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Molecular and functional characterization of arachidonate-releasing and metabolizing enzymes in murine osteoblast-like MC3T3-E1 cells.

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Abbreviations

AA	Arachidonic acid
ADP	Adenosine diphosphate
ARF	ADP Ribosylation Factors
ATP	Adenosine triphosphate
BEL	Bromoenol lactone
cAMP	Cyclic adenosine monophosphate
Co-IP	Co-immunoprecipitation
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
DAG	diacylglycerol
DGKs	Diacylglycerol kinases
GC	Gas chromatography
GPCR	G-protein coupled receptor
GPC	1,2-diarachidonoyl-sn-glycero-3-phosphocholine
GTP	Guanosine-5-triphosphate
IB	Immunoblot
IP3	Inositol-1,4,5-triphosphate
LC	Liquid chromatography
LOX	Lipoxygenase

LPA	Lipoprotein A
LPAAT	lysophosphatidic acid acyltransferases
MAFP	Methyl arachidonyl fluorophosphonate
MAP kinase	Mitogen-activated protein kinase
MS	Mass spectrometry
PC	Phosphatidylcholine
PG	Prostaglandin
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
RTK	Receptor tyrosine kinase

1. Introduction

1.1 Overview

Phospholipases are enzymes responsible for catalyzing the release of fatty acids from phospholipids. There are four major classes of phospholipases: PLA, PLB, PLC and PLD. Each of them cleaves at a different site of the substate (*Figure1.1).*

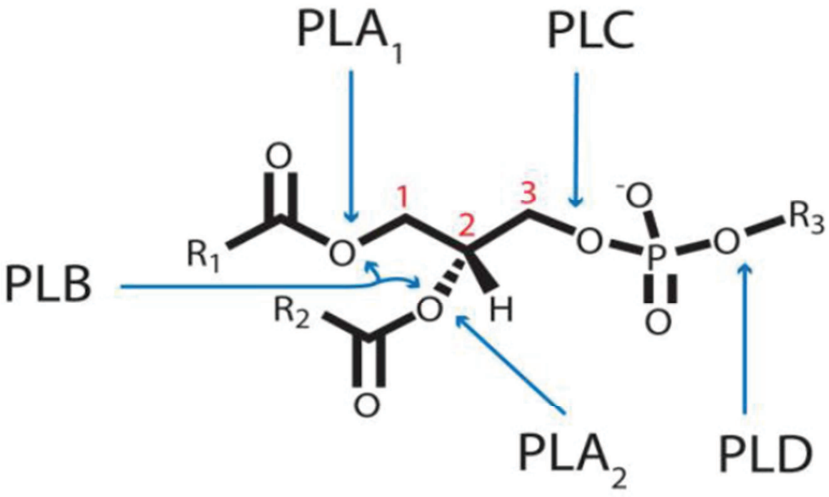

Figure 1.1 Cleavage sites of the phospholipases PLA1, PLA2, PLC and PLD. PLA1, PLA2 and PLC catalyze the hydrolysis of the ester bond from the sn-1, sn-2, and sn-3 carbon, respectively. PLD hydrolyzes the other phosphodiester bond. PLB cleaves both the sn-1 and sn-2 ester bonds [1].

One very important fatty acid released by phospholipases is Arachidonic acid (AA). It then can either be metabolized to eicosanoids or transferred into the extracellular space by diffusing through the plasma membrane. Exogenously-provided AA can be initially incorporated into a variety glycerolipids; which one depends on the concentration of AA provided to cells. Low concentrations of exogenous AA incorporate mainly to 1-acyl-linked phospholipid molecular species, high concentrations of AA accumulate in triglycerides and are also moved into unique phospholipid molecular species such as 1,2-diarachidonoyl-sn-glycero-3-phosphocholine (GPC).[2,3]

1.2 Aracidonic acid

Arachidonic acid is a polyunsaturated omega-6 fatty acid 20:4(ω-6) with the systematic name (5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoic acid. It is present in the phospholipids (especially phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol) of membranes of the body's cells esterified on the *sn-2* position, and is abundant in brain, muscle, and liver. In animal organisms it cannot be synthesized de novo; it has to be taken from dietary sources or generated through the conversion of linoleic acid.

In addition to being involved in cellular signaling as a lipid second messenger involved in the regulation of signaling enzymes, such as Phospholipase C-γ (PLC-γ), PLC-δ, and PKC-α, -β, and -γ isoforms, arachidonic acid is a key inflammatory intermediate and can also act as a vasodilator [4] since it is a precursor molecule in the production of eicosanoids. The bioavailability of free AA undergoes a tight control balancing the AA efflux from the cell as well as deacetylation and reacetylation reactions with subsequent transfer into phospholipid pools [2]. In mammalian cells the eicosanoid substrate production is regulated by the presence of free AA [5].

Arachidonic acid is released from a phospholipid molecule by the enzyme phospholipase A2 (PLA2), which cleaves off the fatty acid, but can also be generated from diacylglycerol (DAG) by diacylglycerol lipase [4]. Arachidonic acid generated for signaling purposes appears to be derived by the action of a phosphatidylcholine-specific cytosolic phospholipase A2, whereas inflammatory arachidonic acid is generated by the action of a low-molecular-weight secretory PLA2. PLA2 is activated by ligand binding to various receptors such as 5-HT2 receptor and the bFGF receptor. Furthermore, some of its forms can be activated by any agent increasing intracellular calcium. Alternatively, arachidonic acid may be cleaved from phospholipids by phospholipase C (PLC) into diacylglycerol (DAG), which subsequently is then cleaved by DAG lipase to arachidonic acid [6]. PLC may be activated also by MAP Kinase.

To fulfill its role as a lipid second messenger arachidonic acid can be metabolized by three different pathways involving three kinds of enzymes: the cyclooxygenase pathway, the 5-lipoxygenase (LOX) pathway and the cytochrome P450 pathway.

1.3 Cyclooxinase-1

Cyclooxygenase-1 (COX-1), also known as prostaglandin G/H synthase 1, is an enzyme encoded by the PTGS1 gene in humans. COX is the central enzyme in the biosynthesis of prostaglandins (PG) from arachidonic acid via the cyclooxygenase pathway mentioned above (figure 1.2). It converts free arachidonic acid to prostaglandin H2 (PGH_2), after being released from membrane phospholipids at the *sn-2* ester binding site by the enzymatic activity of phospholipases. The reaction involves both cyclooxygenase (dioxygenase) and hydroperoxidase (peroxidase) activity. The cyclooxygenase activity incorporates two oxygen molecules into arachidonic acid or alternate polyunsaturated fatty acid substrates, such as linoleic acid and eicosapentaenoic acid. Metabolism of arachidonic acid occurs via formation of prostaglandin G2 (PGG_2), a labile intermediate peroxide, that is subsequently reduced to PGH_2, the corresponding alcohol by the enzyme's hydroperoxidase activity. There are two isozymes of COX encoded by distinct gene products: a constitutive COX-1 and an inducible COX-2. They differ in their regulation of expression and tissue distribution.

A sustained supply of arachidonic acid and induced COX-2 activity are required for the delayed phase of AA metabolism. The delayed phase is the second distinct phase of prostaglandin biosynthesis following the intermediate phase [78].

1.4 Prostaglandins

Prostaglandins (PG) are a group of lipid compounds that are derived enzymatically from fatty acids with important functions throughout the body. PGs are autocrine or paracrine hormones that are locally acting messenger molecules. They are mediators for a variety of strong physiological effects, such as regulating the contraction and relaxation of smooth muscle tissue [9]. In contrast to other hormones they are not produced at a discrete organ but in many tissues throughout the human body. Their target cells are present in the immediate vicinity of their secretion site. They are unsaturated carboxylic acids that consist of 20 carbon atoms, including a 5-carbon ring.

There are various types of prostaglandins, involved in a variety of functions such as vasodilatation, bronchodilatation, platelet aggregation, GI tract smooth muscle contraction,

lipolysis inhibition, gastric acid secretion, and uterus contraction. They are synthesized in the cell from AA which is then brought to either the cyclooxygenase pathway or the lipoxygenase pathway to form either prostaglandins and thromboxanes or leukotriene respectively.

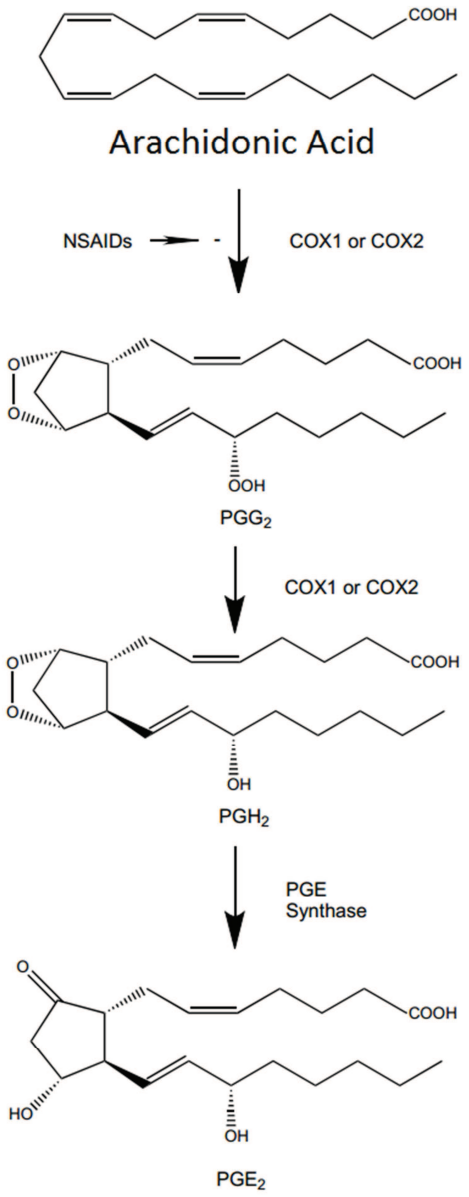

Figure 1.2: Pathway of the prostaglandin PGE_2 synthesis from arachidonic acid. COX-1 and COX2 convert arachidonic acid to PGG_2 and PGH_2. PGH_2 is precursor for different prostaglandins such as PGD_2, PGI_2, PGE_2, PGF_2 and thromboxane TXA_2. Non-steroidal anti-inflammatory drugs (NSAIDs) like acetylsalicylic acid inhibit the COX-1/2 by blocking the channel of the enzyme, thus, preventing arachidonic acid to enter the active site of the enzyme.

1.5 Phospholipase D

Phospholipase D enzymes are transphosphatidylases that play an important role in membrane trafficking, cytoskeletal reorganization, receptor-mediated endocytosis, exocytosis, and cell migration [10] and are involved in various diseases like cancer, and neurodegenration [11]. Hanahan and Chaikoff [12] were the first to describe a PLD-type activity in carrots extracts in 1947. It was not until almost thirty years later that the PLD activity was demonstrated in rats by Saito and Kanfer [13]. The first isolation of the enzyme was successfully performed in 1993 by two different groups. Abousalham et al. [14] isolated and purified PLD from cabbage and Wang et al. [15] from castor bean endosperm.

PLD activities have been described in multiple organisms, such as plants, mammals, bacteria, yeast and even in viruses. In mammalian systems it is found in almost all cell types except leukocytes and a few lymphocyte lines [16,17] and is regulated by a wide variety of growth factors, cytokines, neurotransmitters and other agonists such as phosphatidylinositol-4,5-bisphosphate, protein kinase C and ADP Ribosylation Factor and Rho family GTPases [18] and there are two isoforms of phospholipase D described: PLD1 and PLD2 [19]. All the PLD isoforms are splice variants of the two major isoforms previously mentioned which have a molecular weight of 120 and 100 kDa respectively. The sequence of PLD1 and PLD2 is by 50% identical [20–22]. The highly conserved and catalytic motif of PLD enzymes is designated as HKD and denoted as HxxxxKxD, whereas H is Histidine, K is lysine, D is aspartic acid and x any other amino acid [23]. Both PLD1 and PLD2 in mammalia contain two catalytic motifs (HKD). These motifs are of great importance for the activity of the enzyme since mutagenesis studies showed that point mutations can disrupt their functionality and activity [24]. For the catalysis to take place the first step is that the two HKD motifs associate with each other in order to form a catalytic center; the second step is the formation of a phosphohistidine intermediate [24–26]. The cellular localization of the enzyme remains unclear since many groups describe different locations. For instance Lucocq and Freyberg [27,28] suggest a perinuclear localization such as Golgi or endoplasmic reticulum, however [29] and Hughes et al. [30] have not found Golgi localization. Brown and Huang [31,32] suggested that when PLD is stimulated it translocates to the plasma membrane.

Phospholipase D catalyzes hydrolysis of phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine by mediating the hydrolysis of polar headgroups which are covalently attached to membrane-bound lipids cleaving the phosphodiester bond *(Figure 1.1)* [10]. Phosphatidic acid, which often acts as secondary messenger of transduction of intracellular signals and choline which has little second messenger activity, are the products of this hydrolysis [33].

Phosphatidic acid (PA) has been showed to play an important role in endocytosis, secretion and vesicle trafficking. PA can be converted to other potentially bioactive lipids, including diacylglycerol (DAG) and lysophosphatidic acid [18] by phosphatidic acid phosphohydrolases [34,35]. Diacylglycerol kinases (DGKs) can reconvert DAG via phosphorylation to PA. Another type of enzyme, phospholipase A (PLA) deacylates PA to form the monoacylated form of PA, lysoPA (LPA) which can then be reconverted to PA by lysophosphatidic acid acyltransferases (LPAAT) [35]. LPA is a major extracellular messenger that interacts with certain types of EDG receptors.

Growth factors, cytokines, neurotransmitters and various hormones increase the activity of PLD in many cells. Phosphatidylinositol 4-5-bisphosphate (PIP2) is a factor that affects the activity of PLD directly [36–38]. On PLD2, between the conserved sequences II and III there is a binding site for PIP2 [34]. Its sequence is characterized by conserved residues found both in PLD1 and PLD2. Any mutations that occur on that sequence can cause loss of binding capacity and catalytic activity in PLD1 and PLD2 but the cellular localization is not affected [34].

As mentioned above there are many PLD isoforms which derive from PLD1 and PLD2. PLD1 and PLD2 have been very interesting because of their association with various severe health issues such as cancer, neurodegenerative, cardiovascular and infectious diseases [39]. Pharmacologically, PLD1 and PLD2 are of great importance since their inhibition could play a role in developing novel therapeutics. They are expressed in different cell types and tissues and are activated by receptor tyrosine kinases (RTK) and G-Protein coupled receptors (GPCR*) (Figure 1.3).*

The main difference between those two molecules is their localization. Although the localization of PLD1 and PLD2 is still being investigated it has been shown that PLD1 is mainly found on the inner membranes of cells, for instance in the Golgi complex and in the endosomes [19], whereas

PLD2 is mainly found on the plasma membrane, in lipid rafts [10,33]. PLD1 activation by proteins is necessary whereas PLD2 is intrinsically highly active therefore activation molecules do not have a big effect on its catalytic activity [33]. The mentioned localizations can dynamically change during signaling cascades.

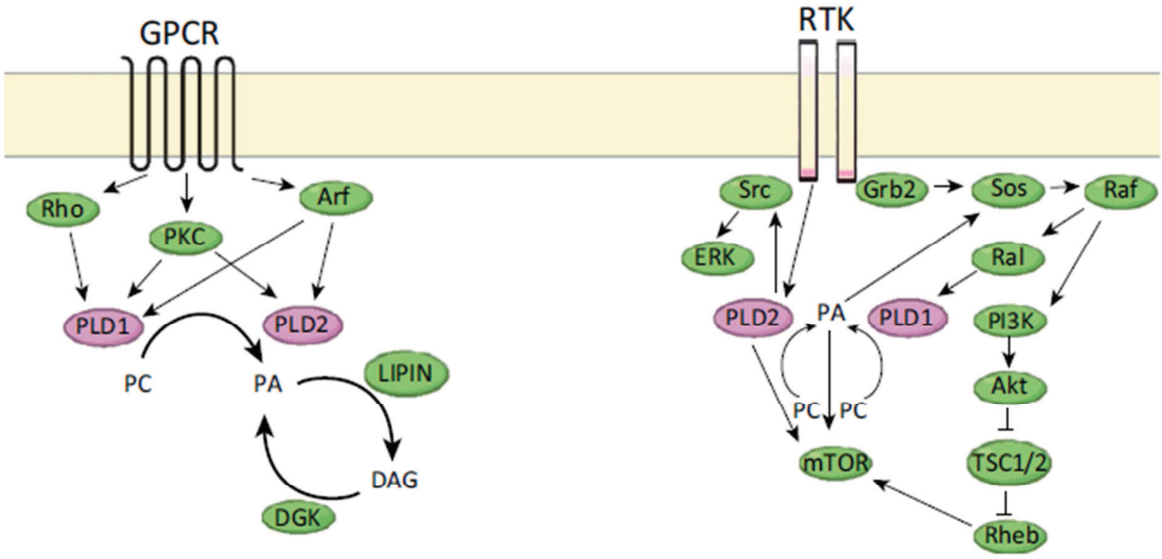

Figure 1.3: Simplified schematic of the signal transduction pathways where phospholipase D (PLD) is involved. G protein-coupled receptors (GPCR) and receptor tyrosine kinases (RTK) are stimulated by various hormones, growth factors, and cytokines and thereby PLD is activated [18]. PLD1 is activated by protein kinase C (PKC) and GTPases RhoA and ARF, whereas PLD2 is assumed to be activated by some of above illustrated factors. PLD2 can also be activated directly by RTKs and PLD1 by Ral. Necessary for PLD activity is the binding to phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2]. PLD2 can stimulate Src activity, and the PA generated by PLD1 and PLD2 can promote mTOR, such as the small GTPase Rheb [40].

PLD3 and PLD4 are endoplasmic reticulum (ER) integral transmembrane proteins. The bulk of the proteins and the catalytical domains are found in the ER lumen, whereas the N-terminus tail is in cytoplasma [41,42]. PLD5 is a rather yet not well investigated molecule for which is assumed that it has no catalytical activity since its catalytic motif is not well conserved [40]. PLD6 is also a transmembrane protein but with its N-terminus on the surface of mitochondria [43]. Ha et al. [44] have reported that PLD6 hydrolyses cardiolipin to PA and can function as an endonuclease.

1.6 Phospholipase A2

1.6.1 Ca^{2+}-independent PLA_2 ($iPLA_2$)

Phospholipase A_2 (PLA_2) belongs to a family of lypolytic enzymes that catalyze the cleavage of fatty acids from the sn-2 position of glycerophospholipids to generate free fatty acids and lysophospholipids. PLA_2 participate in a wide variety of physiological processes, including phospholipid digestion, remodeling of cell membranes and host defense. More than 19 different isoforms of mammalian PLA_2 are classified. They are diverted into five main groups based on sequence, molecular weight, cellular localization, disulfide bonding and requirement of Ca^{2+} including the secreted small molecular weight PLA_2 ($sPLA_2$), subdivided into several groups: IB, II (IIA, IIC, IID, IIE, IIF), III, V, X, and XII, the Ca^{2+}-independent PLA_2 ($iPLA_2$), consisting of two groups VIA and VIB, the Ca^{2+}-dependent arachidonoyl-specific cytosolic PLA_2 ($cPLA_2$), subdivided into six groups: IVA, IVB, IVC, IVD, IVE, and IVF, the platelet activating factor-acetylhydrolases (PAF-AH), subdivided into groups VII (VIIA, VIIB) and VIII (VIIIA, VIIIB) and the lysosomal PLA_2. [45] [46] [47]. The first PLA_2s discovered are the secreted ones. They occur in venoms from various snakes, scorpions, bees, they are components of the pancreatic juices and they are found in many mammalian tissues [48]. Research results indicated that PLA2s play distinct roles in various diseases such as atherosclerosis and diabetes and in several biological processes like inflammation, tissue injury and tissue repair, digestion and host defense [49].

The Ca^{2+}-independent PLA2 (iPLA2) belongs to group VI family of PLA2 and as the name implies, this enzyme does not require Ca^{2+} for being catalytically active. The first member was purified in 1994 and it was the group VIA from macrophages [50]. The best investigated enzyme from group VI is group VIA [51] and it exists in different splice variants [52]. The two active forms are VIA-1 and VIA-2. The group iPLA2 VIA is an 85 kDa protein and it contains eight N-terminal ankyrin repeats which are common motifs and mediate protein-protein interactions, a consensus lipase motif and a catalytic domain. [52]. It shows no fatty acid selectivity.

The group VIA-2 PLA2 is an 88 kDa protein with a similar sequence to the group VIA-1 PLA2. The only two differences are that the eighth ankyrin repeat is interrupted by 54 amino acids and a single amino acid change at position 450. They can act both as phospholipase and transacylase [52]. Burke et al. [53] suggested that the regulation of the activity of group VIA could happen through various mechanisms such as caspase cleavage, ankyrin repeat mediated protein aggregation, ATP binding and calmodulin.

Over the years there were many studies performed using different inhibitors in order to investigate the activity of this enzyme. Bromoenollactone (BEL) was the first inhibitor used by Winsted et al. [54] in an attempt to determine the functions of group VIA. A few years later, another study performed by Song et al. [55] showed that BEL is a rather inappropriate inhibitor because it is not specific for group VIA. A study with monocytes performed by Mishra et al. [56] using antisense oligodeoxyribonucleotide toward group VIA showed that monocyte directionality and recruitment decrease. Animal experiments performed with group VIA deficient mice showed that this enzyme plays a role in bone formation, development of sperm, apoptosis and secretion of insulin [57–60]. Furthermore, specific fluoroketone inhibitors of group VIA were recently developed and applied in animal models (mouse) showing that group VIA in combination with group IVA are involved in axon regeneration and Wallerian degeneration in nerve injury [61]. Acute inhibition has often resulted in decreased rate of incorporation of AA into phospholipids. This is the result of decreased lysoPC which is the main acceptor of free AA [51,62–65]. Therefore iPLA2-VIA plays a role in phospholipid fatty acyl chain deacylation/ reacylation reactions especially in phagocytes. Nevertheless, a study showed no evidence for alternated Lands Cycle when the cells were lacking Ipla2-VIA and therefore one should keep in mind that the cells may be able to use other mechanisms for controlling the levels of lysoPC [57].

Its role in apoptosis has also been investigated. Atsumi et al. [66,67] induced apoptosis in U937 promonocytes with different apoptosis factors, tumor necrosis factor –α or fas ligand plus cycloheximide. This resulted to cleavage of membrane phospholipids mediated by iPLA2-VIA and to the release of AA and other fatty acids. Furthermore, it was observed that the enzyme was cleaved resulting to a smaller truncated enzyme having increased iPLA2 functionality.

Studies with other types of cells regarding apoptosis were also performed. Turk et al. [68] used insulinoma cells and the apoptosis inducer thapsigargin. They observed an acceleration of membrane hydrolysis and of apoptosis through the overexpression of iPLA2-VIA. This study confirmed the results of Atsumi et al. about iPLA2-VIA being cleaved by caspase-3 [68]. The increased rate of apoptosis caused by overexpression of the enzyme was also proven by Perez et al. [69], who used hydrogen peroxide, an oxidative stress agent which promotes apoptosis to investigate the apoptosis effects in insulinoma cells. They also showed that the use of a specific antisense oligonucleotide or the use of an inhibitor in this case methyl arachidonyl fluorophosphates (MAFP) could not decrease apoptosis at longer period of times. Altogether, from these apoptosis results it could be concluded that iPLA2-VIA may also have other roles during apoptosis.

Group VIB (iPLA2γ) is the second $iPLA_2$ isoform which is homologous to iPLA2β in the C-terminal catalytic domain but shows no similarities in N-terminal region; accordingly, $iPLA_2\gamma$ may have distinct regulatory properties from those of iPLA2β [70,71]. It is membrane bound and is localized at the mitochondira, ER and peroxisomes [72]. This localization variability might be a result of specific domains in the structure of the enzyme [73]. It plays a role in chemotaxis, tumorigenesis and cell injury [74], in the assembly of very low density lipoprotein [75], endothelial cell platelet activating factor synthesis [76] and cellular proliferation [77].

Cummings et al suggested [72] that iPLA2γ may also play a role during oxidative stress as it recognizes, cleaves and removes oxidized phospholipids from the ER membrane of renal proximal tubular cells in order to repair or prevent lipid peroxidation. Another study from 2008 by Kinsey [78] also suggested that iPLAγ is of great importance for the protection of the cells against oxidative stress as they showed that is necessary for the maintenance of the function and viability of mitochondria and for the prevention and repair of basal lipid peroxidation. Murakami [79] transfected iPLA2 into HCA-7 cells and observed increased AA release and prostaglandin E2 synthesis via both COX-1 and COX-2, with a concomitant increase in cell growth.

Furthermore, Cohen [80] showed amplification of the Ca^{2+}-independent PLA2 activity and of the release of AA and prostaglandin E2 in glomerular epithelial cells (GEC) that overexpress iPLA2γ. Moreover, the iPLA2 directed inhibitor BEL as well as indomethacin, reversed the attenuation of complement-induced GEC injury resulting from overexpression of iPLA2γ, suggesting that the cytoprotective effect of $iPLA_2\gamma$ was at least in part mediated by generation of prostanoids [80]. Kinsey et al in 2008 [78] observed that expression of shRNA against iPLA2γ increased lipid peroxidation and induced apoptosis in renal cells. Another study showed that, complement mediated activation of iPLA2γ is mediated via ERK and p38 pathways, and phosphorylation of S511 and/or S515 plays a key role in the catalytic activity and signaling of iPLA2γ [81].

The group of Mancuso et al. [73] suggested a role of iPLA2γ in obesity whereby siRNA knockdown of iPLA2γ inhibited adipogenesis and iPLA2γ message, activity and protein mass increased during adipogenesis. In addition they demonstrated the up-regulation of the 63 kDa isoform in adipose tissue of Zucker obese rats.

iPLA2γ contains a consensus site for nucleotide binding and a lipase consensus motif in its C-terminal half [70]. It also contains a potential cAMP-dependent protein kinase, protein kinase C, and extracellular signal-regulated kinase (ERK) phosphorylation sites [81]. Essential for its catalytic activity is the lipase consensus motif GVSTG which consists of the amino acids 481-485 in the C-terminus. Loss of catalytic activity will occur if Ala is substituted by Ser-483 or Asp-627 [82]. iPLA2γ transcription and translation seem to be rather complicated since expression studies in recombinant systems demonstrated the usage of multiple translation initiation codons resulting in the production of different polypeptides [83] with the 63 kDa form mainly localized in peroxisomes [79].

Defining the mechanisms by which iPLA2γ is regulated and activated could increase the opportunities for the development of therapeutics against diseases that their underlying cause is iPLA2γ such as GCE injury.

1.6.2 Group IVA cytosolic phospholipases A2 (cPLA2)

cPLA2's are in general cytosolic 85 kDa calcium dependent PLA2s [84], a subgroup of enzymes that act on the intracellular phospholipid membrane [46]. cPLA2 catalyzes release of arachidonic acid from glycerophospholipids, leading to prostaglandins, thromboxane A2 (TxA2) and leukotrienes [85,86]. It has been purified and cloned [87]. Its primary structure has been elucidated by cDNA cloning and there was no significant homology with that of the 14 kDa secretory PLA2s [88]. Intracellular Ca^{2+} induces the translocation of cPLA2 to membranes through an N-terminal 87 amino acids long Ca^{2+} dependent lipid binding motif [89]. A regulatory phosphorylation site (Ser-505) has been identified that resides within the mitogen-activated protein kinase recognition sequence Pro-X(Leu)-Ser-Pro [90]. Several studies showed that phosphorylation on serine residues increases enzymatic activity [84,90] and Sharp et al. [88] demonstrated that Serine 228 is essential for the catalytic activities of cPLA2 *(Figure.1.4).*

Clark JD et al. [91] have proposed a model for the activation of cPLA2, where the enzymatic activity is regulated by several pathways, including those that control the concentration of calcium, the phosphorylation states and protein levels. When cells are stimulated with a receptor ligand, PLC is activated through either a G-protein dependent or independent process. The activation of PLC leads to the production of DAG and inositol triphosphate (IP3). When the concentration of these messengers rises, PKC is activated and intracellular Ca^{2+} is mobilized which then leads to the activation of cPLA2. An increased Ca^{2+} influx could also lead to cPLA2 activation and translocation to the membrane where its substrate is localized [91]. MAP kinase activation can occur through both PKC-dependent and -independent [92–94], whereby cPLA2 is phosphorylated at Ser-505 by MAP-Kinase leading to increased intrinsic catalytic activity [91]. A platelets study showed that when platelets are stimulated by collagen- and thrombin cPLA2 undergoes phosphorylation independent of PKC and MAPK and verified that Ca^{2+} plays an important regulatory role independent of phosphorylation [87]. Furthermore it was demonstrated that cPLA2 can also be active in the absence of Ca^{2+} if the assay buffer has high enough salt concentrations for enabling the binding of the enzyme with its substrate [95,96]. cPLA2 is in addition able to act as lysophospholipase and transacylase if incubated with lyso-phosphatidylcholine [96,97].

Ohto et al. [46] reported cloning and characterization of a number of novel murine cPLA2s: cPLA2 (group IVD), cPLA2 (group IVE), and cPLA2 (group IVF), that form a gene cluster with cPLA2β (group IVB). The amino acid sequences of cPLA2δ, ε and ζ had a conserved domain structure of cPLA2, i.e. one C2 domain and one lipase domain. The catalytic dyad, Ser and Asp, was conserved along with high conservation for the surrounding residues.

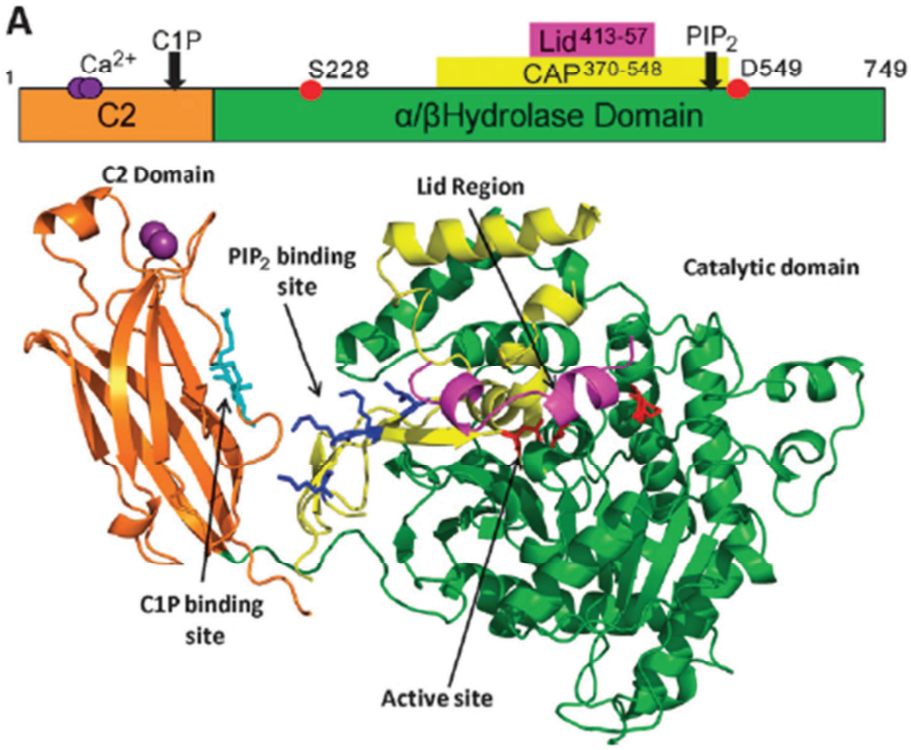

Figure 1.4: Group IVA PLA2 (cPLA2) crystal structure [98]*. The C2 domain is shown in orange, with two bound* Ca^{2+} *ions shown in purple. The catalytic domain is shown on the right with the cap region colored yellow, and the lid region is colored magenta. The active site residues are shown in stick form in red. The PIP2 binding site is shown in dark blue, and the C1P binding site is shown in cyan* [47]*.*

In general, and in relevance with this study phospholipases are a substance class currently being investigated for their role in brain and cardiovascular diseases such as Alzheimer's and stroke, and atherosclerotic vascular disease [99,100] as they could be considered a diagnostic marker or even a pharmacological target.

2. Aim of the thesis

In this thesis our aim was to develop a method for characterization of various phospholipases and to elucidate the role of the BEL inhibitor in the PGE2 synthesis from arachidonic acid under the catalysis of COX-1. Furthermore we investigated additional possibilities of these LC/MS measurements since phospholipases play a catalytical role in various diseases such as Alzheimer's or obesity. Therefore we performed co-immunoprecipitation experiments followed by LC/MS measurements for the proteins verified by co-ip and western blot. The proteins were tagged with inhibitors and derivatization agents like BEL to enable the measurements with gas chromatography and LC/MS. The identified proteins were confirmed through bioinformatical methods.

3. Material and Methods

3.1 Cyclooxygenase 1 enzyme kinetics

3.1.1 Enzyme kinetics

In the cyclooxygenase-1 (COX-1) enzyme kinetics assay 1u cyclooxygenase-1 (Cayman, 70 % purity, 13700 u/mg)) was added to 90 µl COX1 test buffer (30 mM TrisHCl, 0.49 mM glutathione (*Sigma-Aldrich*), 1 mM L-adrenaline (*Fluka*), 1 M hematine (*Sigma-Aldrich*), pH 8.0) containing the blocking agent (R)-Bromoenol lactone (BEL) (*Cayman*), Indomethacin (*Sigma Aldrich)* was used as positive control in different concentrations. In the untreated control 2 µl of DMSO (*Sigma-Aldrich)* were added respectively. For the substrate and the enzyme blank, no arachidonic acid sodium salt COX-1 enzyme was added respectively. After incubation at 25°C for 15 min, 1 µM arachidonic acid sodium salt (*Sigma-Aldrich)* in a NaOH (0.1 M) : MeOH (1:1, V:V) solution was added and samples were gently mixed at 37°C for 30 minutes. The reaction was quenched by adding 0.5 ml 0.05 % ice cold formic acid on ice.

3.1.2 Prostaglandin extraction

1ml of each sample was transferred into polypropylene tubes containing 1 ml of 0.02 % formic acid (*Merck*) and 50 µl Prostaglandin E_2-d4 standard in a concentration of 10 ng/ 50 µl (*Cayman 314010*). The prostaglandins were extracted with 4.5 mL ethyl acetate (*Merck*). The mixture was shaken for 10 minutes and centrifuged at 3500 rpm for 3 minutes. The supernatant was collected and dried under nitrogen stream at 40°C.

3.1.3 Prostaglandin derivatisation

The prostaglandins were derivatized with 50 µl pentaflourobenzyl bromide (PFBBr)-reagent (3.4 % PFBBr (*ABCR*) in acetonitril (*Pierce*)) and 10 µl N.N-diisopropylethylamine (DIPEA) (*Sigma-Aldrich*, 99% purity) (figure 3.1). The reagent was vortexed, incubated at room temperature for 10 minutes and dried under nitrogen stream at 40°C.

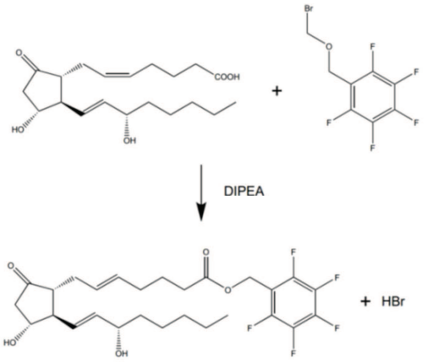

Figure 3.1 Derivatisation of prostaglandin E_2 with PFBBr. The carboxyl group of the prostaglandin is esterified by PFBBr under DIPEA catalysis.

50 µl methylhydroxylamine (MOX) reagent (*Thermo Scientic*) was added, vortexed and incubated at 75°C for 2 hours (figure 3.2). 0.4 ml distilled H_2O and 2.5 ml n-hexane (*Merck*) were added and the samples were shaken for 10 minutes. After centrifugation at 3500 rpm for 3 minutes the supernatant was vaporized under nitrogen stream at 40°C.

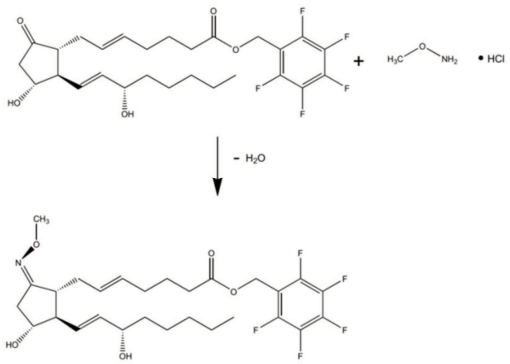

Figure 3.2 Derivatisation of the prostaglandin with MOX. The ketone of the prostaglandin reacts with the methylhydroxylamine (MOX) forming a ketoxime.

The samples were then incubated with 50 µl N-methyl-N-(trimethylsilyl) triuoroacetamide (MSTFA) reagent (MSTFA (ABCR, 97% purity) : pyridine (*Thermo Scientic*, silylation grade) (2:1, V:V), 1% trimethylchlorosilane (TMCS) (*Pierce*)) at room temperature for 20 minutes, prior evaporation under nitrogen stream at 40°C. The pellet was dissolved in 50 µl n-hexane/1% N.O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA), vortexed and transferred into GC-MS vials.

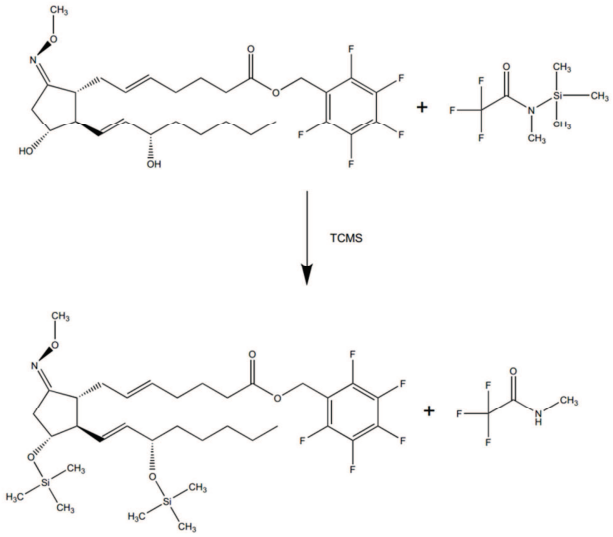

Figure 3.3 *Derivatisation of the prostaglandin with MSTFA. The hydroxyl groups of the prostaglandin are silylated with MSTFA under TMCS catalysis.*

3.1.4 Prostaglandin measurement

Prostaglandin E_2 (PGE_2) was measured via gas chromatography mass – spectrometry (ISQ Single Quadrupole GC-MS, *Thermo scientific*). Injection device was the autosampler AS 200 S. ID-BPX5-0.25; Thermo TraceGOLD TG-SQC 15 m x 0,25 mm was the analytical column used. Helium was used as carrier gas at a constant flow of 1.5 ml/min. 4 µl of each sample was injected splitless for 1 min. The injector temperature was set at 260°C. The GC-MS interface was set at a temperature of 310°C. The temperature program was set as followed: 160°C for 1 min, 40°C/min 310°C, 310°C 4 min. The samples were ionized with methane by negative ion chemical ionization (NICI). NICI was accomplished with an electron energy of 70 eV and an emission current of 0.05 A. Methane was used as moderating gas (1,5 ml/min). The ion source temperature was set to 180°C; Mode SIR. Carboxylate anions formed under NICI conditions by loss of the pentafluorobenzyl moiety were monitored by selected ion monitoring at m/z 524.30 (native PGE_2) and at 528.0 (d_4 PGE_2 standard). The dwell time was set to 0.05 s; SIR span 0.25 amu. FC 43 was used as reference calibration gas. Chromatograms were used for quantification by comparing peak areas of the deuterated standard and the native compound.

3.2. Characterization of various phospholipases

3.2.1 Cell Culture

MC3T3-E1 clonal osteoblastic mouse cells were used for this assay. The cells were maintained in 500 ml α-Minimum Essential Medium (α-MEM 1x, (*gibco life technologies*) containing 5 % fetal calf serum (FCS, Fetal Bovine Serum Gold, *PAA laboratories GmBH*) and 500 µL gentamycin (50 mg/ml*, gibco life technologies*) at 37°C in a humidified atmosphere with 5 % CO_2. The cells were further cultivated in α-MEM 1x containing 5 % FCS without antibiotics. The cells were seeded in a 100 x 100 mm well plate with an initial density of 2 x 10^6 cells and left for 4 days. The medium was then carefully removed from the cells. Then the cells were washed once with PBS pH 7.2 (0.1 M sodium phosphate, 0.15 M sodium chloride, *Apotheke LKH - Univ.-Klinikum Graz*). 600 µl of ice cold IP Lysis/Wash Buffer (2x 50 ml, 0.025 M Tris, 0.15M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, ph 7.4) plus a Complete Mini Tablet (1 in 10 ml Lysis/Wash Buffer - Protease inhibitor cocktail, *Roche*) was added to each dish of the cells. For the samples incubated with the inhibitors MAFP and BEL, the cells were incubated with 50 µM MAFP and 50 µM BEL in 50µl DMSO at 37°C for 20 min.

Chemical composition of the other different tested lysis buffers. Lysis buffer: 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 mM Sodium pyrophosphate, 2 mM Sodium orthovanadate, 10mM Sodium fluoride, 10% Glycerol, 1 % Triton X-100 (*Sigma-Aldrich)* pH 7.4; RAS Buffer: 125 mM HEPES; 750 mM NaCl; 5% Octylphenoxy poly(ethyleneoxy)ethanol IGEPAL® CA-630; 50 mM $MgCl_2$; 5 mM EDTA; 10% glycerol (*Sigma-Aldrich)* pH 7.5; BioRAD Buffer 8 M urea, 2% CHAPS, 50mM DTT, 0.2% Bio- Lyte 3/10 ampholyte, and 0.001% bromophenol blue (trace) *(Bio-RAD)*. U-937 Cell Lysate: sc-2239 (*Santa Cruz Biotechnology)* was used as positive control.

The cells were incubated on ice for 5 min with periodic mixing. The lysate was transferred to a microcentrifuge tube and centrifuged at 13000 g for 10 minutes at 4°C (*SIGMA* 3-30K, Rotor 12154) to pellet the cell debris. The supernatant was then transferred to a new tube and froze at -70°C until use.

For the lysate samples only the SDS-Page and the Western Blot were performed. 30µl of the sample was added to 10µl sample buffer (NuPAGE LDS Sample Buffer 106 mM Tris HCl, 141 mM Tris base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5 (4x), *novex life technologies*) and 3µl reducing agent (NuPAGE Sample Reducing Agent, 500 mM dithiothreitol (10x*), invitrogen*). The samples were incubated at 95°C for 10 minutes (Thermomixer comfort, *eppendorf*), spinned down (Galaxy MiniStar, *VWR*) and then they were allowed to cool down at room temperature before loading on the gel.

3.2.2 Immunoprecipitation

3.2.2.1 Precleaning

100 µL of the Control Agarose Resin (4% crosslinked beaded agarose, *Pierce*) was added into a spin column (*Pierce*). The column was centrifuged at 1000 g for 30 seconds at 4°C (*SIGMA* 3-30K, Rotor 12154) to remove the buffer. 100µl of PBS pH 7.2 (0.1 M sodium phosphate, 0.15 M sodium chloride, *Apotheke LKH - Univ.-Klinikum Graz*) was added, centrifuged at 1000 g for 30 seconds at 4°C and the flow-through was discarded. The column was closed and the supernatant (~1 mg of lysate) was added and incubated at 4°C for 60 minutes with gentle end-over-end mixing. The column was centrifuged at 1000 g for 1 minute at 4°C. The column containing the resin was discarded and the flow-through was saved, which will be added to the immobilized antibody.

3.2.2.2 Immunocomplex formation

10 µg of each antibody was added to the 600 µl lysate and the antibody was incubated with the lysate over night at 4°C.

Used antibodies:

Antibodies for phosphatidylcholine phospholipase D1 (PC-PLD1A and PC-PLD1B)

Molecular Weight of PC-PLD1A: 120 kDa

Molecular Weight of PC-PLD1B: 115 kDa

PC-PLD1 (C-17): sc-17848 (*Santa Cruz Biotechnology)*

PC-PLD1 (S-20): sc-17847 (*Santa Cruz Biotechnology)*

PC-PLD1 (H-160): sc-25512 (*Santa Cruz Biotechnology)*

Antibodies for cytosolic phospholipase A2 (cPLA2)

Molecular Weight of cPLA2: 85

cPLA2 (H-12): sc-376636 (*Santa Cruz Biotechnology)*

cPLA2 (4-4B-3C): sc-454 (*Santa Cruz Biotechnology)*

Antibodies for phosphatidylcholine phospholipase D2 (PC-PLD2A and PC-PLD2B)

Molecular Weight of PC-PLD2: 117 kDa.

PC-PLD2 (V-20): sc-48270 (*Santa Cruz Biotechnology)*

Antibodies for phosphatidylcholine phospholipase D2 (PC-PLD2A; PC-PLD2B and PC-PLD2C)

Molecular Weight of PC-PLD2: 117 kDa

PC-PLD2 (M-20): sc-18527 (*Santa Cruz Biotechnology)*

Antibodies for calcium-independent phospholipase A2 (iPLA2)

Molecular weight 88 kDa

group VI iPLA2 (T-14): sc-14463 (*Santa Cruz Biotechnology)*

Patatin-Like phospholipase Domain Containing 8 (PNPLA8) (Middle Region) antibody ABIN504075 (*antikoerper-online.de*)

Patatin-Like phospholipase Domain Containing 8 (PNPLA8) antibody ABIN1529721 (antikoerper-online.de)

3.2.2.3 Capture of the immune complex

The Protein A/G Agarose (0.55 ml of settled resin supplied as a 50% slurry, *Pierce*) was gently mixed to obtain an even suspension. The pipette tip was cut and 20 µl of the resin slurry was added into the spin column (*Pierce*). The column was placed into a microcentrifuge tube and centrifuged at 1000 g for 1 minute at 4°C and the flow-through was discarded.

3.2.2.4 Elution

The resin was washed twice with 100 µl of cold IP Lysis/Wash Buffer. The flow-through was discarded after each wash. The antibody/lysate sample was added to the Protein A/G Plus Agarose in the spin column and the column was incubated with gentle shaking for 1 hour at 4°C. The column was centrifuged at 1000 g for 30 seconds at 4°C. The column was placed in a new tube, 200 µl of IP Lysis/Wash Buffer was added and centrifuged as above. The resin was washed three times with 200 µl IP Lysis/Wash Buffer and centrifuged as above after each wash. The resin was washed again with 100 µl of 1x Conditioning Buffer (100x neutral-pH buffer, Prod # 1861612, *Thermo Scientific*).

3.2.2.5 Preparation of electrophoreses

55 µl of 2x sample buffer (NuPAGE LDS Sample Buffer 106 mM Tris HCl, 141 mM Tris base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5 (4x), *novex life technologies*) was prepared containing 5 µl reducing agent 1:10 (NuPAGE Sample Reducing Agent, 500 mM dithiothreitol (10x*), invitrogen*). The spin column containing the resin was placed in a collection tube and the prepared sample buffer was added. The column was kept unplugged in the collection tube and incubated at 95°C for 10 minutes (Thermomixer comfort, *eppendorf*). The samples were centrifuged at 1000 g for 1 minute at room temperature (Biofuge pico, *Heraeus instruments*) and then they were allowed to cool down.

3.2.2.6 In solution alkylation

5 µl of 2-Iodoacetamide (150 mM, *Sigma-Aldrich*) or 1 µl 2-Vinylpyridine 97% (*Sigma-Aldrich*) was added respectively to the sample and the sample was incubated for 1 hour in a light-protected environment. Before loading the sample on the SDS-Gel, another 5 µl reducing agent 1:10 was added and incubated for 15 minutes at room temperature.

3.2.3 SDS-PAGE

The sample was loaded onto a 1.5 mm polyacrylamid gel (NuPAGE 4-12% Bis-Tris Gel, *novex life technologies*). Electrophoresis was performed at constant voltage of 130 V for 80 minutes in NuPAGE MES SDS Running Buffer (NP002, *novex life technologies*). XCell SureLock™ Mini-Cell Electrophoresis System II (*invitrogen*) was used for the SDS-Page. 15µl of Novex Sharp Pre-stained Protein Standard (*novex life technologies*) was used to determine the molecular weight of the loaded proteins. Upon completion of the electrophoresis, the gel was either stained with 20 ml SimplyBlue SafeStain (*invitrogen*) per gel for 1 hour at room temperature and distained with Ampuwa (*Fresenius Kabi*) over-night at 4°C or a western blot was performed.

3.2.4 Western Blot

The Western Blots were performed with the Mini Trans-Blot Cell (*Bio-Rad*) and the Power PAC 200 (*Bio-Rad*) system. The proteins were transferred onto a nitrocellulose membrane (0.45 µm pore size, *novex life technologies*) at constant current of 0.3 A for 2 hours in 1x transfer buffer ((10x) - for 1 liter 30.3g Tris, 144.1g Glycin, 10g SDS, pH 8,2 – 8,4) containing 20 % methanol.

3.2.5 Antibody-Probing

Membranes were washed with 1x TBS-T buffer ((10x) for 1 liter 24,2 g Tris, 80 g NaCl, 10 ml Tween 20 (P9416, *Sigma-Aldrich*) pH 7,6) and unspecific binding sites were blocked by a blocking buffer containing 5% non-fat milk (170-6404, *Bio-Rad*) in TBS-T for 30 minutes before shortly washing the membranes again with 1x TBS-T. The primary antibodys were diluted 1:1000 in TBS-T containing 5 % BSA-IgG (*Roth*) free and 0.2 % Gentamycin (50 mg/ml, *gibco life technologies* 15750-037). The membranes were incubated with the antibody solution for 2 hours, gently mixed at room temperature. The membranes were washed again with 1x TBS-T 3 x 5 minutes and incubated with a TBS-T solution containing 5 % non-fat milk and the secondary antibody (0.01 % anti-mouse and anti-goat, 0.03 % anti-rabbit) at room temperature. Then the membranes were washed again with TBS-T 3 x 5 minutes and incubated in horseradish peroxidase substrate (Immobilon Western HRP Substrate, *Millipore*) for 5 minutes and exposed via autoradiography (Amersham Hyperfilm MP, *GE Healthcare*), processing machine CURIX 60 (AGFA), developing concentrate T-Matic 00034 (*Adefo-chemie*), fixing concentrate T-Matic 00091 (*Adefo-chemie*).

The gel bands were cut out of the electrophoresis-gel (coomassie stained) at the appropriate size (e.g. for cPLA2 at 88kDa) in cubes with a edge length of about 1 mm and stored in eppendorf tubes at -20°C until use.

3.2.6 Destaining

The electrophoresis-gel pieces were taken out of the freezer, 150 µl Aqua bidest. pH 5.0-7.0 (Fresenius Kabi) was added to each sample until they reached room temperature. The samples were incubated for 5 min at 25°C under gentle mixing (Thermomixer comfort, eppendorf), centrifuged at 6000 g for 1 minute at room temperature (Biofuge pico, *Heraeus instruments*) and the supernatant was discarded. 150 µl ammonium bicarbonate buffer (100mM $(NH_4)HCO_3$, *Sigma-Aldrich*) was added, incubated, centrifuged as above and the supernatant was discarded. 150 µl acetonitrile 50% (Prod # 89871C, *Thermo Scientific*) in H_2O dd (Aqua Bidest., *Fresenius Kabi*) 1/1 v/v was added, incubated, centrifuged as above, the supernatant was discarded and the last step was repeated once. 150µl acetonitrile 100% was added, incubated, centrifuged as

above and the flow-though was discarded. If the coomassie-stained gels were not completely distained, 150µl acetonitrile 50% was added, incubated at 37°C, centrifuged as above and the supernatant was discarded. The last step was repeated, if necessary, with 100% acetonitrile. The samples were dried in a preheated speedvac (miVAC DNA–concentrator, *GeneVAC*) for 15 minutes at 40°C. The samples were stored at – 20°C until use or the samples were put on ice for further digestion.

3.2.7 Reduction & alkylation

If the samples were alkylated in solution, the following steps were skipped. 60 µl reducing buffer (10 mM dithiothreitol (DTT), *Sigma-Aldrich*) was added to the samples. The samples were incubated for 30 minutes at 56°C with constant shaking, centrifuged at 6000 g for 1 min at room temperature and the supernatant was discarded. 150 µl acetonitrile was added for 2 minutes at 37°C (not longer – critical step), centrifuged shortly ~ 10 seconds (Galaxy MiniStar, *VWR*) at room temperature and the supernatant was discarded. 60 µl of alkylation buffer (55 mM 2-Iodoacetamide, *Sigma-Aldrich*) was added and the samples were incubated for 20 minutes at 37°C with periodic mixing in a light-protected environment, centrifuged shortly at room temperature and the supernatant was discarded. 150 µl 100 mM ammonium bicarbonate buffer was added, incubated for 5 minutes at 37°C with periodic mixing, centrifuged as above and the supernatant was discarded. 150 µl acetonitrile was added, incubated for 15 minutes at 37°C with periodic mixing, centrifuged as above and the supernatant was discarded. The samples were dried in a preheated speedvac for 15 minutes at 40°C.

3.2.8 Enzymatic digestion

A $CaCl_2$ stock solution (120 mM (Merck) in H_2O dd (Aqua Bidest., *Fresenius Kabi*) and a trypsin stock solution (20 µg sequencing grade Trypsin, *Promega* in 200 µl, 1 mM HCl, 67 µl $CaCl_2$ stock solution was added, mixed and stored in 20 µl aliquots at -20°C) was prepared.

The samples were placed on ice. The digestion buffer (H2Odd/ ammonium bicarbonate buffer (NH4HCO3 100 mM in H2O)/ trypsin stock solution 50/50/20 (v/v/v)) was prepared directly before use on ice. The digestion buffer was added in steps of 5 µl, 3 µl, 2 µl, 2 µl up to ~ 14 µl. The gels were allowed to swell between each pipetting step for 5 minutes and buffer was added until the gels were completely re-swelled. The excess of trypsin solution was avoided. The gel pieces were covered with ~20 µl incubation buffer (H_2O dd/ ammonium bicarbonate buffer/ $CaCl_2$ stock solution 65/50/5 (v/v/v)) and incubated while shaking over night at 37°C.

3.2.9 Extraction

20 µl extraction buffer NH_4HCO_3 25 mM (ammonium bicarbonate buffer/ H2Odd 1/3 v/v) was added to the samples, gently shaken for 15 minutes at 37°C, centrifuged at 6000 g for 1 min at room temperature and the supernatant was collected in a new eppendorf-tube. 160 µl acetonitrile was added to the gels, shaken, centrifuge as above and the supernatant was collected in the eppendorf-tube. 5 µl of 5% formic acid (*Sigma-Aldrich*) was added, shaken as above and span down briefly. Another 160 µl acetonitrile was added to the gels, shaken, centrifuged as above and the supernatant was collected in the eppendorf-tube. The eppendorf-tubes were then dried in the preheated speedvac until the samples were dry ~ 4 hours at 40°C. The samples were frozen at -20 until use.

3.2.10 Preparation for LC/MS

100 µl of 0.1% formic acid with 5% acetonitrile was added to the eppendorf-tubes, vortexed (Digital Vortex Mixer, *VWR*) and span down. The supernatent was transferred in autosampler vails for liquid chromatography–mass spectrometry (LC-MS).

3.2.11 Liquid chromatography–mass spectrometry (LC-MS)

The phospholipases were measured via liquid chromatography mass – spectrometry (Q-Exactive Orbitrap, *Thermo Scientific*) and an Accela 1250 Pump with reversed phase Acclaim C18 column.

3.2.11.1 High-performance liquid chromatography (HPLC)

Injection device was the autosampler (50 µl injection volume; Accela open AS. Acclaim Pepmap C 18 (1 x 150 mm, 3µm, 100 Å) was the analytical column used at the temperature of 25°C. For separation a binary gradient consistent of the solvents A - MS grade distilled water (Fluka, *Sigma-Aldrich*) plus 0.1% formic acid (*Sigma-Aldrich*) and B – MS grade methanol (Fluka, *Sigma-Aldrich*) plus 0.1% formic acid (*Sigma-Aldrich*) at a constant flow rate of 50 µl was set. Gradient details are depicted in *Table 3.1* below.

Further setting in the method:

Method finalizing was set to first line conditions. Operating mode was set to low pressure (0 – 7000 PSI), the minimum and the maximum pressure were set to 30 PSI and 1250 PSI respectively.

Table 3.1 Gradient as well as flow rate of both eluents A – MS grade distilled water plus 0.1% formic acid and B – MS grade methanol plus 0.1% formic acid used for chromatographic separation of the protein fragments. The formic acid is needed as an ionization enhancer. The sample was ionized via electrospray ionization (ESI).

Time [min]	A [%]	B [%]
0.00	95.0	5.0
35.00	40.0	60.0
45.00	0.0	100.0
60.00	0.0	100.0
60.10	95.0	5.0
85.00	95.0	5.0

The samples were analyzed in a Q-Exactive Orbitrap mass spectrometer (*Thermo Scientific*) operated in positive ion mode. The electrospray ionization parameters are listed in *Table 3.2*

Table 3.2 Electrospray ionization settings

Sheath gas flow rate	10
Aux gas flow rate	2
Sweep gas flow rate	0
Spray voltage	3.2 kV
Capillary temperature	280°C
Aux gas heater temperature	100°C

3.2.11.2 Method Q- Exactive Orbitrap

Full scan data was acquired in profile mode and the mass range was set from 400 to 1800 mz. The resolution was 140.000 and no lock masses were used. Automatic gain control (AGC – how many ions are allowed to be in the ion trap at the same time) was set to 1e6 and the maximum ion time (IT – how long is the ion trap maximal open) was set to 200 ms. Chromatographic peak width (FWHM) was set to 15 s, and the microscans were set to 1.

Data – dependent MS^2 settings:

The fragmentation method was collision-induced dissociation (CID) and the collision gas was nitrogen. The in-source CID was 0.0 eV and the default charge state was 2.

Further setting in the method:

Mircoscans was set to 1, Resolution to 17.500, AGC target to 1e5, Maximum IT to 60 ms, Loop count to 30, MSX count to 1, TopN to 30, Isolation window to 4.0 m/z, Isolation offset 0.0 m/z, scan range was set from 200 to 2000 m/z. The underfill ratio was set to 0.1 %, the intensity threshold to 1.7e3, the Apex trigger from 8 to 15 s, the charge exclusion to 1 and the peptide match to preferred. Exclude isotopes was enabled with a dynamic exclusion of 10.0 s.

The LC-MS/MS data were analyzed by searching the SwissProt public database with Proteome Discoverer 1.4 (*Thermo Scientific*). Fragment match tolerance used for search was set to 0.1 Da. Fragments used for search: b; b-H_2O; y; y-H_2O; y-NH_3. A carbamidomethyl rest on cysteine residues for the samples derivatized with 2-Iodoacetamide and a pyridylethyl rest on cysteine for the samples derivatized with 2-vinylpyridine, oxidized methionine and acrylamide residues on cysteine was entered as variable modification. For the samples treated with MAFP *(Figure 3.4)* and for the samples treated with BEL *(Figure 3.5)*, MAFP, and BEL and BMK (bromomethyl ketone [6-bromo-2-(1-naphthyl)-5-oxohexanoic acid]) respectively were entered as dynamic modifications on cysteine, lysine, serin, threonine, tyrosin.

3.3.1 Inhibitors

3.3.1.1 Methyl arachidonyl fluorophosphonate (MAFP)

Formal Name: 5Z,8Z,11Z,14Z-eicosatetraenyl-phosphonofluoridic acid methyl ester

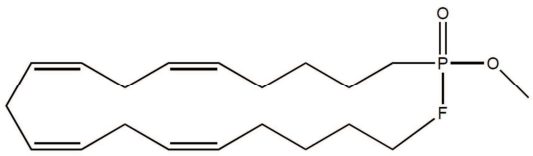

Figure. 3.4 Methyl arachidonyl fluorophosphonate (MAFP). MAFP is a selective, active-site directed, irreversible inhibitor of $cPLA_2$ and [101,102]. It inhibits A23187-induced arachidonic acid release from human platelets with an IC_{50} value of 0.6 µM. The IC_{50} value for inhibition of $iPLA_2$ from P388D1 cells is 0.5 µM [101].

3.3.1.2 R-Bromoenol lactone (BEL)

Formal Name: 6E-(bromoethylene) tetrahydro-3R-(1-naphthalenyl)-2H-pyran-2-one

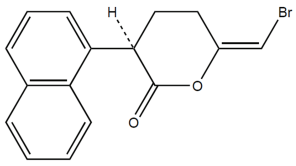

Figure 3.5 R-Bromoenol lactone (BEL) is an irreversible, chiral, mechanism-based inhibitor of calcium - independent phospholipase γ (iPLA$_2$γ). (R)-BEL inhibits human recombinant iPLA$_2$γ with an IC$_{50}$ of approximately 0.6 μM [103].

3.3.1.3 Indometacin

Formal name: Indometacin 1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid

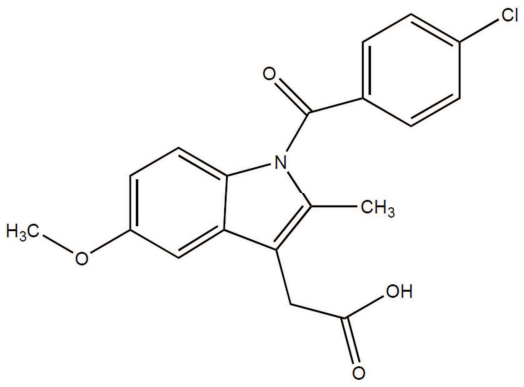

Figure 3.6. *Indometacin is a nonselective competive inhibitor of cyclooxygenase (COX) 1 and 2, enzymes that participate in prostaglandin synthesis from arachidonic acid. Indometacin blocks prostaglandin biosynthesis* [104,105].

4. Results

4.1 COX-1 enzyme kinetics

The enzyme COX-1 is involved in the reaction of arachidonic acid to PGE_2. Arachidonic acid is a membrane bound molecule. To become available for COX-1, arachidonic acid is cleaved from the membrane by phospholipases. To clarify if BEL has an inhibiting effect on COX-1 a time kinetics *(Figure 4.1)* was performed as a first step in order to examine how long COX-1 needs to synthesize PGE_2 from arachidonic acid. The time kinetics showed that after 10 min there is no further increase of the PGE_2 level visible. The Y-axis represents the total amount of PGE_2 in ng.

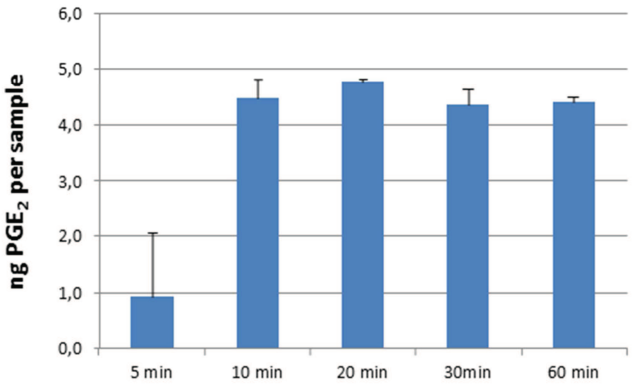

Figure 4.1. Time kinetics of COX-1. 1u COX-1 enzyme was incubated for 15 min at 25°C without inhibitor. 1 µM arachidonic acid sodium salt was added to COX-1. The solution was incubated at 37°C for 5, 10, 20, 30 and 60 min. The reaction was quenched with 0.05 % HCOOH on ice. The PGE2 was extracted, derivatized with PFBBr, MOX and MSTFA and measured via GC-MS (negative ion CI).

The kinetic assay of the used calcium-independent phospholipase A_2 blocker bromoenol lactone (BEL) showed that this blocker does not inhibit the downstream enzyme COX-1 significant at any of the following tested concentrations: 100 nM; 1 µM and 10 µM *(Figure 4.2)* since the concentrations of PGE_2 seem not to influenced by the blocker, while the positive control indomethacin inhibits COX-1 strongly, although the concentration 0.5 µM and 5 µM are significantly lower (p-value < 0.05) than the control (0.5 µM=0.013; 5 µM= 0,046).

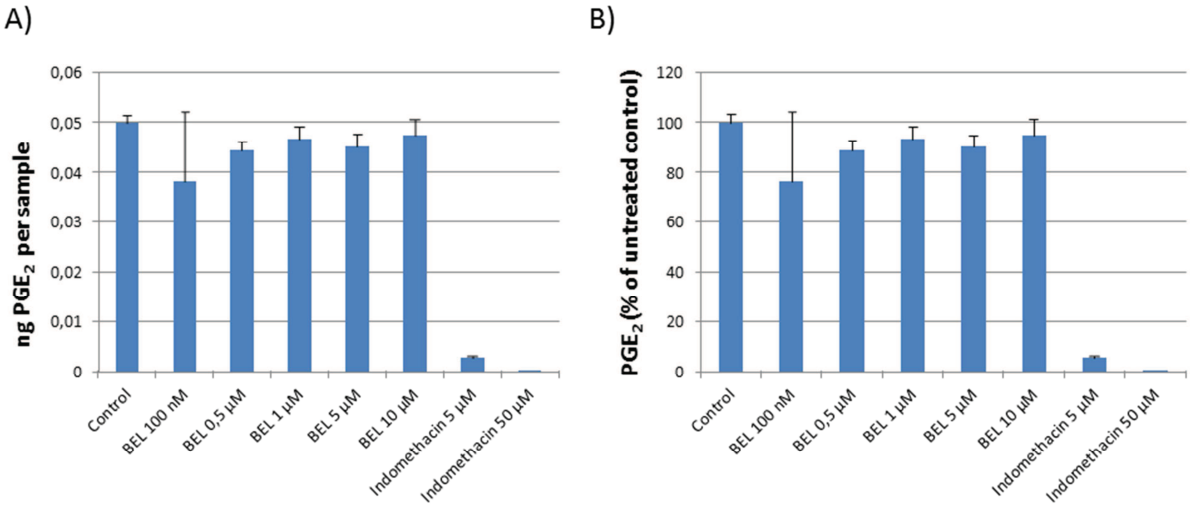

Figure 4.2. Enzyme kinetics of COX-1 in the presence of the calcium-independent phospholipase A_2 blocker bromoenol lactone (BEL). 1u COX-1 enzyme was incubated for 15 min at 25°C without inhibitor as control and with BEL at 100 nM; 0.5 µM; 1 µM; 5 µM and 10 µM. As blocking control the COX-1 blocker Indomethacin was used at 5 µM and 50 µM. 1 µM arachidonic acid sodium salt was added to COX-1. The solution was incubated at 37°C for 30 min. The reaction was quenched with 0.05 % HCOOH on ice. The PGE_2 was extracted, derivatized with PFBBr, MOX and MSTFA and measured via GC-MS (negative ion CI). Data is depicted in absolute PGE_2 concentration (A) and normalized to control (B). p-values for student's t-test (two-tailed, unpaired, independent) were: 100 nM= 0.28; 0.5 µM=0.013; 1 µM= 0,12; 5 µM= 0,046, 10 µM=0.31; Ind 5 µM=5.51e $^{-6}$; Ind 50 µM= 3.23e^{-6}).

4.2 Characterization of phospholipase D

For finding an appropriate antibody which would be able to detect PLD1 in MC3T3 cell lysate we investigated through immunoblotting the following three antibodies PC-PLD1 (S-20): sc-17847; PC-PLD1 (C17) sc-17848; PC-PLD1 (H-160): sc-25512 *(Figure 4.3)*. The PC-PLD1 (S-20) antibody *(Figure 4.3 A)* detected a protein at about 40 kDa and the PC-PLD1 (C17) sc-17848 *(Figure 4.3 B)* antibody showed no specify. Both antibodies were rejected since they could not detect the wanted protein. Although the third antibody *(Figure 4.3 C)*, PC-PLD1 (H-160): sc-25512 delivered a band at approximately the correct height, it was not further used because it was not specific enough.

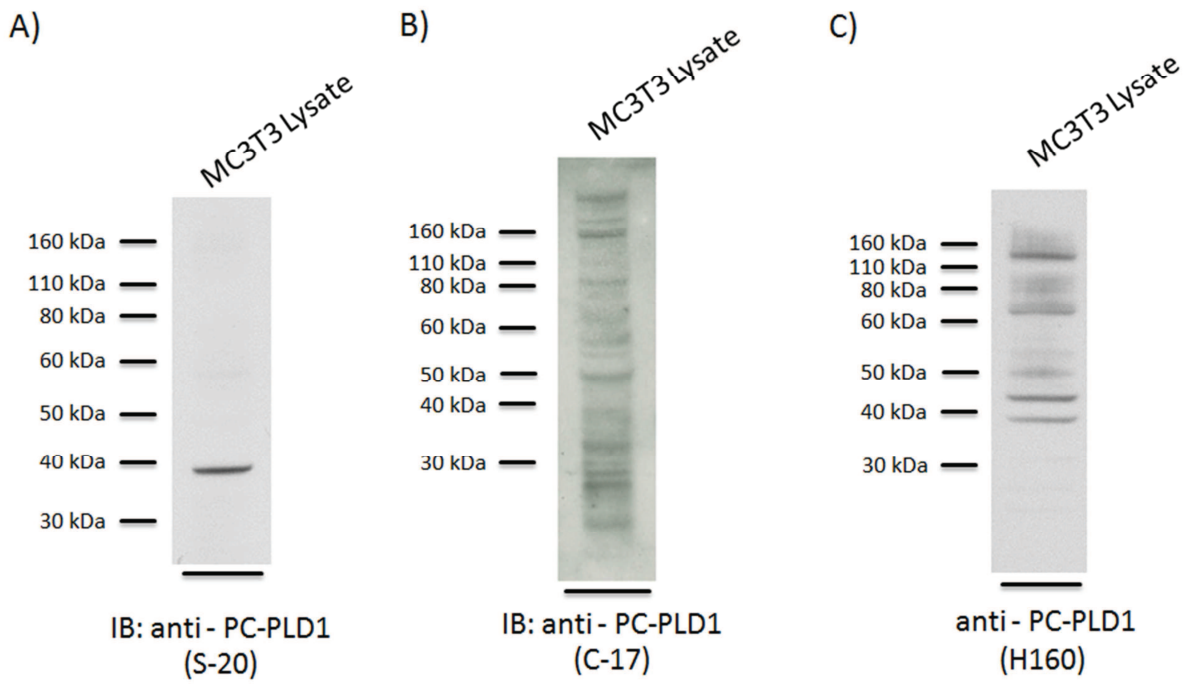

Figure 4.3. Immunoblot analysis of phosphatidylcholine phospholipase D1 (PC-PLD1) in MC3T3-E1 cells. Immunoblot (IB) of phosphatidylcholine phospholipase D1 (PC-PLD$_1$) (A;B;C) from lysates of MC3T3-E1 cells. For the IB the used antibodies were: A) PC-PLD1 (S-20): sc-17847; B) PC-PLD1 (C17) sc-17848; C) PC-PLD1 (H-160): sc-25512. The molecular weight of PC-PLD1a is 120 kDa and the molecular weight of PC-PLD1b is 115 kDa.

To examine whether the consistency of the lysate buffer could potentially affect the yield of PLD2 during the IP, four different lysate buffers were tested and their outcome was compared with the U-937 cell lysate and against each other *(Figure 4.4)*. There was no significant difference between the various lysate buffers and therefore we processed with the same lysate buffer (IP Buffer). The antibody used for the immunoblot comparing the different lysate buffers was PC-PLD2 (V-20): sc-48270 and it detected the PLD2. Another antibody, PC-PLD2 (V-20): sc-48270 was tested for its ability to detect PLD2 but it was unsuccessful.

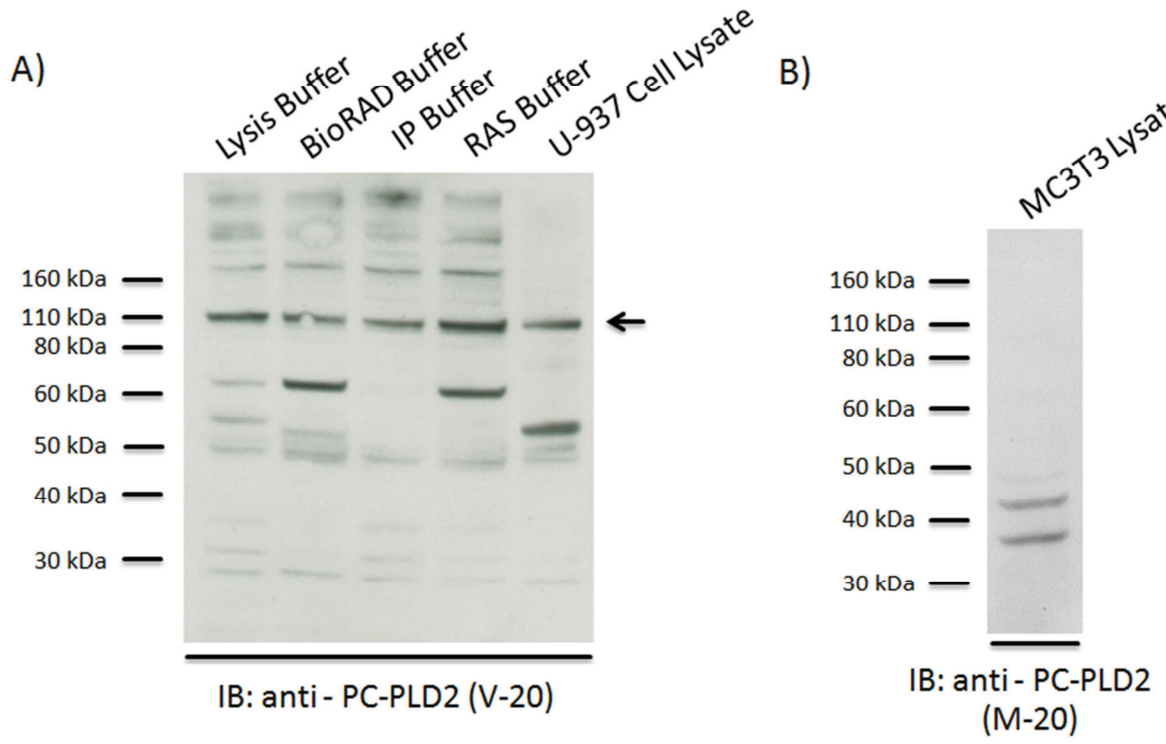

Figure 4.4. Immunoblot analysis and lysis buffer test of phosphatidylcholine phospholipase D2 (PC-PLD2) in MC3T3 cells. Immunoblot (IB) of phosphatidylcholine phospholipase D2 (PC-PLD2) from lysates of MC3T3-E1 cells. A) Lysis buffer test. The chemical composition of the various buffers is described in the material and method part. U-937 cell lysate was used as positive control. For the IB the used antibody was PC-PLD2 (V-20): sc-48270. B) For the IB the used antibody was PC-PLD2 (M-20): sc-18527. PC-PLD2 has a molecular weight of 117 kDa.

In order to be able to analyze PLD2 through mass spectrometry an IP had to be performed *(Figure 4.5).* For both the IP and afterwards the immunoblot the same antibody was used: PC-PLD2 (V-20): sc-48270 but there was no protein detected at the desired weight of 117 kDa.

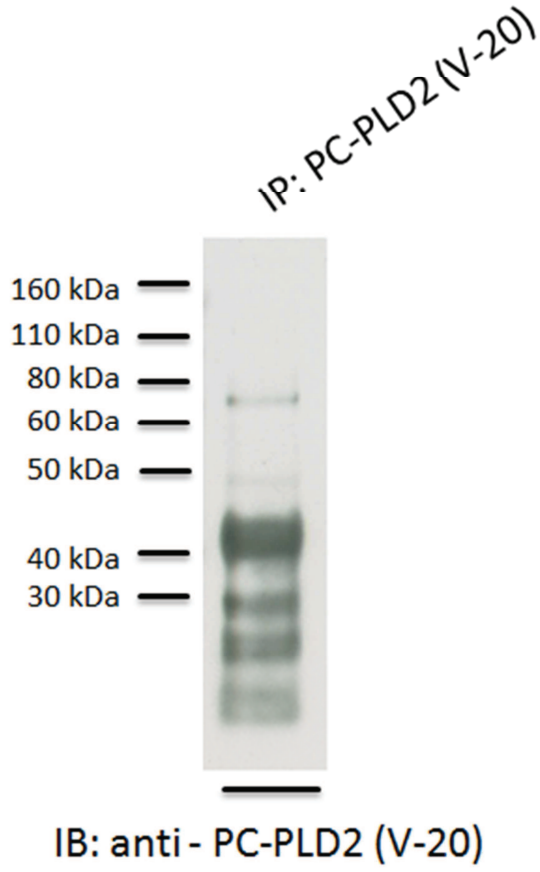

Figure 4.5. Immunoprecipitation of PC-PLD2 in MC3T3-E1 cells. Immunoprecipitation (IP) of phosphatidylcholine phospholipase D2 from lysates of MC3T3-E1 cells and subsequent immunoblot (IB) analysis. The same antibody was used both for the IP and the IB: PC-PLD2 (V-20): sc-48270. PC-PLD2 has a molecular weight of 117 kDa.

4.3 Characterization of phospholipase A2

As next, an IP for iPLA2 was performed in order to be able to characterize it through mass-spectrometry *(Figure 4.6).* The antibody used for the IP was group VI iPLA2 (T-14): sc-14463. Although iPLA2 was clearly visible on the immunoblot, the bands on the coomassie stained gel that was used for the excision of the protein for the mass spectrometric analysis were rather weak and hard to identify. The mass spectrometry analysis did not deliver a significant match.

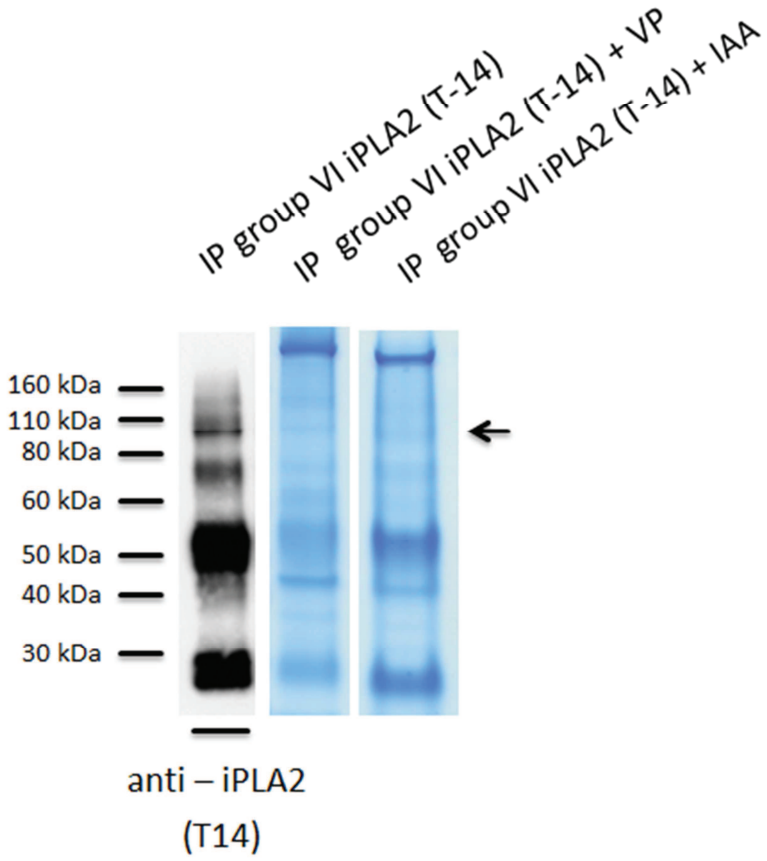

Figure 4.6. Immunoprecipitation of iPLA2 in MC3T3-E1 cells. Immunoprecipitation (IP) of calcium-independent phospholipase A2 (iPLA2) from lysates of MC3T3-E1 cells and subsequent immunoblot (IB) analysis. IB analysis (left lane) and coomassie staining (right and middle lane). For the IP and the IB the same antibody, group VI iPLA2 (T-14): sc-14463 was used. The molecular weight of iPLA2 is 88 kDa. The samples were derivatized with 2-vinylpyridine (VP) (middle lane) or iodoacetamide (IAA) (right lane) in solution. The molecular weight $iPLA_2$ is 88 kDa. The bands (arrow) were cut out and digested with trypsin and measured via LC-MS.

The group VI iPLA2 (T-14): sc-14463 antibody detected two proteins between 80 kDa and 110 kDa *(Figure 4.7 A).* Since iPLA2 has a molecular weight of 88 kDa and the two bands were almost overlapping it was assumed that iPLA2 was the upper band. Three IP´s were performed using this antibody. All the conditions were the same except the derivatization method. 2-vinylpyridine (VP) and iodoacetamide (IAA) derivatizations were performed in solution before gel electrophoresis *(Figure 4.7 B).* Iodoacetamide (IAA) was also used for in gel derivatization *(Figure 4.7 C).* Although none of the above IP´s seemed to be successful since there was no band visible at the correct weight of 88 kDa, and talking into consideration the positive result of the immunoblot the part of the gel between 80 kDa and 110 kDa was excised for further analysis. The mass spectrometry analysis was negative for iPLA2.

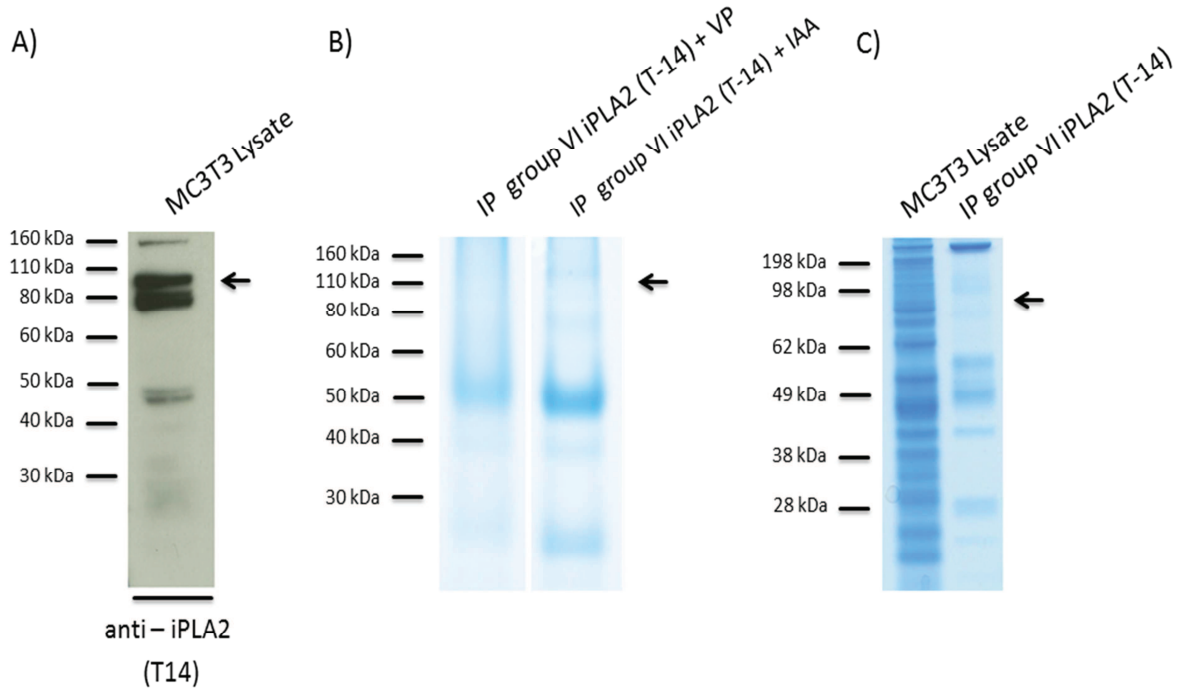

Figure 4.7. Immunoblot analysis and immunoprecipitation of iPLA2 in MC3T3 cells. Immunoprecipitation (IP) of calcium-independent phospholipase A2 (iPLA2) from lysates of MC3T3-E1 cells and subsequent immunoblot (IB) analysis. A) IB analysis from lysates of MC3T3-E1 cells. For the IB the used antibody was group VI iPLA2 (T-14): sc-14463. B) Immunoprecipitation of iPLA2 (coomassie staining). The samples were derivatized with 2-vinylpyridine (VP) or iodoacetamide (IAA) in solutions. C) Native MC3T3-E1 cell lysate (left lane) (coomassie stained) and immunoprecipitation of iPLA2 with group VI iPLA2 (T-14): sc-14463 antibody (coomassie stained). The samples were derivatized with IAA in gel. The molecular weight iPLA2 is 88 kDa. The bands (arrow) were cut out and digested with trypsin and measured via LC-MS.

MC3T3-E1 cell lysates were used for the detection of iPLA2γ with the Patatin-Like phospholipase Domain Containing 8 (PNPLA8) (Middle Region) antibody ABIN504075 *(Figure 4.8 A)* and Patatin-Like phospholipase Domain Containing 8 (PNPLA8) antibody ABIN1529721 *(Figure 4.8 B)*. None of the above mentioned antibodies were able to detect iPLA2γ. The iPLA2γ ABIN504075 antibody detected weakly a protein below 80 and another one above 110 kDa *(Figure 4.8 A*). Since iPLAγ has a molecular weight of 88 kDa no IP was performed.

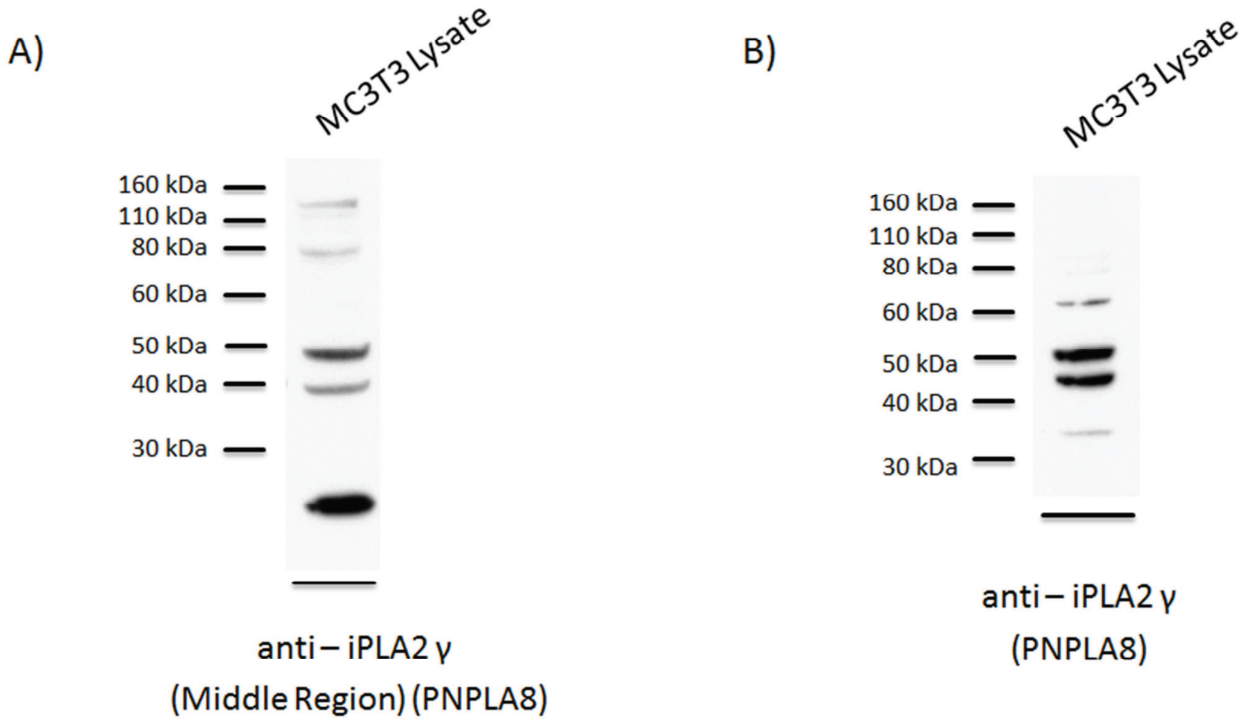

Figure 4.8. Immunoblot analysis of iPLA2γ in MC3T3 cells. Immunoblot (IB) from lysates of MC3T3-E1 cells. For the IB of calcium-independent phospholipase A2 γ (iPLA2γ) the used antibody was A: Patatin-Like phospholipase Domain Containing 8 (PNPLA8) (Middle Region) antibody ABIN504075 and B: Patatin-Like phospholipase Domain Containing 8 (PNPLA8) antibody ABIN1529721. The molecular weight of iPLA2γ is 88 kDa.

The calcium-dependent phospholipase A2 (cPLA2) was successfully immunoprecipitated with the cPLA2 (H-12): sc-376636 antibody and detected by immunoblot with the cPLA2 (4-4B-3C): sc-454 antibody and by coomassie staining *(Figure 4.9).* The coomassie gel was subsequently derivatized with iodoacetamide and was used for in gel digestion with either trypsin or chymotrypsin and LC-MS analysis of both samples followed. The sequence coverage was 31.02% (14.97% for trypsin and 19.52% for chymotrypsin) indicating that the immunoprecipitated protein was indeed cPLA2 *(Figure 4.10)*. In addition the high confidence mass spectra are illustrated in *Figure 4.11*.

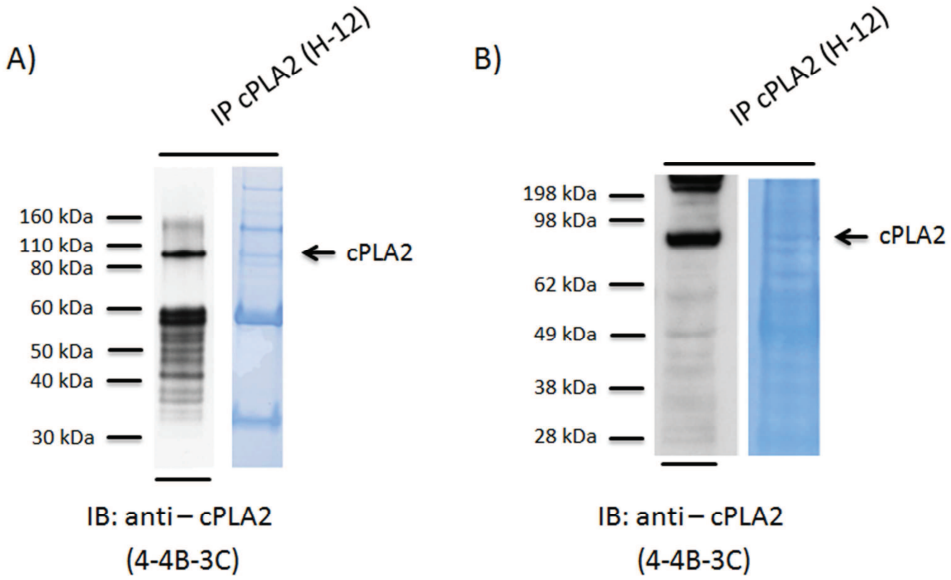

Figure 4.9. Immunoprecipitation of cPLA2 in MC3T3 cells. Immunoprecipitation (IP) of calcium-dependent phospholipase A2 from lysates of MC3T3-E1 cells and subsequent immunoblot (IB) analysis; IB analysis (left lane) and coomassie staining (right lane). For the IP´s the antibody that was used was cPLA2 (H-12): sc-376636 and for the IB it was cPLA2 (4-4B-3C): sc-454. The molecular weight of cPLA2 is 85 kDa. The bands indicated by the arrow were excised and digested with A) chymotrypsin or B) trypsin and measured via LC-MS.

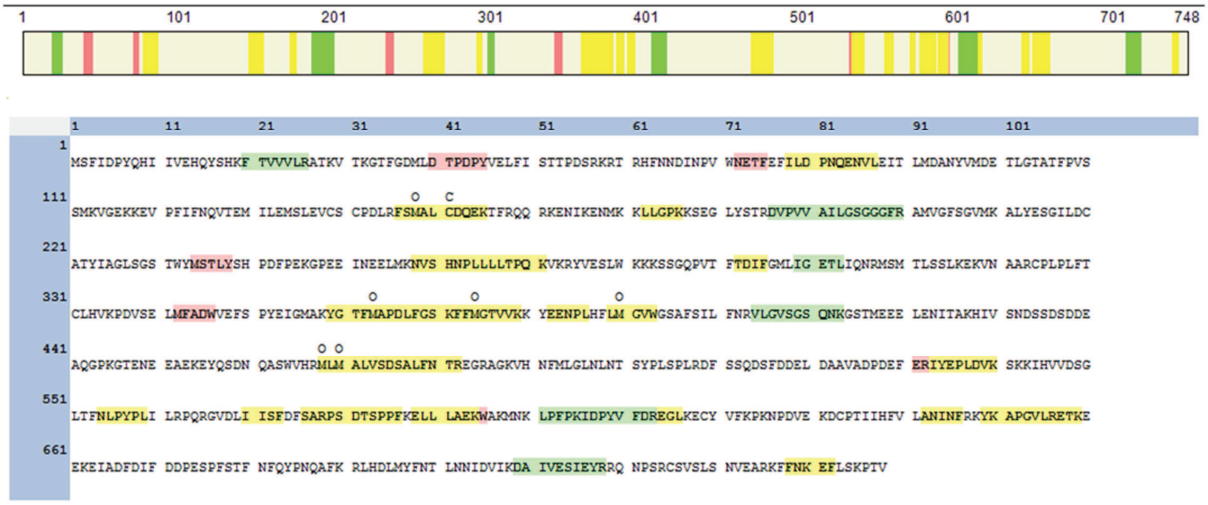

Figure. 4.10. Sequence coverage of the cytosolic phospholipase A2 (Mus musculus). Merged results of the trypsin and the chymotrypsin digested cPLA2. The red color displays peptides of low confidence, the yellow displays peptides of modest confidence and green displays peptides of high confidence. O indicates an oxidation of methionine and C a carbaminomethyl modification of cysteine. The sequence coverage was 31.02 % (14.97 % for trypsin and 19.52 % for chymotrypsin).

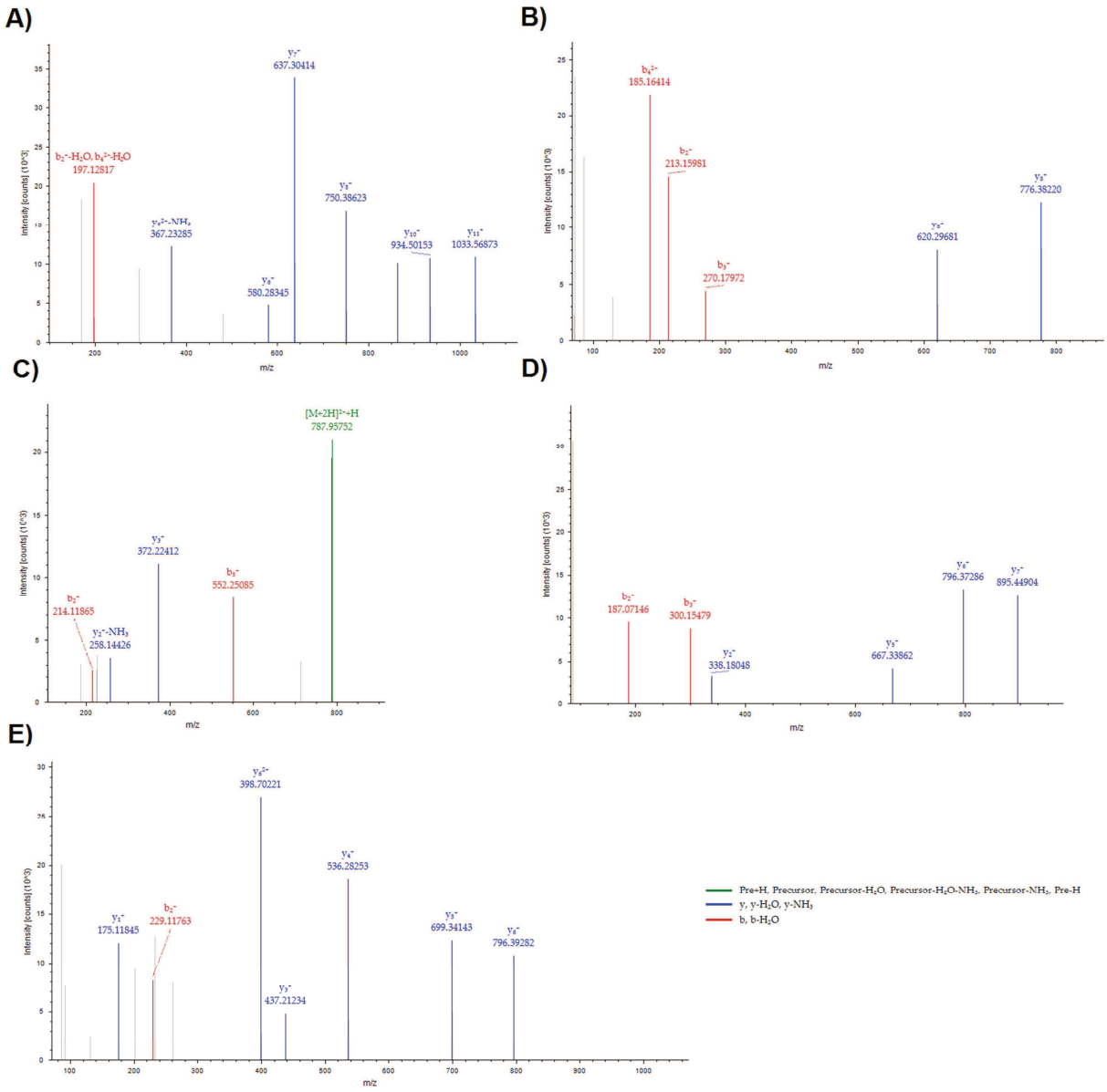

Figure 4.11 Exemplary mass spectra of peptides with high confidence of from cytosolic phospholipase A2 (cPLA2) from Mus musculus. The cell extracts were analyzed by positive ion ESI/MS/MS using Q- Exactive Orbitrap. A) Sequence: DVPVVAILGSGGGFR, Charge +2, Monoisotopic m/z: 722.401 Da, MH+: 1443.79473 Da. B) Sequence: VLGVSGSQNK, Charge:+2 Monoisotopic m/z: 494.77451 Da, MH+: 988.54173 Da. C) Sequence: NVSHNPLLLLTPQK, Charge:+2, Monoisotopic m/z: 787.45587 Da, MH+: 1573.90447 Da. D) Sequence: DAIVESIEYR, Charge:+2, Monoisotopic m/z: 597.80347, MH+: 1194.59966 Da. E) Sequence: IDPYVFDR, Charge: +2, Monoisotopic m/z: 512.75769 Da, MH+: 1024.50810 Da. The green peaks indicate the precursor ions, the blue peaks the y-ions and the red peaks the b-ions.

Since it was possible to immunoprecipitate and analyze via mass spectrometry cPLA2 we further wanted to investigate whether it is also possible to detect and analyze the complex built when MAFP and BEL inhibitors bind to cPLA2. The immunoblot and the IP were both successful. Nevertheless the IP signals were rather weak *(Figure 4.12)* in comparison to the non-modified cPLA2 *(Figure 4.9).* The MS analysis showed no significant results.

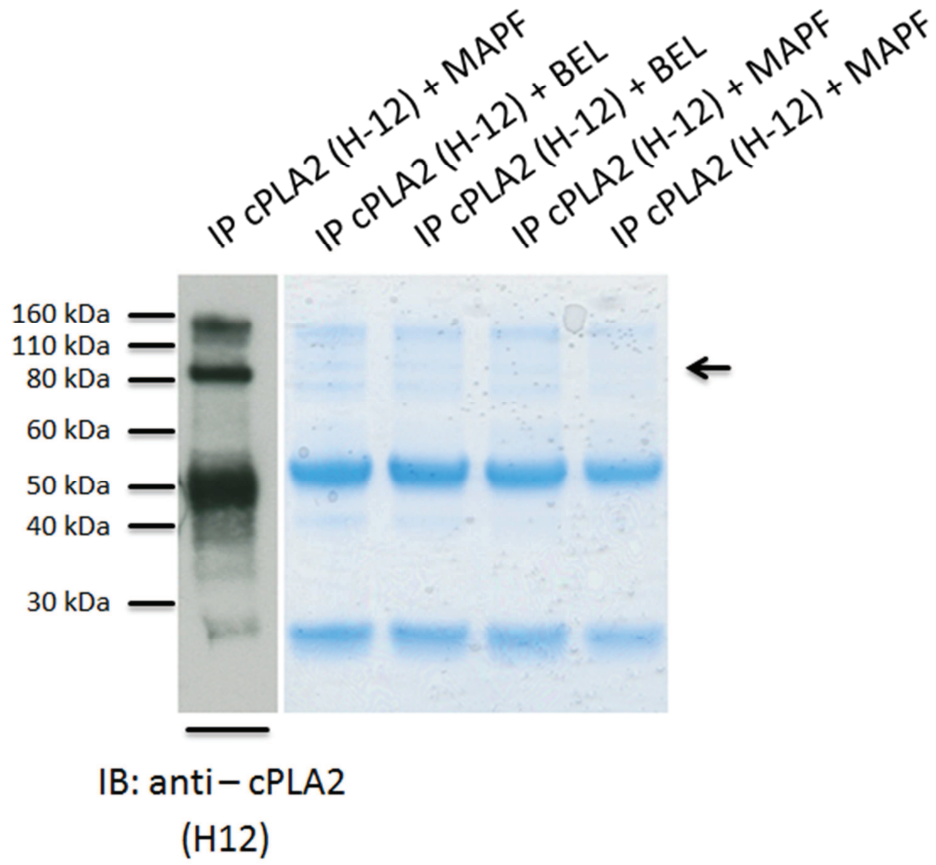

Figure 4.12. Immunoprecipitation of cPLA2 incubated with MAFP and BEL in MC3T3-E1 cells. Immunoprecipitation (IP) of calcium-dependent phospholipase A2 from lysates of MC3T3-E1 cells harvested with 50 µM methyl arachidonyl fluorophosphonate (MAFP) and 50 µM bromoenol lactone (BEL) and subsequent immunoblot (IB) analysis. IB analysis (first lane) and coomassie staining (second to fifth lane). For the IP and the IB the same antibody cPLA2 (H-12): sc-376636 was used. For the IB a lysate incubated with MAFP was used. The samples were further derivatized with iodoacetamide in gel. The molecular weight of cPLA2 is 85 kDa. The band (arrow) was cut out and digested with trypsin and measured via LC-MS.

5. Discussion

The time kinetics *(Figure 4.1)* was performed in order to examine how long COX-1 needs to synthesize PGE_2 from arachidonic acid and whether time is a factor that could potentially influence the reaction between COX-1 and arachidonic acid. The time kinetics showed that after 10 minutes there is no further increase or decrease of the PGE_2 level. This means that the incubation time of 30 minutes which was used for our experiments should be sufficient and should have no negative influence at the outcome of the experiment. The longest time the kinetics was performed was for 60 minutes. We assumed that if there is no significant difference between the four different time points (10, 20, 30 and 60 minutes) after the addition of arachidonic acid then there is no reason to investigate any additional time points or change the incubation time in our experiments as it seems like the 30 minutes have no negative influence in the production of PGE_2 from arachidonic acid.

The kinetic assay of the phospholipase A2 blocker BEL *(Figure 4.2)* showed that this blocker does not inhibit the downstream enzyme COX-1 at the following the tested concentrations 100 nM, 1 µM, 10 µM. 0,5 µM and 5 µM were also tested and showed a statistically significant inhibition of COX-1 with p=0.013 and p=0,046 respectively. This could suggest that the enzyme funtions better at a specfic ratio of enzyme to substrate concentration. Nevertheless a p-value of 0.046 could easily be proven non significant through repetition of the experiment. For performing this experiment two different control samples were used; one was not treated with any kind of inhibitor (untreated) and one was treated with a known inhibitor of COX-1, Indomethacin (positive control). Since COX-1 is in vivo a membrane bound enzyme and it is less active in solubilized form [106], the untreated control was used with the purpose of testing whether COX-1 requires cofactors like hematin for activation. The BEL inhibitor blocks the phospholipase A2 in an irreversible mechanistic manner. If BEL inhibits COX-1 in the same way as it inhibits the phospholipase A2, then it can be assumed that the Ki of BEL is much higher the Km of the arachidonic acid - COX-1 complex. Since three of the five tested concentrations showed no significant inhibition, the Ki of BEL must be a 10-fold or more, higher than the Km. If Ki was

lower than Km then this would suggest a strong inhibition. The experiment should be repeated in order to verify the results.

In order to analyze various phospholipases via LC-MS/MS, the respective phospholipase had to be isolated from cell culture through immunoprecipitation. Unfortunately there is a lack of antibodies which would work both for western blot and immunoprecipitation delivering clean and specific results. For this reason, different antibodies had to be tested until an appropriate one was found.

The first phospholipase to be investigated was PLD1. The first step in order to isolate PLD1 was to find an antibody which would be able to detect PLD1 in MC3T3 cell lysate through western blot. The following three antibodies PC-PLD1 (S-20): sc-17847, PC-PLD1 (C17) sc-17848 and PC-PLD1 (H-160): sc-25512 *(Figure 4.3)* were used. The PC-PLD1 (S-20) antibody *(Figure 4.3 A)* detected a protein at about 40 kDa and the PC-PLD1 (C17) sc-17848 *(Figure 4.3 B)* antibody showed no specificity and could not be further used. Although the third antibody *(Figure 4.3 C)*, PC-PLD1 (H-160): sc-25512 delivered a band at approximately the correct height of 120 kDa, it was not further used because it was not specific enough since there were also many other bands to be observed with approximately the same thickness.

The buffer used for lysing the cells is always a factor that could have a big impact on the lysis outcome and the availability of the respective protein. Thus, a lysis buffer test was considered to be necessary. Four different lysis buffers were tested and their outcome was compared with the U-937 cell lysate and against each other *(Figure 4.4 A).* There was no significant difference between the various lysis buffers and therefore we proceeded using the same lysis buffer (IP Buffer). The antibody used for the immunoblot comparing the different lysis buffers was PC-PLD2 (V-20): sc-48270 and it was successful at detecting PLD2. The same antibody was then used also for an immunoprecipitation (*Figure 4.5)* but it did not deliver the expected protein band, indicating that the ip did not work and the antibody was not appropriate. Another antibody, PC-PLD2 (V-20): sc-48270 was tested for its ability to detect PLD2 but it was rather unsuccessful since the detected proteins were significantly smaller than PLD2 (*Figure 4.5 B)*.

Several antibodies were also tested for the detection of iPLA2. The first antibody was group VI iPLA2 (T-14): sc-14463 and it detected iPLA2 on the immunoblot. Subsequently, immunoprecipitation was performed using the same antibody (*Figure 4.6)* and the immunoblot was positive. Therefore after the derivatization with two different substances (Iodacetamide or 2-vinylpyridine) the respective protein bands were excised from coomassie stained gels and were digested for subsequent mass spectrometric analysis. The analysis did not deliver a match. A further antibody the group VI iPLA2 (T-14): sc-14463 was used and it detected two proteins between 80 kDa and 110 kDa *(Figure 4.7 A).* Since iPLA2 has a molecular weight of 88 kDa and the two bands were almost overlapping it was assumed that iPLA2 was the upper band. Following, three IP´s were performed with varying derivatization methods (2-vinylpyridine, iodoacetamide in solution *figure 4.7B* and iodoacetamide in gel *figure 4.7 C*) using this antibody. None of the above IP´s seemed to be successful since there was no band visible at the correct weight of 88 kDa. Nevertheless, taking into consideration the positive result of the immunoblot the part of the gel between 80 kDa and 110 kDa was excised for further analysis. The mass spectrometry analysis was negative for iPLA2.

It is assumed that the amount of the immunoprecipitated protein was too low since the protein bands on the coomassie gels were not easy to identify. The identification difficulty could lead to false or imprecise excision of the bands that could the lead to insufficient amount of protein for the mass spectrometric analysis.

Two antibodies were tested for the detection of iPLA2γ, the patatin-Like phospholipase Domain Containing 8 (PNPLA8) (Middle Region) antibody ABIN504075 *(Figure 4.8 A)* and the patatin-Like phospholipase Domain Containing 8 (PNPLA8) antibody ABIN1529721 *(Figure 4.8 B)*. The iPLA2γ ABIN504075 antibody detected weakly a protein below 80 and another one above 110 kDa *(Figure 4.8 A*). iPLAγ has a molecular weight of 88 kDa, therefore the previously mentioned antibody was not able to detect iPLA2γ. The other antibody, ABIN1529721, did not detect the wished protein either. Thus no immunoprecipitation could be performed.

The cPLA2 was successfully immunoprecipitated with the cPLA2 (H-12): sc-376636 antibody and detected by immunoblot with the cPLA2 (4-4B-3C): sc-454 antibody and by coomassie staining *(Figure 4.9)*. Derivatization with iodoacetamide and trypsin/ chymotrypsin digestion followed. The LC-MS/MS demonstrated a match with a merged sequence coverage of 31.02% (14.97% for trypsin and 19.52% for chymotrypsin) indicating that the immunoprecipitated protein was indeed cPLA2 *(Figure 4.10); (Figure 4.11).* Although in general a sequence coverage of approximately 30% is rather low, it was in this case sufficient as a preliminary result. The antibody used, cPLA2 (H-12) was the only phospholipase antibody that was specific and delivered clear and distinct protein bands which could be excised precisely. Finding an appropriate antibody was the first step. Now the parameters and the settings of the experiment should be improved in order to make it possible to reach a higher and more significant sequence coverage.

It is known that cPLA2 can build complexes with MAFP and BEL inhibitors. Taking this into consideration an immunoblot and immunoprecipitation from lysates of MC3T3-E1 cells harvested with 50 µM methyl arachidonyl fluorophosphonate (MAFP) and 50 µM bromoenol lactone (BEL) were performed using the above mentioned antibody *(Figure 4.12)*. Although both the immunoblot and the immunoprecipitation were successful, the protein bands were not as distinct as with the unmodified cPLA2 *(Figure 4.9)*. The mass spectrometric analysis showed no match. It could be assumed that the immunoprecipitation efficiency was decreased through the inhibitors binding on the cPLA2, since the binding of another molecule on the protein could negatively affect the binding capacity of the antibody.

6. Conclusions

The identification and characterization of various phospholipases from MC3T3-E1 lysates through liquid chromatography - mass spectrometry was the main aim of this thesis. This part was proven to be a difficult task since we were able to detect and measure only one of the phospholipases that were investigated: $cPLA_2$. This shows that there is still a lot that need to be done in order to be in the position to use phospholipases as a diagnostic tool or as a pharmacological target.

The second aim of this thesis was to examine through gas chromatography mass spectrometry whether the phospholipase A2 inhibitor BEL inhibits the downstream enzyme COX-1. It was concluded that BEL does not have an effect on COX-1 although it was considered to be an appropriate candidate due to its ability to inhibit PLA2.

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