLD1 while the PX (phox), PH-like domain (PH), CR (conserved regions), $HxKx_4Dx_6GSxN$ motifs (HKD motifs) and CT carboxyl terminus are found in both PLD isozymes. Interaction sites for PKC, Rho, PIP_3 and PIP_2 are as indicated.

The rest of the amino termini was shown by Sung et al. [21] to be required for activation of PLD1 by PKCα, closer determined to be in the 1-49 sequence and also in the 216-318 sequence containing the PH domain [28], but not for activation by ADP-ribosylation factor (ARF) and RhoA. There is also evidence for the N-terminal hPLD1b involvement in actin-PLD interactions notably at serine 2 [29]. For conserved region III it has been demonstrated through mutagenesis

experiments [16] that it is almost as critical as the HKD motifs. Conserved region I has some critical position for PLD function, whereas others appear to be dispensable [16]. The loop region that is unique for PLD1 has been shown to mediate inhibition of the enzyme [21]. The carboxy terminus has been shown to be intolerant to modification, thereby important for enzymatic activity [21]. It is also shown that RhoA interacts whit this part of the PLD1 enzyme [30]. Sung et al. [21] showed that the NH_2-terminal 308 amino acids are necessary for the characteristic high basal activity of the PLD2 isoform.

There is evidence that PLD1 is glycosylated *in vivo.* However, PLD1 is not an integral protein, and a role for the glycosylation in its membrane association would be unusual [31].

1.1.2. Regulation

A large number of agonists as mentioned above increase the activity of PLD in many cells and tissues, which implies that different mechanisms may be involved in the regulation of PLD. Many of these agonist acts through receptors coupled to the heterotrimeric G proteins or through receptors with tyrosine kinase activity. In either case it is clear that the signals caused by activated G proteins, tyrosine kinase activity or autophosphorylation of the receptors must be transmitted in some way to the PLD isoenzymes. Since most receptors coupled to G proteins or possessing tyrosine kinase activity are capable of inducing significant PIP_2 hydrolysis and thereby PKC activation, PKC has been proposed to mediate many of these signals. However, PKC activation cannot entirely explain the actions of these agonists on PLD, and PLD activity has also been shown to be regulated by small G proteins, PIP_2, Ca^{2+}, protein tyrosine kinases (PTKs) and other kinases.

In vitro PLD1 has a low basal activity and is readily activated by PKC, ARF and Rho family members, while in contrast PLD2 shows a constitutive high basal activity as mentioned above.

1.1.2.1. PKC

Tumour-promoting phorbol esters such as phorbol 12-myristate 13- acetate (PMA) stimulate PLD in a large number of tissues and cell lines [32]. This indicates that the enzyme is regulated by PKC and this is supported by numerous studies showing that specific and non-specific inhibitors of PKC partly or totally inhibits the agonist-induced stimulation of PLD in many cell types (for review see Exton, [33]).

The loss of PLD activation by inhibiting PKC indicates that PLD is activated downstream of PKC. It was shown that overexpressing PKCα in Swiss/3T3 cells gave a constitutively high PLD activity [34], which could be further increased by the addition of both PMA and PDGF. A different approach was used in Madin–Darby canine kidney cells [35], where depletion of PKCα and PKCβ by the use of antisense cDNA decreased the activity of PLD in these cells.

The mechanism of activation by PKC could be expected to involve phosphorylation of PLD, but there is some controversy concerning this matter. Min et al. [31] have shown *in vitro* that rPLD1 can be directly phosporylated by PKCα and βII, but that the phosphorylation inhibits the PLD activity. This has also been shown for PLD2 phosphorylation by PKCα [36]. Further studies propose a dual mechanism involving both phosphorylation and protein-protein interactions [37]. rPLD1 overexpressed in Sf9 cells is shown to be serine/threonine phosphorylated in response to PMA treatment [38]. Kim et al. [39] suggest that phosphorylation by PKCα plays a pivotal role in PLD1 regulation in vivo and identified serine 2, threonine 147 and serine 561 as phosphorylation sites, but the physiological significance of this findings remain unclear. Others [11, 40] states that rPKC stimulates PLD in the absence of ATP and that PLD activation is independent of PKC kinase activity. This indicates that PKC regulates PLD activity through direct molecular interactions. This hypothesis is supported by PMA dependent co-immunoprecipitation of PKCα and PLD1 [41]. We also found co-immunoprecipitation of PKCα with both PLD1 and 2 in C3H10T1/2 fibroblast, and the co-precipitation was independent of

PLD/PKC activation by PMA/PDGF [42]. Coelution of the regulatory domain of trypsinated PKCα with stimulatory PLD activity indicates that this domain contains a site for PLD complexation [40]. In Swiss 3T3 fibroblasts PLD was found to be associated with both the PDGF-receptor and PKCα, the association was independent of addition of vanadate, a tyrosine phosphatase inhibitor [43]. If protein-protein interactions is a major mechanism by which conventional (c)PKC activates PLD, then one could believe that translocation of cPKC to membranes containing PLD activity would be sufficient for PLD activation. Kim et al. [44] have found that upon treatment with PMA, PKCα translocates from cytosol to the membrane fraction where PLD1 also resides in the 3Y1fibroblast cells. It has also been reported that PKCα translocates to the perinuclear region to activate PLD1 [45] and previously by the same group that the initial activation of PLD1 by PMA was highly correlated with the binding of PKCα, and again that phosphorylation of PLD1 was associated with inactivation of the enzyme [46]. Most studies concern PLD and cPKCs, but lately it has been reported that also novel PKC isoforms can participate in the activation of PLD [47] and there are increasing evidence that PLD-PA might be involved in activation of atypical PCKs [48].

Thus, the evidence is strong for direct PLD/PKC interactions; however, the exact activation mechanisms seem complex. There are discrepancies in the reported mechanisms involved and probably involvement of different mechanisms in order of involvement of different isoenzymes, subcellular localization and function, and yet unknown factors such as a 220 kDa protein that also co-immunoprecipitates with PLD/PKC [38] can not be excluded.

1.1.2.2. Small G proteins

The requirement for protein factors from both the plasma membrane and the cytosol for obtaining full PLD activity were shown amongst others by Olson et al. [49].

The protein factors have been identified as small monomeric GTPases like Rho [50] and ARF [51] belonging to the Ras superfamily. Synergistic interactions between PKC, ARF and Rho in activating PLD have been reported [11, 40], suggesting that these three classes of regulators interact with different sites on the enzyme.

The ARF-family consists of small proteins, where Sar1, ARF1 and ARF6 are the best-characterized members. ARF-family members are involved in membrane traffic and in organizing the cytoskeleton as reviewed [52]. Brown et al. [51] identified the ARF that activated PLD to be ARF1 and ARF3, and the myristoylated rARF1 was found to be a better activator of PLD than the non-myristoylated form. However, PLD can be activated by all six members of the ARF-family [53, 54]. Purified enzymes of both PLD1 splice variants are highly activated by ARF1 [11]. *In vitro* studies of PLD2 have shown that its high basal activity was largely insensitive to ARF and Rho [8, 9]. However, it has been shown that hPLD2 can be activated by ARF [55], although to a much lesser extent than that seen with PLD1. PLD activity found in cytosol from HL60 cells was also regulated by ARF, whereas the Rho proteins RhoA and Cdc42 were ineffective [56]. However, PLD activity found in the cytoskeleton from the same cell line [57] required both ARF1, a Rho-family member and PKC for full activity. Observations that ARF translocates from the cytoplasma to the membrane is associated with observations of increased PLD activity, which suggest that an association of ARF with the membrane is necessary for PLD activation [58, 59]. Kim et al. [60] have reported that RalA and ARF1 synergistically stimulated PLD1 activity, and that both RalA and ARF1 interact directly with PLD1. RalA has also been reported to control calcium-regulated exocytosis by interacting with ARF6 dependent PLD1 [61]. The interaction site of PLD with ARF has been suggested to be in

the carboxyl terminus region of PLD [21]. Most studies identify ARF1 and ARF6 as involved in PLD regulation. ARF1 is localized to the Golgi complex and is required for proper Golgi structure and function while ARF6 localizes to the plasma membrane where it may be involved in vesicular transport and organization of the actin cytoskeleton [52]. PLD-PA is reported to be involved in vesicle transport from ER to Golgi [62], and PLD is reported to be involved in intracellular transport [63-65] and actin remodeling [29, 66-68]. However, it should be mentioned that ARF also can be involved in PIP_2 synthesis as reviewed [52], which is important in PLD activation as discussed later.

The Rho-family regulates cell morphology, cell cycle progression, gene transcription and cell transformation and comprises now 20 family members in mammalian cells, where the most studied are Rac1, Cdc42 and RhoA [69]. Initial evidence for a Rho family member to be required for PLD activation came from studies employing Rho-GDP dissociation inhibitor (GDI) [70]. Rho-GDI caused a nearly complete inhibition of PLD activation by GTPγS in human neutrophil membranes. The activation of PLD could be restored in Rho-GDI treated membranes by the addition of RhoA and to a lesser extent by Rac1 and CdC42 [56]. As in the case of ARF, agonist-induced PLD activation is associated with RhoA translocation to the membrane [71]. The characterization of PLD activation by muscarinic stimulation of HEK cells transfected with m3mACh3, demonstrates that both ARF and Rho are involved in receptor-PLD coupling [59, 72]. Such synergism suggests that ARF and RhoA interact with the same PLD molecule. RhoA is reported to interact with the carboxyl terminus of PLD1 [16, 30]. There exist a myriad of possibly indirect Rho PLD activation mechanisms where one is via PIP_2 [73].

1.1.2.3. PIP_2

Brown et al. observed in 1993 that PIP_2 stimulated mammalian PLD and this has been confirmed in several other studies, and PIP_2 is now generally included as a cofactor to *in vitro* PLD-assays.

The addition of neomycin, an aminoglycoside antibiotic that binds PIP_2, was shown to inhibit *in vitro* PLD activity in rat brain membranes [74]. Then Pertile et al. [75] showed that an inhibitory antibody to phosphatidylinositol 4-kinase reduced the levels of PIP_2, with a coincident decline in GTPγS-stimulated PLD activity, PIP_2 was suggested also as an *in vivo* regulator of PLD activity. It is reported that PLD1a and PLD1b are activated by both PIP_2 and PIP_3 whereas other acidic phospholipids were ineffective stimulators [11]. It is also reported that both hPLD2 [55] and rPLD2 [8] have a requirement for PIP_2. As already mentioned PLD's PIP_2-binding sites can be involved both in PLD catalysis and localization [76].

1.1.2.4. Ca^{2+}-ions

Although Ca^{2+}-ions can stimulate the activity of certain PLD isoforms, the concentration required are often well above the physiological range (for review see Exton, [33]), or the stimulation is not observed in the presence of physiological Mg^{2+} concentrations [11]. However depletion of cytoplasmic Ca^{2+}-ions by treatment with chelators such as EGTA or BAPTA results in inhibition of PLD activation by various agonists [33]. Since Ca^{2+}-dependent proteins like PKC regulate PLD, it is unlikely that Ca^{2+}-ions directly control the PLD enzyme.

1.1.2.5. Inhibitory factors

Several proteins have been reported to inhibit PLD activity [77, 78], some of those identified are the cytoskeletal protein fodrin [79], the clatrin assembly protein AP3 [80] and synaptojanin [81], where both fodrin and synoptojanin acts by decreasing the availability of PIP_2 [81, 82].

Ceramides (C_6 and C_2) inhibit PLD activity in agonist- or PMA-treated cells [83-86] and also block the stimulation of PLD by GTPγS in cell extracts with PMA, ARF or RhoA [86, 87]. Ceramides also inhibit the translocation of ARF, RhoA and Ca^{2+}-dependent PKC, so that the inhibition of PLD activity by ceramides could be due to these effects.

It has also been reported that the βγ subunits of heterotrimeric G proteins can inhibit both PLD1 and 2 *in vitro* and PLD activity *in vivo* in MDA-MB-231 cells [88].

The inhibitory factors explain why investigators have had problems with determining PLD activity in crude cells and extracts and leaves the possibility that negative regulation can play an important role in PLD control mechanisms. There are no known specific PLD inhibitors in intact cells.

1.1.2.6. Other phosphorylation-dependent mechanisms

There is considerable evidence that soluble tyrosine kinases can activate PLD [89, 90]. Slaaby et al. [91] have shown that PLD2 complexes with the EGF-receptor and undergoes tyrosine phosphorylation at a single site, identified to Tyr-11 in EGF-stimulated HEK293 cells. Min et al. [43] have, as mentioned above, reported a constitutive association between the PDGF-receptor and PKCα in H_2O_2-stimulated Swiss 3T3 cells. This stimulation also showed a concentration- and time-dependent tyrosine phosphorylation of rPLD that coincided with PLD activation. The oncogenic tyrosine kinase v-src has been reported to activate PLD in a PKC-independent manner [92]. In RBL-2H3 cells PLD2, but not PLD1, is phosphorylated through the Src kinases Fyn and Fgr, and that this phosphorylation appears to regulate PLD2 activation and degranulation in FcεRI- stimulated cells [93]. The same group also shows that protein kinase A (PKA), Ca^{2+}/calmodulin-dependent kinase II and PKC synergistically regulate PLD1 and 2 and secretion

[94]. A calmodulin-dependent kinase has also been reported in the signaling pathways for PLD activation in renal epithelial cells downstream of $G\alpha_{12/13}$/Rho/F-actin [95]. PKA is reported to act both by stimulating and inhibiting PLD activity [96-100], notably indirectly by inhibiting RhoA membrane translocation [100-102] or activation via ERK1/2 [99].

1.1.3. PLD localization

The subcellular localization of PLD seems to differ in cells (for review, see [2, 3]). In general, PLD1 is often found in perinuclear membrane structures while PLD2 is found at the plasma membrane, but PLD1 has also been reported to be present in the plasma membrane area. In fact, several groups report translocation of PLD to the plasma membrane upon cell stimulation [103-105] and there is also evidence of PLD1 recycling between the plasma membrane and intracellular vesicles [27], which might explain the varying reports of localization. It is also thought that the different locations of PLD determines the function of the enzyme [2].

1.1.4. Physiological roles

Degradation of cellular membrane phospholipids by phospholipases alters the composition and properties of the membrane such as charge, packing and fluidity which influence activities of membrane-associated proteins [106]. Phospholipid degradation, which also produces changes in membrane structure, therefore represents means to modulate and initiate signal transducing processes in addition to the cellular messengers produced.

The main effector of PLD activity is PA. PA may act as a signal transducer by direct interactions or as protein membrane recruitment site. PA production has been shown to be important for

vesicle transport and cytoskeletal rearrangements, and PA can also be further metabolized to diacylglycerol (DAG) and lyso-PA.

PA is reported to modulate many enzymes and proteins in vitro [32, 107, 108]. Potential targets of PA include neutrophil NADPH oxidase [108], GAP [109], PLCγ [110], PKCζ [111], phosphatidylinositol 4-phosphate 5-kinase (PI4P 5K) (type I) [112], mammalian target of rapamycin (mTOR) and Raf-1 kinase [113].

The role of PLD in mitogenesis and DNA synthesis has been demonstrated in PDGF-stimulated Balb/c 3T3 cells [114]. The mitogenic effect of PA has been explained [115] by its ability to inhibit the activity of GAP [109], which functions to turn off the Ras monomeric GTPase [116]. We have, however, demonstrated that PLD activation is involved in PDGF-induced ERK1 activation and c-fos expression [42], suggesting other mechanisms for PLD involvement in mitosis. The role for PLD in mitogenic pathways has been reviewed and also places Raf-kinase and mTOR as potential downstream targets [117].

The role of PLD-PA in regulating PI4P 5K which produces PIP_2 has emerged as a key downstream event of PLD activity [2, 118]. PI4P 5K appears to be linked to many of the same cellular functions and small G proteins as PLD. PLD has a definite role in vesicular trafficking through its association with small G proteins [112] and possibly also PIP_2, which as discussed above, might regulate both PLD localization and catalysis. The role of PIP_2 and small GTPases in PLD signaling has recently been reviewed [119]. It is hypothesized that the co-regulation between PLD/PA and PI4P 5K/PIP_2 leads to a local and explosive generation of these lipids with signaling and possibly fusogenic properties, which may then govern signal transduction and especially membrane trafficking and changes in the actin cytoskeleton. It is reported that the interaction of PLD1/ARF1 is the selective one in contrast to the binding of PI4P 5K/ARF, and it has been suggested that the PLD/ARF binding is the critical one in the formation of optimal triplets of ARF/PLD/ PI4P 5K [120].

Experiments using inactive PLD mutants and RNA interference can indicate that the PLD isoforms may have different roles in cells with PLD1 suggested involved in agonist- induced secretion/exocytosis, [64, 105, 121], cell adhesion and migration [122, 123], while several reports places PLD2 in regulating endocytosis and especially recycling of membrane receptors [124-127].

Furthermore, dephosphorylation of PA by phosphatidate phosphohydrolase gives DAG, which is a potential PKC activator [128].There is substantial evidence for a sustained production of DAG generated via PA [129], whether this DAG can activate PKC isoenzymes are disputed as reviewed [48]. PA generated by the PLD reaction, is also a substrate for PLA_2, which generates free fatty acids and lyso-PA. Lyso-PA has been shown to be an important extracellular signal produced by activated platelets and other cell lines [130, 131]. However, the production of lyso-PA from PLD derived PA remains to be demonstrated in vivo. Taken together, the role for PA as a pre-cursor for lipid-signaling molecules needs further illumination.

1.1.5. PLD in platelets

In the presence of ethanol, thrombin induces formation of PtdEth in human platelets, which demonstrates that a physiological agonist can activate PLD in these cells [132]. There is also evidence for PLD activation by other platelet agonists [133-135] and different activation mechanisms have been proposed:

The thrombin-induced activation of PLD is markedly inhibited by ADP scavengers (apyrase, phosphocreatine-creatine kimase) [136, 137] while ADP itself does not activate platelet PLD [133, 136], suggesting that secreted ADP amplifies the thrombin-induced PLD activation, and that thrombin and ADP activates PLD in a synergistic manner. The thromboxane mimetic U46619 also activates PLD in an ADP-sensitive manner [136]. The particulate agonist collagen

gave more activation of platelet PLD than thrombin, and in a thromboxane-insenstive manner; choline was released in parallel with aggregation [133]. Sphingosine, a PKC inhibitor, inhibited both thrombin and collagen-induced activation of PLD, aggregation and ATP secretion in a parallel manner [133]. In contrast, only a slight inhibition of thrombin-induced PLD activity by the PKC inhibitor staurosporine was found in an other study, which estimated that 13% of PA produced in the thrombin-platelet activation originated from the PLD reaction, which was thought to be stimulated by intracellular mobilization of Ca^{2+} [134]. We reported that addition of extracellular Ca^{2+} potentiated thrombin-induced PLD activity, while extracellular Ca^{2+} alone was unable to induce PLD activity [137].

In permeabilised platelets the GTPγS-induced formation of PA paralleled serotonin secretion in platelets, suggesting involvement of a G protein in dense-granule secretion and PLD activation [138]. Using quercetin, a flavonoid shown to inhibit platelet activity [139-143], dense-granule secretion and PLD activity was inhibited in permeabilised platelets [144]. However, the PLD activation seemed to be a more slow process than dense-granule secretion and addition of exogenouse PA alone had no effect, indicating that PLD is not essential for dense-granule secretion, but a modulatory role was proposed as was also the case for PLC and PLA_2 activities [144]. Both thrombin- and GTPγS- induced activation of PLD in intact and permeabilised platelets, respectively, are markedly inhibited by a variety of protein tyrosine kinase inhibitors, suggesting involvement of both G proteins and protein tyrosine phosphorylation, particularly $pp60^{src}$, in the activation mechanism(s) [145]. Prevention of platelet aggregation by blocking fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ by RGDS, or use of platelets from a thrombosthenic patient lacking this integrin, did not prevent PLD stimulation by thrombin or PMA, while the specific PKC inhibitor Ro-31-8220 completely blocked PLD activation [135]. In contrast, we recently found that RGDS, which prevents fibrinogen binding, could inhibit thrombin-induced PtdEth formation in platelets pre-labeled with [^{3}H]arechidonic acid [137], and previously PLD activation in platelets by high density lipoprotein (HDL_3) has been reported to depend on

ligation of integrin $\alpha_{IIb}\beta_3$ [146] indicating that fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ can be important for activation of PLD in platelets.

Others have shown that platelets possess a specific receptor for low density proteins (LDL) with high affinity for the cholesterol moiety of LDL that directly activates PLD [147]. It has also been demonstrated that incorporation of cholesterol in platelets stimulates both PLD and PLA_2 [148].

While the above characteristics are confined to human platelets, PLD activity has also been demonstrated in rabbit platelets. Thus, membranes from PMA-treated rabbit platelets contain a GTPγS-activatable PLD, that is thought to produce PA exclusively as substrate for a PA-specific PLA_2 [149]. This involves PLD in the formation of eicosanoids. Platelet membranes contain a PLD that is activated by PMA and GTPγS in a synergistic manner and which is inhibited by staurosporine at low concentration but activated by staurosporine at high concentration, suggesting that PMA may activate PLD by a phosphorylation-independent mechanism [150].

Platelets contain phosphatidate phosphohydrolase (lipid phosphate phosphatase) [151] which splits PA to DAG and inorganic phosphate. DAG is produced directly in the PLC reaction and this DAG originates from PIP_2 and consists almost exclusively of sn-1-stearoyl-2-arachidonoylglycerol, which is effectively converted to the corresponding PA in platelets by DAG kinase [152]. The production of DAG in platelets upon thrombin stimulation is controversial as some groups report a biphasic production while others report a monophasic, transient production (for references, see [152]).

We have previously reported a biphasic production of DAG [152] and we have found, as others have for other cell-lines, that the second peak disappears in the presence of ethanol and also with the use of inhibitors of autocrine stimulation, IAS (Figure 3), which we have previously found to inhibit PLD activity in platelets [137], indicating a role for PLD in the sustained DAG production also in these cells.

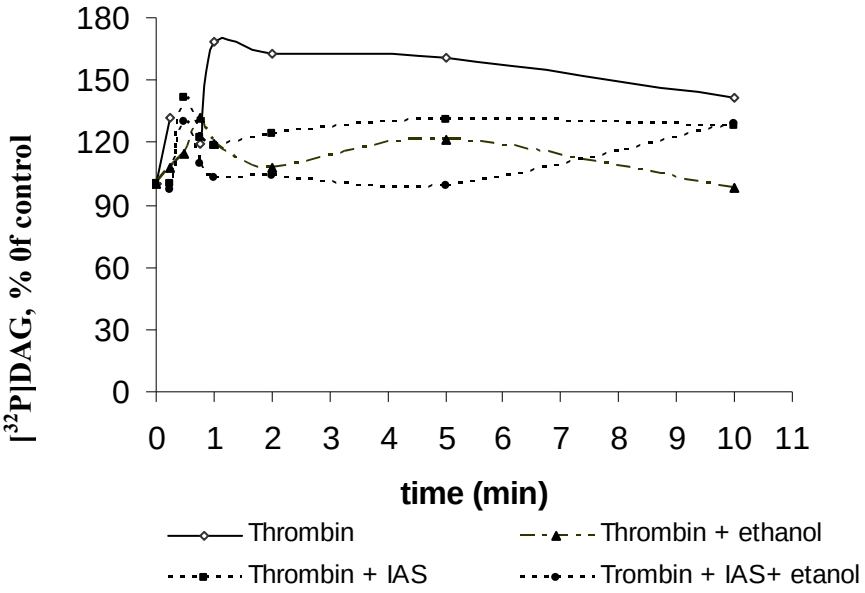

Figure 3: The effect of ethanol and IAS on DAG production in human platelets. The procedure was performed as described [152]. Thrombin (0.5 U/ml) added to gel-filtered human platelets in the presence or not of 0.4 % ethanol or inhibitors of autocrine stimulation (IAS) and the reaction stopped at the indicated times. Total lipid was extracted and DAG phosphorylated to PA with [γ^{32}P]ATP. PA was separated by thin layer chromatography (TLC) and its radioactivity measured by instant imager. IAS are; the ADP-removing system, creatine phosphate/creatine phosphokinase (CP/CPK 5 mM/ 10 U/ ml, Sigma Chemical Co. St. Louis, MO), the selective thromboxane A_2 (TXA_2) antagonist SQ 29.548 (150 μM, Research Biochemicals International, MA, USA) and the peptide RGDS (150 μM, Calbiochem, San Diego, CA, USA) that prevents fibrinogen binding, all used as described [153]. Control value was 6580 cpm. The experiment is representative of four others

A recent report using propranolol to inhibit lipid phosphate phosphatase-1 (LPP-1), inhibited PAR1-mediated aggregation and sustained Rap1 activation, and the same effects was observed when 1-butanol was added, indicating a role for PLD-PA and DAG produced from PA in the PAR1-mediated aggregation via Rap1. Propranolol inhibited PKC-mediated aggregation and

Rap-1 activation totally. Propranolol also inhibited $\alpha_{IIB}\beta_3$ activation, α-granule and lysosomal secretion mediated by both PAR1 and PAR4 [154].

We have found evidence for the presence of both PLD1 and PLD2 in platelets both by immunoprecipitation and by immunohistochemical studies. In resting platelets both isoenzymes seemed to be localized throughout the cells. PLD1 seemed to be up-concentrated in some areas as the imaging studies show PLD1 in dots, which we hypothesize being granules, while PLD2 seems to be all over the cells. By addition of thrombin both isoenzymes rapidly translocate to the plasma membrane area; this translocation seems to be a primary response to thrombin as it is independent of the use of IAS as described above (Figure 3) [137]. Our most recent study indicates different activation mechanisms for the two isoenzymes as PLD1 membrane translocation was inhibited by PKA activation with forskolin and a specific PKA activator while PLD2 was not [155].

As reviewed above there is evidence for PLD involvement both in aggregation and secretion. In our hands we propose a role for PLD in F-actin regulation and secretion of lysosomal glycosidases as these processes are partially affected both by ethanol and the inhibitors of autocrine stimulation that inhibit PLD activity [137], and these platelet responses also correlated with the more slower process of PLD activation than the rapid dense-granule secretion. The finding that propranolol also inhibits lysosomal secretion [154] might suggest involvement of DAG produced from PLD-PA. As no specific inhibitors of PLD are known, studies rely on the use of alcohols as an initial approach to identify PLD involvement. The use of alcohols to establish PLD's role in platelets is however difficult as high concentration gives adverse negative effects on platelets and low concentration will allow PLD-PA production.

In conclusion, the mechanisms for activation of PLD in platelets correlate well with findings for the enzyme in other cells placing PKC and small G proteins as important PLD activators. The assumed pathways of PLD activation in platelets are depicted in Figure 4A and B. However, the exact mechanisms for PLD activation in platelets remain unclear. Findings from platelets taken

together with results from other cell-lines; good candidates for PLDs involvement are vesicle transportation (notably secretion) and cytoskeletal rearrangements.

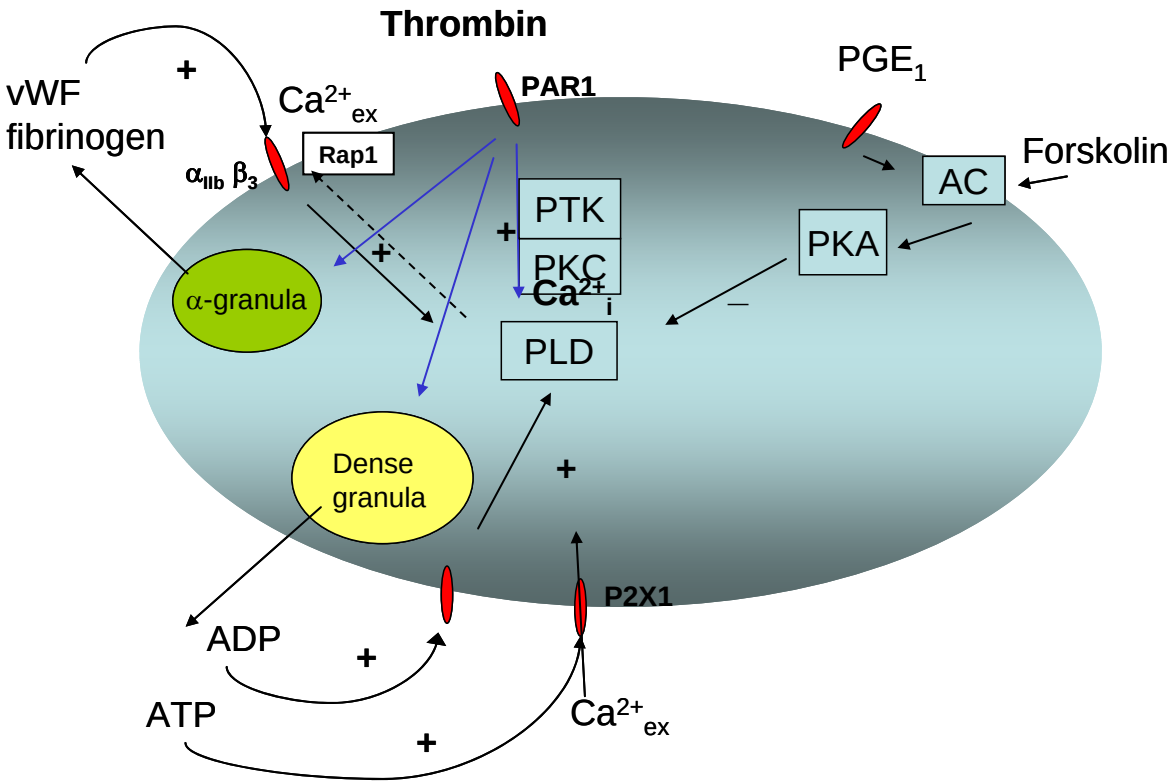

Figure 4A: Mechanism of thrombin induced PLD activation in platelets. PKC, protein tyrosine kinases (PTK) and Ca^{2+} are shown to be involved in the activation of PLD by thrombin, Ca^{2+} probably via indirect mechanisms such as PKC. Secreted ADP and the binding of fibrinogen to its receptor are necessary for full thrombin-induced activation of PLD. Thrombin induces translocation of PLD to the plasma membrane area, independently of ADP/fibrinogen binding. PLD activity has been implicated in aggregation and Rap1 activation mediated by the thrombin receptor PAR1. Rap1 is reported involved in activation of the fibrinogen receptor ($\alpha_{IIb}\beta_3$). PGE_1, forskolin and direct PKA activators inhibit thrombin-induced PLD activation and the translocation of PLD1.

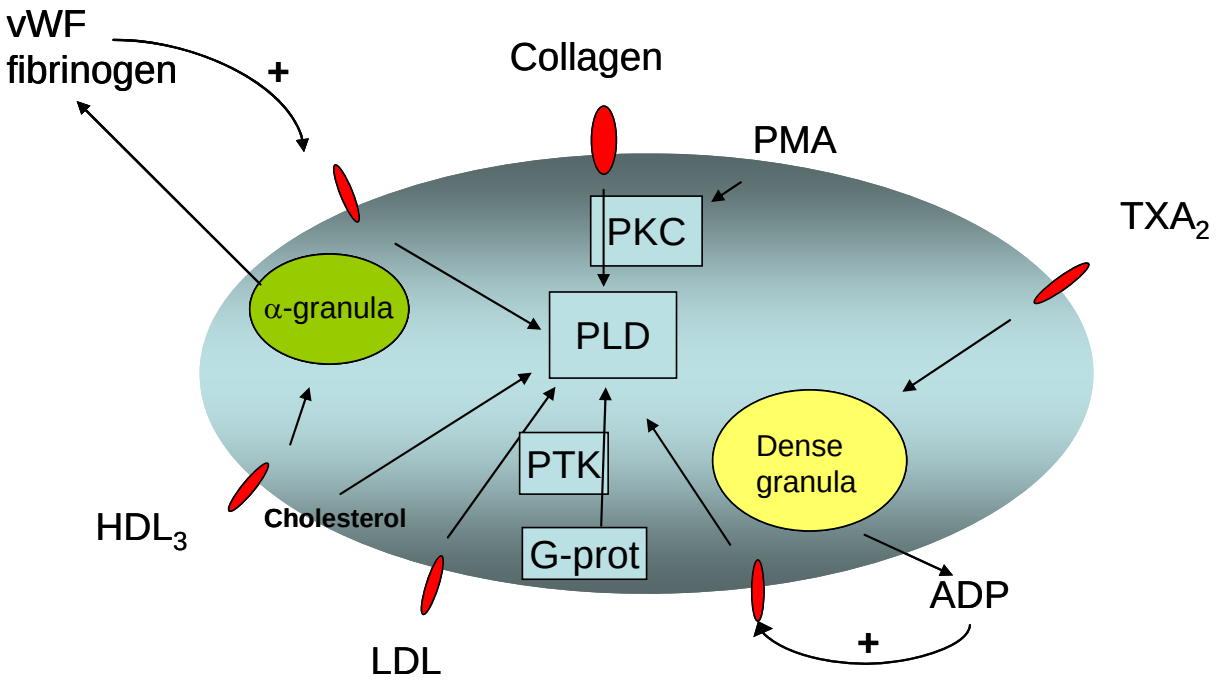

Figure 4B: Other mechanisms for PLD activation in platelets. Collagen has been reported to activate PLD in a PKC dependent pathway and direct PKC stimulation by PMA increased PLD activity. Activation of the thromboxane receptors lead to an ADP dependent activation of PLD, while activation by high density lipoprotein (HDL_3) was dependent of fibrinogen binding. Low density proteins (LDL) were reported to activate PLD directly and the incorporation of cholesterol in platelet membranes was observed to lead to PLD activation. The addition of GTPγS to permabilized platelets also activates PLD implying G proteins in the activation of PLD in a protein tyrosine kinase dependent mechanism.

1.2. Human Platelets

1.2.1. Platelets in haemostasis and thrombosis

Platelets are the smallest cellular components of blood. They are anucleate, discoid cells which circulate along the vessel wall, 1 μm thick and 3 μm in diameter. Platelets are produced by the megakaryocytes in the bone marrow [156], and sequestered after about 9 days of circulation in man by the reticuloendothelial system. Their main function is in the normal haemostatic process, but they are also actively involved in thrombosis, restenosis and in inflammatory reactions. Platelets interact with other components in blood and with components in the vessel wall during haemostasis and these complex interactions are influenced by the rate of blood flow, which are slower at the vessel wall than in the centre, creating a shearing effect between adjacent layers of fluid. The shear rate (S^{-1}) is expressed as difference in flow velocity as a function of distance from the wall, the highest wall shear rate in normal circulation occurs in small arterioles and have been estimated to vary between 500-5000 S^{-1} [157]. Upon rupture of a vessel wall the initial deposition of platelets is predominantly mediated by the interactions of platelet glycoprotein (GP) Ib-IX-V with von Willebrand Factor (vWF) at high shear rates and the GPVI and integrin $\alpha_2\beta_1$ with exposed collagen fibers at low shear rates. As platelets adhere to collagen they change shape to spheres with long pseudopods and secrete a number of substances from three distinct storage granules, the dense granules (ATP, ADP, serotonin, Ca^{2+}), α-granules (growth factors, coagulation factors, many glycoproteins) and lysosomes (mostly acid glycosidases). Upon adhesion to the collagen fibers they also activate PLA_2 that liberates arachidonic acid (AA) esterified in glycerophospholipids; the free AA is rapidly oxygenated to prostaglandins, thromboxanes and leukotriens. The secreted ADP and serotonin as well as the thromboxane A_2 (TXA_2) produced during platelet stimulation are potent platelet agonists that activate bypassing platelets that in turn aggregate with the adhered platelets and with each other.

In this way a platelet plug is growing that partially stops the loss of blood from the damaged vessel (primary haemostasis). The adhesion, secretion, TXA_2 formation and aggregation described above takes 1-3 min within which time the extracellular coagulation cascade reaches the stage of thrombin formation. Thrombin is also a potent platelet agonist that both potentiates the activation of platelets and causes formation of fibrin strands between platelets and around the plug, making it non-permeable (secondary haemostasis).

Platelet activation leads to release of cell activators like TXA_2, platelet factor 4, platelet-derived growth factor (PDGF) and 12-HETE and exposure of P-selectin and CD40 on the platelet surface, all factors which may facilitate monocyte activation and strengthen the intercellular interactions, also important for the coagulation process [158].

The initial aggregation under high shear rates differs from the process described above as it occurs between discoid platelets and is mediated by formation of membrane tethers and involves the adhesive functions of both GP1b and $\alpha_{IIb}\beta_3$ receptors and vWF and fibronectin in addition to fibrinogen, (details are reviewed in [159]).

In the microcirculation ruptures of the wall of small vessels occur continuously and the haemostatic process is vital in order to prevent blood loss in vital organs such as brain and heart. When the number of circulating platelets is low (<20 000/μL) or the platelets have impaired functions; the growth of the platelet plugs is too slow to cause proper haemostasis, which causes local ischemia in the brain, which for example, might cause stroke.

Hyperactive platelets and/or pathological deposits of various cells and substances on the vessel wall (i.e. cholesterol) resulting in plaque formation or other forms of arterial occlusion, which result in high shear stress which might lead to activation-independent platelet aggregation mediated by soluble vWF facilitated adhesion can lead to unwanted plug formation, the so-called thromboembolism. It is believed that under pathological high shear stress, vWF links platelets

together transiently until activation occurs [159], and one believes that platelets during thrombosis are activated in the same way as in haemostasis. The pathological thrombotic plug leads to reduced supply of blood downstream of the thrombus. Such platelet thrombi form exclusively in the arteries. The platelet plug can also dissociate and lead to emboli elsewhere. Proper platelet function is therefore vital for normal body function.

1.2.2. Platelet responses

When platelets are activated in the haemostatic process, a number of responses are revealed that can be observed in vitro. The responses include: 1) Adhesion: the mechanism of platelet binding to foreign surfaces, like collagen. 2) Shape change: a rapid process in which the platelets change form from discs to spheres presenting pseudopods, involving rearrangements in the platelet cytoskeleton and depending on actin polymerization. The resting platelets have four distinct actin structures: membrane skeleton, filaments of the central core, radiating filaments that connect the core to the membrane and monomeric actin in the cytoplasm. Platelet activation cause formation of a ring structure which separates from the membrane skeleton and move to the centre of the cells; this is called the contractile ring and is formed from components of the membrane skeleton and associated with rounding of platelets. Next filopodia protrude and during the adhesion process lamellipodia become spread out while stress-like fibers coalesce internally [160]. During platelet shape change cryptic integrin receptors are exposed on the platelet surface. 3) Aggregation: platelets stick to each other mediated by binding of fibrinogen and also vWF to the $\alpha_{IIb}\beta_3$-receptors. The aggregation process takes 2-3 min to be complete. Two types of aggregation may be observed in vitro depending of the potency and concentration of the agonist involved, a reversible aggregation without secretion and an irreversible associated with

secretion. Thus, a biphasic response can be observed by the use of some activating factors, where the second phase results from autocrine stimulation. 4) Secretion: as mentioned, platelets contain three types of granules. The granules differ from each other by the agonist strength necessary to induce their secretion, the speed and the degree of secretion. Dense- and α-granules can be induced by week agonists such as ADP and TXA_2; it is rapid and 100% completed in 1-2 min. Lysosomal secretion is a more slow process and occur only after stimulation with a strong agonist like collagen or thrombin and is never completed [161].

In addition, platelets expose neoisotopes, shed microparticles, display procoagulant activity and participates in the retraction of fibrin clots as described [161].

1.2.3. Mechanisms of platelet activation and inhibition

1.2.3.1. Activation

In the haemostatic process described above, collagen, thrombin, ADP, TXA_2 and serotonin participate as platelet agonists, and they all have distinct receptors on the platelet surface. These receptors are of two main types: 1) G protein-coupled receptors (GPCRs), receptors that consist of seven transmembrane domains that are linked to and activate heterotrimeric G proteins upon agonist occupation, and 2) Adhesion receptors often linked to soluble protein tyrosine kinases of the Src family. An overview of the receptors mentioned in the text and their main effector systems are presented in table 1.

Table 1: Platelet receptors are divided into two subgroups: G protein-coupled receptors (GPCP) and adhesion receptors. GPCR are listed with the G proteins coupled to each receptor and their main effector systems. All receptors lead to activated platelets except the PGI_2-receptor which inhibits platelets by activating adenylat cyclase (AC). Abbreviations: protease activated receptor (PAR), purinergic receptors (P2Y), thromboxane receptor (TP), prostaglandin (PG), glycoprotein (GP), von Willebrand factor (vWF), thromboxane A_2 (TXA_2), phospholipase C (PLC), Rho kinase (ROCK), phosphoinositide 3-kinase (PI3K), protein tyrosine kinase (PTK).

Receptor category	**Receptors**	**Agonist**	**G-proteins**	**Receptor-coupled effector system**
GPCP	PAR1	Thrombin	G_q, $G_{12/13}$, G_i	PLCβII (+), Rho/ROCK (+), AC $(-)_{Gi\alpha}$ and PI3K $(+)_{Gi\beta\gamma}$
GPCP	PAR2	Thrombin	G_q, $G_{12/13}$, G_i	PLCβII (+), Rho/ROCK (+), AC $(-)_{Gi\alpha}$ and PI3K $(+)_{Gi\beta\gamma}$
GPCP	$P2Y_1$	ADP	G_q	PLCβII (+)
GPCP	$P2Y_{12}$	ADP	G_i	AC $(-)_{Gi\alpha}$ and PI3K $(+)_{Gi\beta\gamma}$
GPCP	TP	TXA_2	G_q, $G_{12/13}$	PLCβII (+), Rho/ROCK (+)
GPCP	PGI_2	PGI_2, PGE_1	G_s	AC (+)
Adhesion	GP IV	Collagen	-	PTK (+)
Adhesion	GP1b	vWF	-	PTK (+)
Adhesion	$\alpha_{IIb}\beta_3$	Fibrinogen, vWF	-	PTK (+)

1.2.3.1.1. G protein-coupled receptors

Agonists that activate platelets through GPCRs include thrombin, ADP, TXA_2 and serotonin amongst others. Signaling via GPCR in platelets involve three major G protein mediated pathways that are initiated by activation of $G_{q,}$ G_{13} and G_i. Thrombin is the most efficient platelet activator of the above-mentioned agonists; thrombin is the main effector protease of the coagulation system, and act by cleaving of protease-activated receptors (PARs). Cleaving of the receptors induce conformational changes leading to activation of the coupled G proteins. In human platelets thrombin activate platelets through the PAR1 and PAR4 receptors [162], which couple to $G_{q,}$ G_{12}/ G_{13} and sometimes also to G_i family members, as reviewed [163] . Reports indicate that PAR1 mediates platelet activation at low thrombin concentrations, while PAR4 mediates the response to high thrombin concentrations [162, 164, 165]. TXA_2, produced as described above functions as an autocrine stimulator during platelet activation by binding to the TXA_2 receptor (TP) coupled to G_q and G_{12}/G_{13} [166-169]. Platelet activation by ADP is mediated by the two purinergic receptors $P2Y_1$ and $P2Y_{12}$, where $P2Y_1$ is reported to be coupled to G_q and $P2Y_{12}$ to G_{i2} as reviewed [170].

The exact role of the individual G proteins have been difficult to establish in platelets as all mediators in turn can increase the formation and release of thrombin, ADP and TXA_2; their effects are amplified by all major heterotrimeric G protein pathways; however, the use of mice platelets lacking different G proteins have lead to new knowledge. Thus Moers et al. [171] showed that G_q or G_{13} was required to induce some platelet activation, whereas the activation of G_i -mediated signaling alone was insufficient to induce activation of mouse platelets. G_i-activation on the other hand seemed important for obtaining full platelet activation through G_q and G_{13}. As reviewed [172] G_{13}- mainly mediates signals leading to shape change, while G_q and G_i pathways mainly are involved in activation of aggregation and secretion . However, one should bear in mind that human and mouse platelets are different i.e. human platelets present

PAR1 and PAR4 receptors while mouse platelets PAR3 and 4 [173], and results observed are not necessarily interchangeable.

The G_q family activates phospholipase Cβ, where especially βII and βIII are present in platelets. These PLCs are specific for polyphosphoinositides (PPIs) and hydrolyze phosphatidylinositol 4, 5-*bis*phosphate (PIP_2) to membrane-bound diacylglycerol (DAG) and cytoplasmic inositol 1, 4, 5-*tris*phosphate (IP_3). IP_3 binds to specific receptors in the dense tubular system (DTS, counterpart to endoplasmic reticulum in other cells) which causes release of stored Ca^{2+} to the cytoplasm. The free, cytoplasmic Ca^{2+} has many functions; it is involved in activation of a range of enzymes like PKC, PLC, PLA_2 and Ca^{2+}-calmodulin-dependent protein kinase (CAMK). Activated CAMK in turn activates myosin light chain kinase (MLCK) by phosphorylation, and the active MLCK phosphorylates myosin light chain (MLC). Phosphorylated MLC combines with actin and makes contractile actomyosin bundles (thick filaments), the contraction of which may be of importance in shape change [174] and exocytotic secretion of granule contents. Ca^{2+} also activates a number of proteases of which calpain has been shown to proteolyse PKC, thus terminating its activity [175].

DAG produced by PLC can activates protein kinase C isoforms. PKC activity in platelets is discussed in detail below.

Apart from activating classical PKC (cPKC), in mouse platelets DAG and calcium ions can also activate guanine nucleotide exchange factor (CalDAG-GEF1), which in turn activate Rap1 [176]. Mouse platelets that lack CalDAG-GEF1 are severely compromised in integrin-dependent aggregation [176].

Other small G proteins are also candidates for activation by G_q. The Rho/ Rho kinase pathways which lead to MLC activation, as described below, has been suggested in the $P2Y_1$-mediated activation [177] and is also reported involved in the disruption of the microtubular ring leading

to the rapid shape change of platelets from disc to spheres [178]. Thrombin-induced PLC activity and Ca^{2+} was demonstrated to be essential for Rac activity, while Cdc42 activation was independent of PLC activation [179]. PAR-1 stimulation rapidly activates both Rac and Cdc42, and upon activation Rac associates with the plasma membrane and the association between Cdc42 and actin increases, indicating different signaling roles for these GTPases [180]. Downstream targets for Rac are PI4P 5K and p21-activated kinase (PAK). Activated PI4P 5K generates $PI(4,5)P_2$ which induces uncapping of actin filament ends [181]. ADP activated Rac and its effector PAK via its $P2Y_1$ receptor, through a G_q-dependent pathway [182]. Thrombin was reported to activate Cdc42 and Rac1, which both activated PAK in another study. Activated PAK dissociated from the cortical/actin binding protein cortactin which might trigger early shape change was reported [183]. The use of mice platelets deficient in Rac and Rac inhibition in human platelets blocked platelet dense-granule secretion, which was partly responsible for diminished aggregation [184].

G_{13} has been shown to activate /regulate several signaling pathways where the Rho/Rho kinase pathway is the best established. The $G_{\alpha 13}$ subunit directly interacts with and activate p115 Rho guanine exchange factor [185], thus leading to activated RhoA. It has been established that RhoA activates Rho kinase (ROCK) which inhibits myosin light chain phosphatase, thus also contributing to the activation of MLC and shape change independently of calcium [186]. The RhoA-specific ADP-ribosyltransferase, C3 toxin [187], inhibits thrombin induced platelet aggregation [188, 189]. It has also been reported that RhoA inactivation decreased the adhesion of agonist-stimulated platelets to fibrinogen [190]. However, it has been shown that inhibition of RhoA (C3 exoenzyme) or its downstream effector Rho kinase had no effect on integrin $\alpha_{IIb}\beta_3$ activation induced by soluble agonists or adhesive substrates, but that RhoA regulated the stability of integrin $\alpha_{IIb}\beta_3$ adhesion contacts under conditions of high shear stress [191]. It is reported that both RhoA and the Rho effector Rho kinase activate and mediate PI4P 5K membrane translocation in thrombin-activated platelets [192]. It has also been demonstrated that

PAR1- mediated $G_{\alpha 13}$ activation can mobilize intracellular calcium in a Rho kinase-independent mechanism and that G_{13} signaling is required for shape change, aggregation and secretion mediated by PAR1 [193].

G_{i2} is the main member of the G_i family present in platelets. The $G_{i2\alpha}$ subunit is linked to the inhibition of adenylyl cyclase, inhibiting the cAMP production in platelets, whereas one important role for the $G_{\beta\gamma}$ subunit is to activate PI3K. The two isoforms involved in platelets are believed to be PI3Kγ and PI3Kβ. One downstream target for PI3K is Akt. In mice deficient in PI3Kγ phosphorylation of Akt does not occur, which indicate that Akt is a PI3Kγ effector [194]. G_i- induced Akt phosphorylation has been reported potentiated by the $G_{12/13}$ pathway via the activation of Src kinase [195]. It has recently been reported that thrombin activation of platelets mediated by PARs causes rapid Akt phosphorylation independently of ADP secretion, but that ADP/PI3K were required for the maintenance of this phosphorylation. Activated Akt regulates platelet function by modulating secretion and the $\alpha_{2b}\beta_3$ activation [196].

G_i also activates Rap1b via PI3K and further studies indicate PIP_3 as the primary regulator of Rap1b activation [197], which is critical for fibrinogen receptor activation, leading to aggregation.

It has been reported that thrombin-induced $cPLA_2$ activity leading to TXA_2 generation was potentiated by secreted ADP via the $P2Y_{12}$ receptor through regulation of ERK1/2 activation [198].

1.2.3.1.2. Platelet adhesion receptors

There are three types of adhesion receptors: the leucine-rich glycoproteins (GP), integrins and immunoglobulin. The main agonists involved in adhesion, and aggregation using these receptors

are vWF, collagen and fibrinogen and the main receptors involved are the GP Ib-V-IX complex, GP VI and $\alpha_{IIb}\beta_3$, respectively.

Collagen has the ability to bind to several platelet receptors where GP VI is considered to be the main signaling receptor. The GP VI receptor is coupled to the disulfide-linked Fc receptor (FcR) γ- chain homodimer. Stimulation of platelets with collagen induces tyrosine phosphorylation of the FcR γ-chain immunoreceptor tyrosine based activation motif (ITAM). The Src kinases Fyn and Lyn are involved in this initial activation step [199, 200]. The phosphorylation of ITAM promotes association between the FcR γ-chain and the tyrosine kinase Syk [201]. Collagen (type I) stimulates platelets through phosphorylation of PLCγ, and Syk kinase is reported to be upstream of PLCγ. PLCγ stimulation leads to production of DAG and IP_3, which acts as described above. Collagen activation mediated by GP VI, involve PI3K signaling as reviewed [202]; $PI(3,4,5)P_3$ is reported to interact directly with PLCγ2 [203] indicating involvement upstream of PLC. In fact both PI3K and PLCγ2 are reported to be involved in formation of a signalosome together with adaptors and other effector proteins downstream of Syk, some of the factors involved are the transmembrane adapter LAT, the cytosolic adapters SLP-76 and Gads and Vav 1/3, a Rho/Rac family GEF, which also can act as an adaptor through its multidomaine structure including SH2 and SH3 domains, the purpose of this LAT signalosome is probably to modulate and support signaling via PLCγ2, one of the major effector enzymes in the GP VI signaling cascade [204, 205].

There are evidence of GP VI activation leading to activation of several small G proteins: Vav is constitutively associated with Grb2 (growth factor receptor bound protein 2) [206]. Grb2 is an adaptor protein comprised of a central SH2 domain and two flanking SH3 domains, and it forms a stable complex with Sos (“Son of sevenless”) via the SH3 domains, Sos is a dual specificity guanine nucleotide exchange factor (GEF) that regulates both Ras and Rho family

GTPases, thereby linking recruitment of Grb2 to activation of Ras. The downstream effectors of Ras are believed to be well characterized [161]. Activated Ras recruits the Ser/Thr kinase Raf to the membrane where it is activated. Raf then phosphorylates MAP kinase kinases (MEKs) that in turn phosphorylate MAP kinases (ERKs) on serine, threonine and tyrosine residues. ERKs can activate $cPLA_2$ in platelets leading to TXA_2 as reported above. However, a later study reporting Ras activation by GP VI stimulation demonstrates that Ras is not necessarily coupled to ERK in human platelets [207]. Phosphorylated Vav acts as an exchange protein for Rac-1 [208], a member of the Rho-family. The exact steps from Vav to Rac are still not clear in platelets. Collagen was reported to rapidly activate both Cdc42 and Rac, where Cdc42 activation was independent of PLC and Rac dependent of both PLC and PI3K [179]. ARF6 has also been reported involved in the collagen signaling cascade upstream of the Rho-family of GTPases [209].

GP VI can also mediate signals in FcRγ-independent mechanisms involving calmodulin (calcium pathway) [210].

Ultimately, the engagement of GP VI up-regulates platelet integrins, including the collagen receptor $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ involved in aggregation (inside-out signaling).

vWF is involved in the primary adhesion of platelets and binds to the GP Ib subunit of the GP Ib-V-IX complex. It can also interact with the $\alpha_{IIb}\beta_3$ receptor contributing to platelet aggregation. Signaling through the GP Ib-V-IX complex is suggested to involve phosphorylation of the FcRγ-chain and the downstream effects as described for collagen, and phosphorylation of another ITAM-containing receptor, FcγRIIA, a low affinity receptor for IgG [211]. This phosphorylation is mediated by a Src-kinase [212] and probably involves PLC at some point [211].

Pyk2 (proline-rich tyrosine kinase 2) also called RAFTK (related adhesion focal tyrosine kinase) is reported to be activated in response to vWF, downstream of Syk and probably independent of phospholipase signaling [213]. Pyk2 is a cytosolic tyrosine kinase which has two proline-rich domains which may interact with SH3 domains in other proteins, and it has at least two tyrosines that become phosphorylated, Y882 (a Grb2 binding site) and Y402 (a binding site for members of the Src family). This makes Pyk2 a signaling-complex, which in vWF mediated platelet activation is believed associated with 14-3-3, Src, FAK, PI3K and talin, where PI3K and talin effect the activation of integrins [211].

PI3K effectors involved are probably Akt1 and Akt2 reported to activate the NO-cGMP-PKG pathway leading to integrin activation mediated by p38/ERK [214].

Another pathway leading to fibrinogen receptor activation by vWF involves Src/ERK-mediated TXA_2 generation, which was reported to be an absolute requirement for aggregation [215].

The GP Ib-V-IX complex is reported constitutively associated with a number of intracellular proteins which include actin-binding proteins and calmodulin amongst others. These interactions may explain GP Ib-V-IX involvement in adhesion, cytoskeletal reorganization and transmembrane signaling as reviewed in detail [211].

Agonist-induced integrin activation and ligand binding is in general proposed to initiate formation of phosphotyrosine signaling complexes mediating the outside-in signaling. This topic has recently been reviewed [205, 216] and the initial tyrosine kinases so far established to be involved are Syk and Src [217-219] and the activation mechanisms involved suggested as: Src dependent activation of Syk, leading to activation of a signalosome similar to the one established for GP IV signaling, except for involvement of ITAM and LAT, containing SLP76, Vav 1/3, PLCγ2 and PI3K. Syk independent pathway, where focal adhesion kinase (FAK) has been shown to interact with the cytoplasmic domain of β_1, β_2 and β_3 integrins and becomes

autophosphorylated following integrin engagement [220]. Src is bound via a SH2 domain at Y397 to FAK [221]. It has been demonstrated in Cho cells that Src and $\alpha_{IIb}\beta_3$ form distinct signaling complexes with either FAK or Syk [222]. FAK interacts with Grb2 [223], and is thereby linked to the Ras pathway as described above. Rac is considered a downstream effector of Syk/Vav in integrin signaling, leading to actin polymerization/reorganization [216].

1.2.3.1.3. Protein kinase C

Both soluble agonists and adhesion molecules activate platelets through PLC, thus increasing intracellular calcium and DAGs, which activate PKC. PKC seems to be central in regulating platelet activity and function; the use of broad-spectrum PKC inhibitors has given PKC a role in calcium entry, granule secretion, $\alpha_{IIb}\beta_3$ activation, and outside-inn signaling. PKC is a protein serine/threonine kinase which exist in at least 10 isoforms divided in three subgroups: conventional PKC (cPKC), comprises the α-, βI-, βII- and γ- isoforms activated by DAG and Ca^{2+}, novel PKC (nPKC) which are the δ-, ε-, η- and θ- isoforms activated by DAG and the atypical PKC (aPKC) comprising the ζ-, ι-, λ- and μ-isoforms that are insensitive to DAGs and Ca^{2+}. In platelets multiple isoforms of PKC are expressed [224, 225], and it is presumed that each isoform plays one or more distinct roles, although the exact roles of each isozyme have not yet been elucidated. However, progress have been made by the use of more specific inhibitors and platelets deficient in PKCβ, δ and θ. PKCα have been reported to be essential for α- and dense-granule secretion [226] and directly involved in Ca^{2+}-induced aggregation [227]. The importance of PKCα in $\alpha_{IIb}\beta_3$ activation and aggregation is underscored by the significant inhibition of broad-spectrum PKC inhibitors, while platelets lacking PKCβ, δ or θ showed no decrease in aggregation. Others have shown that PKCα activation was important for both GP VI and GP Ib-IX-V mediated dense granule secretion and aggregation. The PKCα activity was

dependent of both PLC and Syk, while PKCα itself negatively regulated Src [228]. The use of mice lacking PKCβ or θ have implied that these isoforms are involved in $\alpha_{IIb}\beta_3$ outside-in signaling playing distinct but essential roles as reviewed [229]. PKCβ was also reported to co-immunoprecipitate with $\alpha_{IIb}\beta_3$ probably mediated by RACK1 in another study [230]. Several roles for PKCδ have been proposed by the use of the specific inhibitor rotlerin [224, 231]. However, the use of murine platelets lacking PKCδ questions the specificity of this inhibitor [232] as it exhibits many of the same effects in the absence of PKCδ. PKCδ appears to regulate filopodia dynamics negatively, probably by inhibiting VASP phosphorylation by a cPKC at Ser^{157} [232]. Another study showed that Gp VI and GP Ib-IX-V mediated signaling leads to interaction of Fyn and PKCδ, which involves tyrosine phosphorylation of PKCδ [225].

Activated PKC's main substrate is pleckstrin (P47) that is rapidly phosphorylated during platelet activation on multiple sites and may function in inhibiting PIP_2 hydrolysis. PKC also phosphorylates the PKC-specific substrates MARCKS (myristoylated, alanine-rich C-kinase). MARCKS is known to bind actin and cross-link actin filaments, which is inhibited by PKC phosphorylation and is suggested to play a role in dense granule secretion [233, 234].

Figure 5 summaries the major activation pathways in platelets and the possible involvement of PLD in these pathways. One should bear in mind that in platelets during haemostasis the receptors and pathways work together in synergy to enhance or dampen signals elicited for the proper platelet response.

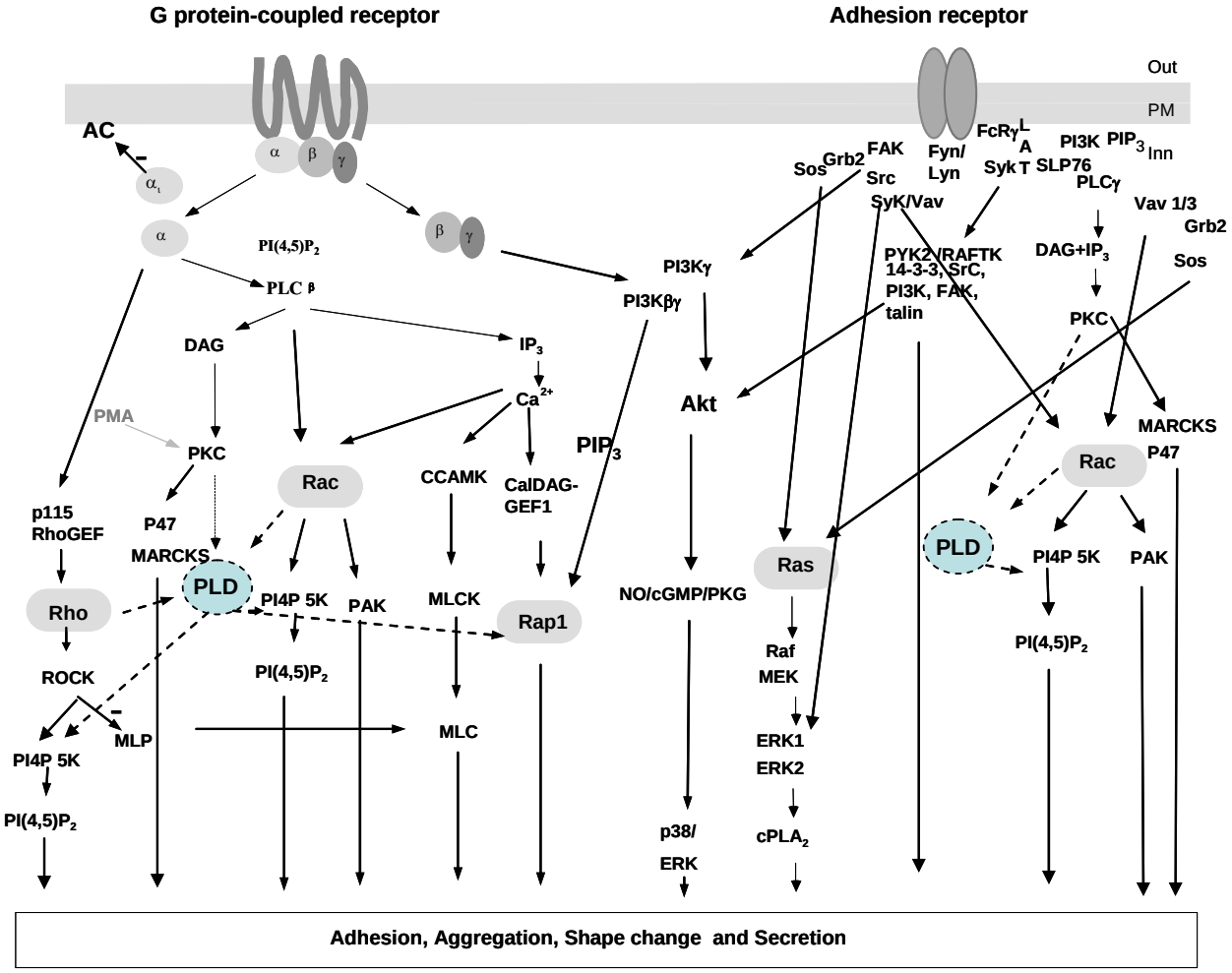

Figure 5: Major signaling pathway following activation of adhesion or G protein-coupled receptors in platelets, adapted from [161]. Se the text for more details and abbreviations. This figure is meant to give an overview of the signaling pathways and their complexity, it does not show all factors or pathways involved. Solid arrows represent known interactions and broken arrows proposed.

1.2.3.2. Inhibition

The most known system that inhibits platelet activation is elevation of cAMP, but the exact mechanism(s) by which cAMP exerts its action is not yet known. The level of cAMP is controlled by the activities of adenylyl cyclase (AC) and cyclic nucleotide phosphodiesterase

(PDE). Prostacyclin (PGI_2), PGD_2 and adenosine are physiological activators of AC that work through G_s. However, the interaction of most all platelet agonists with their receptors activates G_i thereby inhibiting AC, so AC is controlled by the relative concentration of agonists and AC activators. Thrombin is also reported to regulate the cAMP concentration by phosphorylation/activation of PDE3A involving Akt signaling pathway [235]. Many antiplatelet drugs (e.g. persantin) are PDE inhibitors, which elevate the cAMP level. cAMP activates protein kinase A that phosphorylates many platelet proteins, including a Ca^{2+}-ATPase in the DTS which pumps cytosolic Ca^{2+} back into the DTS, thereby suggesting one mechanism for cAMP's inhibitory effect. Elevation of cAMP also slows down the PPI cycle, thus decreasing the amounts of PIP_2 available to PLC early in the signaling cascade [236].

Nitric oxide (NO) released from the endothelium, is another physiologically important platelet antagonist. NO leads to an elevation of cGMP [237]. cGMP activates cGMP-dependent protein kinase (PKG), but there is also reports of cGMP involvement in the cAMP pathway by inhibiting PDE3A [238-240]. It has been reported that NO mediates inhibition of thrombin-induced platelet shape change via PKA, an inhibition that correlated to an increase in cGMP in compartments close to PDE3A, PKA and VASP (vasodilator-stimulated phosphoprotein) [237]. VASP is phosphorylated on Ser^{157} by PKA and Ser^{239} by PKG; VASP is a key regulator of filopodia dynamics and negatively regulate platelets through these kinases. A recent study identifies a novel target for NO and PGI_2 mediated signals via PKG/PKA: the phosphorylation of Rap1GAP2, which inhibits Rap1GAP2 binding to 14-3-3, that eventually leads to early termination of Rap1 signaling, which is involved in integrin activation and platelet adhesion [241].

In addition, inhibition of production of the autocoid TXA_2 by cyclooxygenase inhibitors (eg. acetylsalicylate) is also a much-used means of inhibiting platelet activation that has been used in many antithrombotic trials.

1.2.3.3. Autocrine stimulation and inhibition

Activation of platelets by a primary agonist such as thrombin or collagen leads to secretion of a number of substances and the synthesis of several platelet antagonists, which enhance the primary signal, a phenomenon known as autocrine stimulation. However, PDGF has an inhibitory effect, which makes PDGF an autocrine inhibitor of platelets, as illustrated in Figure 6.

Thus, the primary signal acts through multiple receptors and effector systems and crosstalk between different signaling pathways leads to multiple platelet responses as discussed above. In order to distinguish between primary and autocrine effects different inhibitors of autocrine stimulation (IAS) have been developed. The combination of creatine phosphate (CP)/ creatinphosphate-kinase (CPK) will catalyze conversion of ADP to ATP. Activated $\alpha_{IIb}\beta_3$ has the ability to bind fibrinogen, vWF, fibronectin and trombospondin, all of these ligands contain an Arg-Gly-Asp-Ser- (RGDS) sequence recognizes by the receptor, thus the peptide RGDS is a useful inhibitor of binding to the fibrinogen receptor inhibiting aggregation and outside-in signaling. Inhibitors for TXA_2, Serotonin and PAF are SQ 29,548, cyproheptadine and BN 52021 respectively. An important issue is that all of these inhibitors act extracellularly [161].

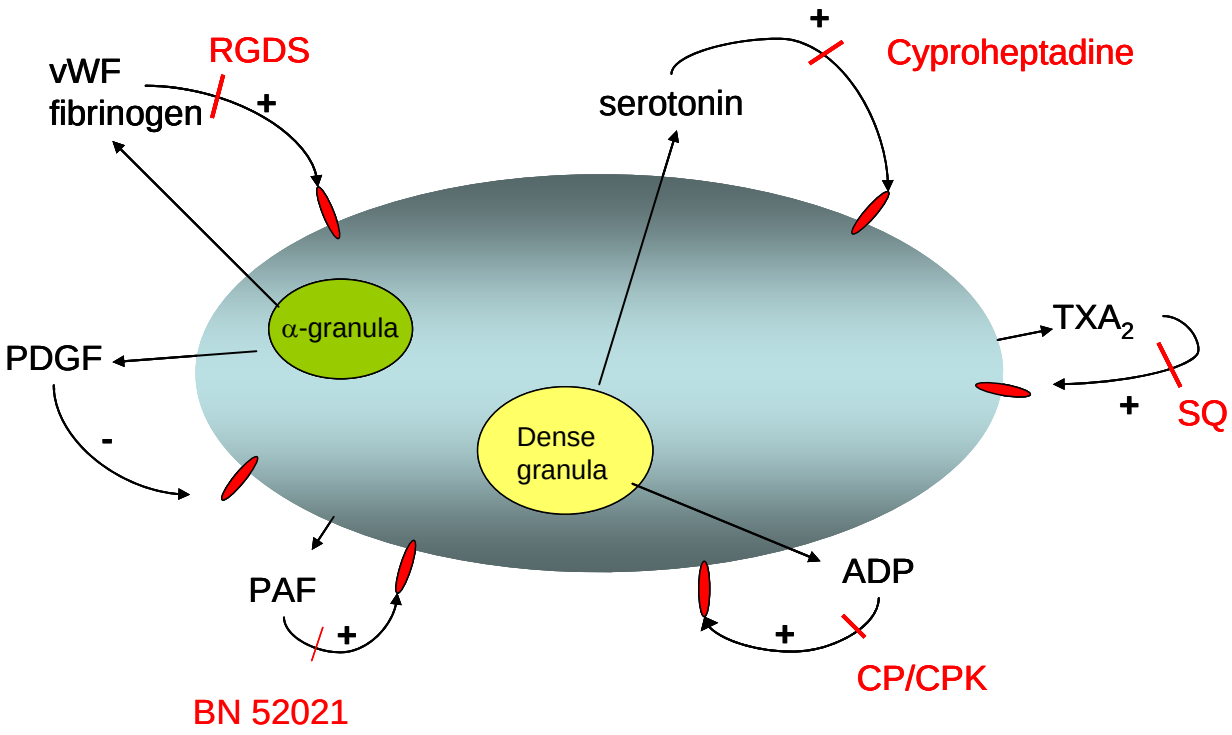

Figure 6: Platelet autocrine stimulation and inhibition as a result of addition of a primary agonist. Inhibitors of the different pathways are indicated in red and described in more details in the text.

2. Aims of the study

In the last decades great progresses have been made in the field of phospholipids, and it is now recognized that both the head group and the acyl chains are important for lipid functions. The lipid composition of membranes determines their properties such as charge and fluidity, membrane fusion processes, protein localization and activation. It is therefore of major interest to study the enzymes able to modulate these properties.

Phosphatidylcholine, the main substrate for PLD, is the most abundant phospholipid in eukaryote cells, and the PLD product PA is recognized as a signaling lipid, implied in protein translocation to and localization in/at the membrane and is probably important for membrane fusion as discussed above. Thus PLD is an interesting enzyme to study.

When this project was started it seemed clear that PLD was present in platelets and activated by agonists that evoke platelet responses (as reviewed in 1.5). However, many of the reported findings were contradictory (e.g. requirement for integrin ligation and autocrine stimulation, time courses of PtdEth, etc.). In addition most of the studies where PLD activity has been measured, have been performed in the presence of alcohol, which might have severe inhibitory effects on platelet functions. Equally importantly, use of alcohol to measure PLD activity eliminates production of PA, the physiological cellular product of the PLD reaction, which has been postulated to be an important signaling molecule in platelets. Therefore our initial goal was to establish a PLD assay without the use of alcohol by measuring released [^{14}C]choline as an indicator of PLD activity, but this seemed difficult as discussed in the appendix.
We have previously in our lab studied the PLD enzyme in C3H10T1/2 fibroblasts, especially the interaction between PLD and PKC, which we wanted to explore further in platelets. In addition, little is generally known about PLD's exact role and activation mechanisms in eukaryotic cells.

Thus our main goal was to gain more knowledge about activation mechanisms and functional roles of PLD, and the partial aims of this study is described in detail for each paper below.

Paper 1

In this study the main goal was to investigate thrombin-induced PLD activity and its dependence of autocrine stimulation using specific, extracellular inhibitors. A secondary goal was to study possible roles for PLD in platelet function. However, as no specific inhibitors for PLD exist at present, this had to be done by time and dose correlation studies. We also wanted to establish the presence of PLD isoenzymes in platelets and, if possible, their subcellular localization both by cellular fractionation and immunohistochemistry.

Paper 2

The initial work with establishing a choline release assay for measuring PLD activity, indicated that PLD could be activated by the platelet antagonist PGE_1, which we wanted to investigate further in this study. As PGE_1 is a platelet inhibitor we also wanted to se if it could inhibit the thrombin-induced PLD activity and PLD translocation (found in Paper 1). PGE_1 is believed to inhibit thrombin-induced platelets activity by increasing cAMP. To study the mechanisms involved we included other substance, which affects this pathway in both the PLD activity measurements and immunohistochemical studies. The additional substances used were: forskolin (direct activator of adenylate cyclase), PKA inhibitors (Rp-8-Br- cAMPS and Rp –cAMPS), PKA activator (Sp-5,6-DCL-cBIMPS), PKG inhibitor (Rp-8-pCPT-cGMPS) and PKG activator (8Br-PET-cGMP).

Manuscript for paper 3

The main objective of this work was to study the relationship between PKC isoenzymes and PLD1 and 2. We wanted to investigate if PKC activity was involved in thrombin-induced PLD

activity and translocation, and to study a possible association between the two enzymes by immunoprecipitation, as previously found in fibroblasts, and if this association was dependent of addition of thrombin, PMA, PGE_1 or forskolin. One of the main effectors for thrombin-induced platelet activation is PLCβII leading to PKC activity amongst others, therefore a second objective were to investigated if this enzyme also associated with the PLDs using immunoprecipitation and the same substances as for the PKC interactions. Additionally, we wanted to compare the localization of PKC isoenzymes and PLCβII with PLD in resting and thrombin-treated platelets by immunohistochemistry and to observe if the localization in thrombin-treated platelets were affected by the presence of forskolin as we found for PLD (Paper 2).

3. Summary of results

In the first paper we report the presence of a basal PLD activity and a thrombin-induced PLD activity in human platelets. We used [^{3}H]arachidonic acid-labeled platelets and incubated the labeled platelets with 0.4% ethanol in order to measure PLD activity as the formation of [^{3}H]labeled PtdEth. The thrombin-induced PLD activity in this system was immediate and did not level of until after 10 min. Thrombin-induced PLD activity was increased by presence of extracellular calcium, while calcium alone had no effect. When adding the calcium chelator EDTA together with thrombin extracellularly, the PLD activity decreased compared with thrombin alone.

In order to study if activation of PLD was a primary effect of thrombin or dependent of thrombin-induced autocrine stimulation we included three inhibitors of autocrine stimulation (IAS); RGDS a peptide inhibiting binding to the $\alpha_{IIb}\beta_3$ receptor (which prevents fibrinogen binding) and subsequently the outside-in signaling, CP/CPK which removes the secreted ADP and SQ that inhibits binding of TXA_2 to its receptor. We found that the thrombin-induced PLD activity was greatly inhibited by IAS, notably by RGDS and CP/CPK in synergy.

In this study (Paper 1) we also investigated potential roles for PLD in platelets; knowing that extracellular calcium and IAS affected thrombin-induced PLD activity, we used these parameters together with dose dependencies, time course and effects of ethanol in order to try to correlate platelets responses such as shape change (measured by F-actin formation), and secretion (lysosomal and dense-granules) with PLD activity.

Dense granule secretion is a rapid platelet response; the secretion was independent of extracellular calcium, IAS and ethanol and therefore did not correspond well with PLD activity.

The lysosomal secretion is a more slow process than the dense-granule secretion, and the response to different thrombin concentrations at 300 sec was almost identical of that observed for PLD, which was also the case for the effect of calcium. In addition, lysosomal secretion was inhibited by CP/CPK and ethanol, making this secretion a possible candidate for PLD involvement. F-actin formation in platelets is an ongoing event in activated platelets and seems to be dependent more on the time since stimulation than the thrombin concentrations used. F-actin formation was independent of the presence of extracellular calcium; however, its removal by EDTA seemed slightly inhibitory. The addition of IAS had little effect of the F-actin formation, with the exceptions of RGDS with 10 min of thrombin incubation. The addition of ethanol led to an increase in F-actin formation, which might suggested a regulatory role for PLD in this particular process.

We established the presence of both PLD 1 and 2 isoenzymes in platelets (Paper 1) and we found them to be localized predominantly in the cytosolic fraction, with traces present both in the low-speed (cytosolic actin filaments) and high-speed (membrane cytoskeleton) fractions. Further we studied PLD localization by the use of immunohistochemistry: thus, in resting platelets we found that PLD1 and PLD2 were present all over the platelets, although with PLD1 localized in dots. When thrombin was added, both isoforms rapidly translocated to the plasma membrane areas. The translocations were independent of extracellular calcium, PLD-PA or autocrine stimulation.

In order to establish a PLD assay by measuring choline-release, we added the platelet antagonist PGE_1 to avoid aggregation during a centrifugation step and often we observed an elevated level of released choline, when starting the PLD assay. In Paper 2, we found that PGE_1-induced choline release and also increased PtdEth formation. This effect was slightly increased by extracellular calcium and only 50 % of the activity obtained by thrombin.

As PGE_1 is a well-known platelet antagonist, we wanted to study its effect on the thrombin-induced PLD activity. PGE_1 inhibited thrombin-induced PLD activity partially (40%), as was

found for forskolin (a direct activator of adenylate cyclase) and a direct activator of PKA (Sp-5, 6-DCL-cBIMPS). The direct activator of PKG (8Br-PET-cGMP) had no effect on the thrombin-induced PLD activity. The inhibitory effect of forskolin on PLD activated by thrombin could be partially abolished by addition of PKA inhibitors (Rp-8-Br- cAMPS and Rp –cAMPS).

Forskolin, PGE_1, the PKA activator, but not the PKG activator inhibited thrombin-induced PLD1 translocation. Although PGE_1 was able to activate PLD, it was not able to induce PLD translocation to the plasma membrane (Paper 2).

In order to study the relationship between PLD and PKC (manuscript for Paper 3), we established that PMA induced PLD activity in our PtdEth assay, which was markedly increased by the addition of extracellular calcium. PMA, was able to induce the same level of PLD activity as thrombin, however, it did not have the same effect on PLD translocation as PMA only induced translocation of PLD1.

Both the thrombin-induced PLD activity and translocation were independent of the PKC inhibitor Ro-32-0432. Immunoprecipitation studies showed that PLD 1 and 2 were associated with the PKC isoforms PKCα, βI, βII and δ. The association between PLD isozymes and PKCα was constitutive, whereas the associations between PLDs and the other isoforms varied with the factors added (thrombin, PMA, PGE_1 and forskolin). The localization of the PKC isoenzymes was investigated by immunohistochemistry; in resting platelets the PKC isoenzymes were localized all through the platelet cytosol as observed for the PLDs, except for PKCβII, which showed a distinct different more central localization. Upon addition of thrombin, PKCα, βI, and δ moved towards the plasma membrane area, while βII became even more centralized. PKCβI was the one corresponding best with PLD translocation. Thrombin-induced PKC translocation was inhibited by forskolin. Activation of platelets by thrombin leads to activation of PLCβII, which is considered to be upstream of PKC, thus, we wanted to se if this enzyme also could associate with PLDs. PLCβII co- immunoprecipitaded with both PLD1 and 2 in unstimulated as

well as in activated platelets. PLCβII was also observed to have the same localization as PLD in unstimulated and thrombin-activated platelets as observed by immunohistochemistry; however, PLCβII translocation was independent of forskolin.

4. General discussion and future perspectives

4.1. Measuring PLD activity

In the present work we initially wanted to measure PLD activity as released labeled choline, but as discussed in the appendix this appeared complicated since choline seemingly was re-metabolized in platelets. The product of the transphosphatidyl reaction in the presence of ethanol (PtdEth) is considered to be un-metabolized and is therefore a good measure of PLD activity and most studies uses the detection of radiolabeled PtdEth or PtdBut, which is the product in the presence of 1-butanol, as a measure of PLD activity. The use of butanol to measure PLD activity or to inhibit the formation of PA has an advantage over ethanol that one can use 2-butanol or *tert*-butanol to control for adverse effects as they are not substrates in the PLD reaction. However, most studies in platelets use ethanol. In our hands, with 10 min pre-incubation of butanol, we found a decrease in both PLD and PLA_2 activity with 1-butanol compared to using ethanol and that *tert*-butanol had a severe inhibitory effect on PLA_2 (unpublished results). A recent report using butanol to inhibit PA-production in platelets observed no inhibition with *tert*-butanol on aggregation and Rap1 activation using 3 min pre-incubation [154]. On the other hand, it is also reported that 1-butanol interferes with PLD1 and PKCα association and inhibits PLD1 basal activity after 2 min of incubation in COS-7 cells [242]. Therefore, great care should be taken in choosing the alcohol, time of incubation and concentrations in each study in order to obtain reliable results.

In our studies we preferred to label with arachidonic acid after testing several fatty acids (Paper 1), and by using this method we found non-corresponding results with studies using different labeling [135]. One should bear in mind that by labeling the acyl chains one probably measures parts of PLD activity as PLD in other cells shows substrate specificity [243-246] and which has

been shown for platelet PLC and PLA_2 [247]. In our study we also tried to label the glycerol backbone to avoid the possible effects of labeling only parts of PtdEth formation, [^{3}H]labeled glycerol in our hands did not incorporate well in platelets and was therefore not an option. As discussed (Paper 1), the labeling of cellular lipids is anisotropic; thus the possibility also exists that findings only reflect the availability of the labeled-lipids to the PLD enzyme. It will be of great interest to establish a PLD assay in our lab in which one measure all PtdEth produced and the specific acyl chains by mass spectrometry and not a selective labeled part of the population as probably done by different means of labeling and ultimately to develop an assay without the use of alcohol, since many enzymes are regulated by the concentration of substrates, it can not be excluded that the removal of PA does not give a full picture of PLD activation and regulation mechanisms. PA produced by PLD1 has been reported involved in activation of PLD2 [117].

4.2. Activation mechanisms and PLDs role in platelets

The so far reported activation mechanisms of PLD in platelets are depicted in Figure 4AB and the involvement of PLD in platelet activation pathways are proposed in Figure 5. As mentioned in the introduction, a recent report suggests PAR1 signaling to be dependent of a PLD-PA pathway [154] .It would be of interest in the further study of thrombin-induced PLD activity to establish which PAR receptors are involved as emerging evidence reports that they act by different mechanisms [154, 248], this can be done by specific peptides. Although PAR1 mediated activity was the only one affected by 1-butanol [154], this does not exclude PAR4 in activating PLD. PLD is activated by high concentrations of thrombin which are proposed to act via PAR4 [162, 164, 165]. Another aspect is to identify the G protein pathways involved, since PLD are implied downstream of $G_{12/13}$ and the Rho pathway in other cells [249]. PAR1 reported

to be dependent of PLD-PA was in another study reported to activate human platelets through a $G_{i/o}$/PI3K signaling pathway [248]. As mentioned in the introduction, PLD possesses a PIP_3 binding site, making PI3K a possible factor in PLD activation, which would be interesting to study further in platelets. We find co-localization with PLCβII that suggest involvement of the G_q pathway.

It would also be interesting to study which ADP receptors contribute to the autocrine stimulation of PLD and to use this knew knowledge to continue correlation studies in wait for a specific inhibitor.

The immunohistochemical studies can be improved by using electron microscopy and immunogoldlabeling, which might enable us to find the exact localization of the PLD enzymes. The subcellular localization of PLD can also contribute to more knowledge of PLDs role in cells. Co-localization studies by immunohistochemistry can be continued by fluorescence resonance energy transfer (FRET), a more accurate method for identifying proteins in proximity to each order.

To look into the concept of a signaling platform one could do an immunoprecipitation with PLD1 and PLD2 antibodies and a proteomic study of the immunoprecipitates in order to look for the presence of other proteins.

The mechanism underlying PGE_1-induced PLD activity also remains to be determined.

To conclude, this work establishes the presence of both PLD1 and PLD2 in platelets, which are both translocated to the plasma membrane area in thrombin-treated cells; this translocation seems important for PLD activation as loss of translocation coincides with loss of activity. However, translocation is not sufficient for maximal PLD activity since translocation is independent of autocrine stimulation while the PLD activity is not. We show that PLD1 and PLD2 have different mechanism of activation in platelets as PKA activity only inhibits PLD1

translocation and as PMA only induces translocation of PLD1. Finally, we suggest that PKC activity and association is implicated in regulating PLDs in platelets possibly involving a signaling complex including PLCβII, and that different isoforms of PKC interacts with PLD1 and 2 in a different manner, which implies different regulation mechanisms and probably different roles for the two PLD isoenzymes. However, major tasks remains to establish exact roles and regulation of PLD enzymes in platelets.

5. Appendix-Introductory results and observations

Initially we wanted to study PLD activity as the release of [^{14}C]choline to avoid the use of alcohol and its possibly negative effects on platelet functions.

The exact procedure for measuring PLD activity by release of [^{14}C]choline was as described in the methods in paper 2. In short, gel-filtered platelets suspended in modified Tyrode's solution (without Ca^{2+}) were incubated with [^{14}C]choline for 120 min. at 37^{o}C, centrifuged in the presence of 25 μM PGE_1 and resuspended in modified Tyrode's solution before they were stimulated with 1 U/ml thrombin. We observed an immediate increase in the [^{14}C]choline production, which leveled off after a few minutes (Figure 7); the same effect was observed with the natural agonist collagen (50 μg/ml) while PMA (170 nM) only gave 50 % of the effect obtained by thrombin and collagen.

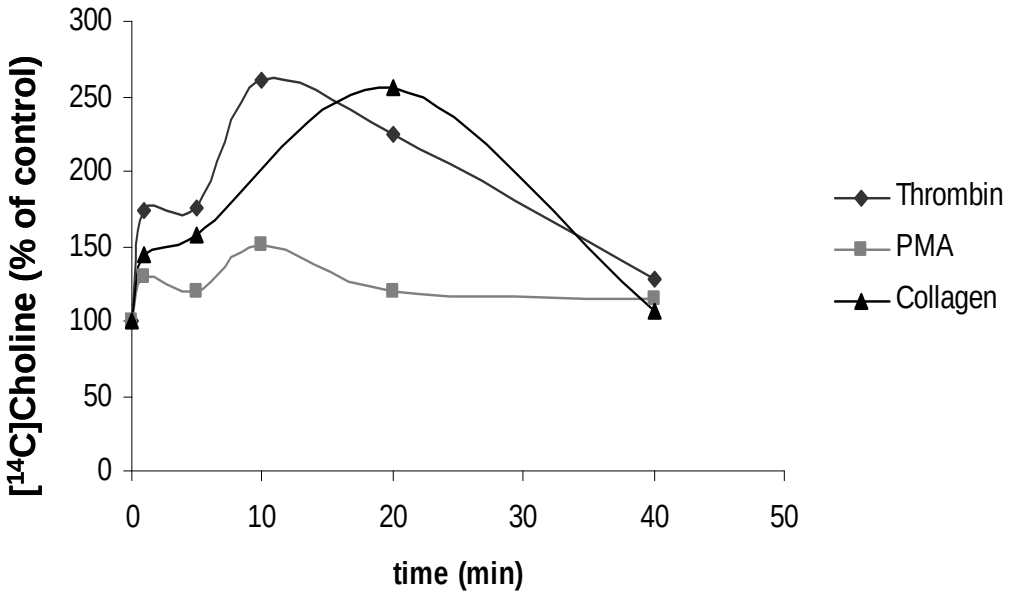

Figure 7. Stimulation of [^{14}C]choline-prelabeled platelets with PMA, thrombin and collagen. GFP were prelabeled for 2 h with [^{14}C]choline, washed in the presence of PGE_1 and resuspended in Tyrode's-buffer before the incubation with 170 nM PMA, 1 U/ml thrombin or 50 µg/ml collagen at 37 ^{o}C for the indicated times. [^{14}C]choline was isolated by thin-layer chromatography (TLC) from the water phase of chloroform/methanol extracts of the platelets and the radioactivity determined by Instant Imager. Control value (set to 100) was ≈ 23 cpm. Similar results were obtained in two other experiments**.**

[^{14}C]Choline-labeled platelets were separated from the media by centrifugation after stimulation with thrombin to investigate the localization of the newly formed choline. Choline appeared to leak rapidly out of the platelets (Figure 8) before re-entering the cells, and the total amount of free, labeled choline decreased with time.

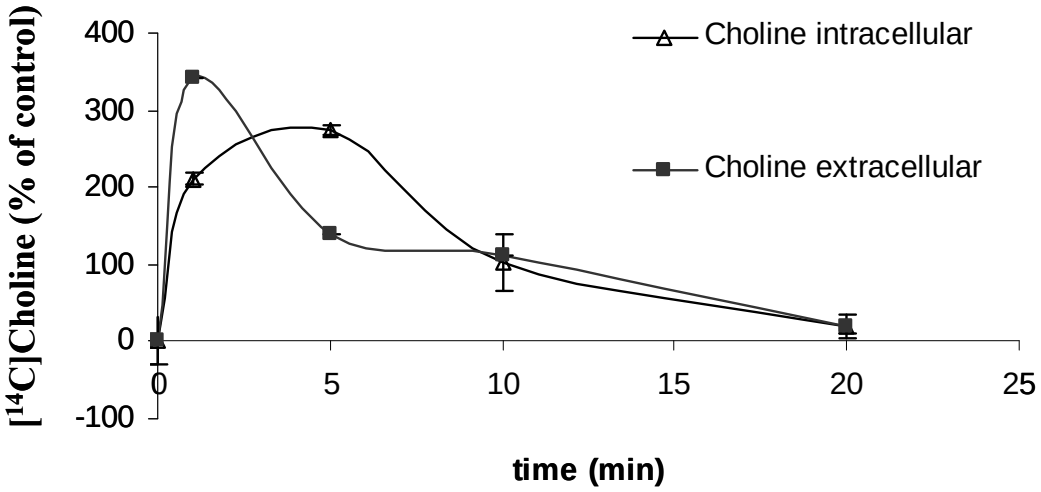

Figure 8: Choline produced by thrombin stimulation leaks rapidly out of the platelets. GFP were prelabeled for 2 h with [^{14}C]choline, and the platelets isolated, washed and resuspended as described in Figure 7, before the addition of 1 U/ ml thrombin for the indicated time. Platelets and medium were separated by centrifugation before extraction. [^{14}C]choline was isolated by TLC from the water phase of platelet extracts and the radioactivity determined by Instant Imager. Control values were 4 cpm intracellular and 2 cpm

extracellular. Two parallels were performed and the standard deviations calculated as indicated by the vertical bars. Similar results were obtained in five other experiments.

In these experiments we used a relatively high dose of thrombin (1 U/ml), but we observed release of choline at concentrations down to 0.1 U/ml; we also performed the assay with shorter time intervals, and already 15 sec after stimulation choline was released and leaking out of the cells. It was excluded that this release was due to secretion of [^{14}C]choline as we observed no incorporation into the granules.

PLD activity in human platelets as measured by the release of choline, has previously been shown to be stimulated by thrombin, collagen and PMA [133], as we have observed in our experiments. However, the time course studies showed fluctuations in the choline level, which was different from donor to donor; this made it difficult to choose a fixed time for performing the PLD assays. It also seemed that choline can be re-metabolized by the platelets, since the total amount of free, labeled choline decreased by time (Figure 7 and 8). This is different from our previous studies with fibroblasts where PLD-produced choline is not reutilized [250]. Choline can be phosphorylated to participate in de novo-PC synthesis, and choline generated by PLD isoforms can participate in synthesis of acetylcholine [251], which is present in platelets [252].

Another interesting fact was the observation that choline leaked out of the platelets before disappearing from the extracellular space, probably by re-uptake (Figure 8). Choline is a charged hydrophilic cation, and is transported by mechanisms driven by Na-gradients in other cells [253-255], but no mechanisms of this is so far known for platelets. We suspected that this presence of labeled choline in the extracellular space could be due to incorporation into granules and subsequent secretion as choline is an amide and could be stored in dense granules as is the case for serotonin [256], histamine [257], tyramine and tryptamine (Holmsen, unpublished

results), but we found this to be untrue as we observed no choline incorporation in the granule fraction.

In our choline release experiments we included PGE_1 to avoid aggregation during platelet preparation, however later experiments (Paper 2) showed that PGE_1 also could act as a PLD agonist, which has also been shown in human erythroleukemia cells [258, 259].

The choline release assay is also very time-consuming and taken together with the findings that choline was seemingly metabolized by platelets; we concluded that this assay was not optimal as a frequently used assay to screen for factors and concentrations that may affect PLD activity, but rather to give additional information to the PtdEth assay.

Although knowing that ethanol can affect the platelet activities, we preferred to measure PLD activity as the PLD-specific product PtdEth.

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