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Paramjit S. Tappia
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Phospholipases in Health and Disease

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ISBN 978-1-4939-0463-1 ISBN 978-1-4939-0464-8 (eBook)
DOI 10.1007/978-1-4939-0464-8
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2014933051

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

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This book is dedicated to Prof. Grant Pierce for his outstanding leadership in promoting cardiovascular research and education. Dr. Pierce is the founder of the Canadian Centre for Agri-Food Research in Health and Medicine at St. Boniface Hospital in Winnipeg. His work on diabetic cardiomyopathy and ischemic heart disease has been pioneering and highly cited.

In addition, he has written/edited eight books on cardiovascular health. He has received awards from the American Heart Association, the International Society for Heart Research, the Heart and Stroke Foundation of Manitoba, and Canadian Institutes for Health Research in recognition of research excellence. Dr. Pierce has served on the Editorial Boards of several cardiovascular journals and has served as Assistant Editor of Molecular and Cellular Biochemistry for over two decades. He is Coeditor of the Canadian Journal of Physiology and Pharmacology. Dr. Pierce is an elected Fellow of different international scientific organizations including the American College of Cardiology, the International Academy of Cardiovascular Sciences, the Royal Society of Medicine (London), and the Royal Society of Canada.

Preface

Phospholipids were originally considered as plasma membrane components that primarily provided cellular structural and functional integrity. However, these are now also recognized as the source of molecules that act as biological mediators of cell function. Some of these mediators serve as extracellular lipid-signaling molecules while others act as intracellular second messengers that regulate effector enzymes. The activation of phospholipases is a primary step in the generation of lipid mediators and the initiation of intracellular signal transduction pathways in a variety of cell types. Neurotransmitters, hormones, and growth factors evoke intracellular responses by activating phospholipases. Most of these mediators are produced upon activation of many different forms of phospholipase A, phospholipase C, and phospholipase D.

The contribution of different phospholipases and their related signaling mechanisms to altered function during different pathophysiological conditions is not completely understood. Resolution of this issue is essential for both the understanding of different disease conditions and for determining if components of the phospholipid-signaling pathways could serve as appropriate therapeutic targets. Furthermore, the interaction between the different lipid molecules and the different phospholipases adds to the complexity of phospholipid-signaling mechanisms. While phospholipases also reside in the cytosolic compartment of the cell, these must migrate to a membrane compartment where there physiological substrates reside. Indeed, phospholipases were considered to localize primarily to the plasma membrane; however, they are also located in intracellular compartments including the cytoskeleton, endo(sarco)-plasmic reticulum, the Golgi apparatus, and the nucleus.

This book has been compiled to present a comprehensive and up-to-date view of the phospholipase research field. A wide range of topics covered here are of interest to basic research scientists, clinicians, and graduate students, who are devoted to the study of human health and disease. Furthermore, these chapters are directed towards increasing our understanding of novel strategies for the prevention/treatment of different diseases. Twenty three chapters in this book are organized into four parts.

The first part consisting of four chapters discusses general aspect of phospholipases. The subsequent three parts are designed to specifically highlight the most characterized forms of the phospholipases. The second part consists of seven chapters and covers the role and function of phospholipase A in different pathophysiological conditions. Phospholipase A continues to be the subject of considerable interest in the field, since it hydrolyzes membrane phospholipids to produce substrates for the biosynthesis of prostaglandins, thromboxanes, leukotrienes, and other oxygenated metabolites of arachidonic acid as well as platelet-activating factor. Some of the products of phospholipase A activity also serve as molecules for the activation of intracellular signal transduction pathways.

The third part comprises nine chapters and is focussed on phospholipase C which is believed to play a central role in transmembrane signaling. The first signal-activated phospholipase that was established as a key player in signal transduction was a phosphoinositide-specific phospholipase C. The phosphoinositide-specific phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate two second messenger molecules, namely, diacylglycerol and inositol 1,4,5-trisphosphate known to regulate a diverse range of cell function through the activation of various forms of protein kinase C enzymes as well as mobilization of Ca^{2+} from intracellular stores.

The fourth part has three chapters concerning phospholipase D, which is present in a variety of different cells. In fact, phospholipase D was originally discovered in plants and the first indication of its presence in mammalian cells was by Kanfer and his colleagues almost three decades ago. This phospholipase hydrolyzes membrane phospholipids to produce phosphatidic acid and releases the free polar head group. Although phosphatidic acid is central to glycerolipid metabolism, it is also considered as an important lipid signaling molecule involved in a wide range of cellular processes, including vesicular trafficking, cytoskeletal organization as well as cell growth, proliferation, and survival. This part is relatively short; however, the subject matter highlighting the unique features of this particular phospholipase is also referred to in the first part.

In summary, this book covers a broad range of topics related to general aspects of the different phospholipases and their role in cell function pertaining to human health and disease. We hope that the reader will understand that membrane phospholipids are a rich source of lipid-signaling molecules that are produced through receptor-mediated activation of phospholipases and serve as second messengers. Furthermore, the underlying message presented in this book is that the activation of phospholipases is of fundamental importance in signal transduction affecting cell function under normal and diseased conditions.

We would like to take this opportunity to offer our sincerest gratitude to all eminent authors for their outstanding contributions. We thank them also for their willingness to be part of this book, as without their expertise, this project would not

have been possible. The time and efforts of both Dr. Vijayan Elimban and Ms. Eva Little of the Institute of Cardiovascular Sciences at St. Boniface Hospital Research, University of Manitoba are gratefully acknowledged. Our appreciation is also extended to Ms. Rita Beck and Ms. Diana Ventimiglia as well as the staff at the Springer Media, New York for their understanding and assistance in the preparation of this book.

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Part I
Phospholipases: General Aspects

Chapter 1
Phospholipases in Health and Disease

Yong Ryoul Yang, Hyun-Jun Jang, Sung Ho Ryu, and Pann-Ghill Suh

Abstract Phospholipids are a class of complex lipids that are composed of two fatty acids, a glycerol unit, a phosphate group, and a polar molecule. Phospholipids include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol, which are major components of cell membranes. They are hydrolyzed by various lipolytic enzymes, including phospholipase C, phospholipase D, and phospholipase A. Enzymatic processing of phospholipids by phospholipases converts these molecules into lipid mediators or second messengers that regulate a variety of physiological and pathophysiological functions. Thus, dysregulation of phospholipases contributes to a number of human diseases and these phospholipases have been identified as therapeutic targets for prevention and treatment of diseases.

Keywords Phospholipase • Phospholipid • Phospholipase C • Phospholipase D • Phospholipase A • Brain disorder • Cancer • Immune system dysfunction • Metabolic disease • Atherosclerosis • Arthritis • Kidney dysfunction • Platelet dysfunction

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_1,

1.1 Characteristics and Cellular Signaling of Phospholipases

PI-PLC: Phosphoinositide-specific phospholipase C (PLC) hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to generate second messengers, inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), in ligand-mediated signal transduction (Fig. 1.1). DAG activates protein kinase C (PKC) and IP_3 binding to its receptor triggers the release of calcium ions from intracellular stores like ER (endoplasmic reticulum). PLC-mediated signaling pathways regulate diverse biological functions. Firstly, Hokin et al. suggested evidence of PLC activity in 1953. They observed specific hydrolysis of phospholipids in pigeon pancreas slices after cholinergic stimulation [1]. In 1983, Sterb et al. reported that IP_3 generated from PIP_2 hydrolysis induces mobilization of intracellular calcium in pancreatic acinar cells [2]. To date, 13 mammal PLC isozymes have been identified and are divided into six subtypes: PLC-β(1–4), γ(1,2), δ(1,3,4), ε, ζ, and η (1,2) (Fig. 1.2). PLC isozymes commonly have highly conserved X and Y domains which is responsible for PIP_2 hydrolysis. Each PLCs contain diverse regulatory domains including the C2 domain, the EF-hand motif, and the pleckstrin homology (PH) domain. Notably, each PLC subtype has a unique domain and PLC isozymes are differentially expressed in different tissues. These unique domains and different expression patterns contribute to the specific regulatory mechanisms and functional diversity of PLC isozymes [3].

PLC-β subtypes are activated by G protein-coupled receptor (GPCR) through several mechanisms. In contrast, PLC-γ subtypes are activated by receptor tyrosine kinase (RTK). Upon growth factor stimulation, PLC-γ is recruited to activated growth factor receptors via SH2 domain–phosphotyrosine interaction and then subjected to phosphorylation by RTK [3]. PLC-ε can be activated by both GPCR and RTK activation with distinct activation mechanisms [4]. It has been suggested that

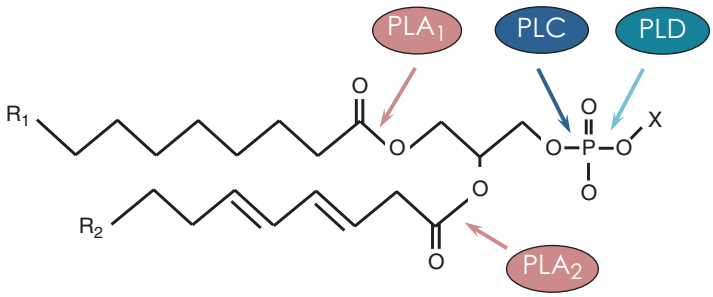

Fig. 1.1 Phospholipid structure and the site of action of phospholipases. Phospholipids are composed of a glycerol-3-phosphate esterified at the sn-1 and sn-2 positions to nonpolar fatty acids (R1 and R2, respectively) and at the phosphoryl group to a polar head group, X. Phospholipase A1 and phospholipase A2 cleave the acyl ester bonds at sn-1 and sn-2, respectively. Phospholipase C cleaves the glycerophosphate bond, whereas phospholipase D removes the head group, X. PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D

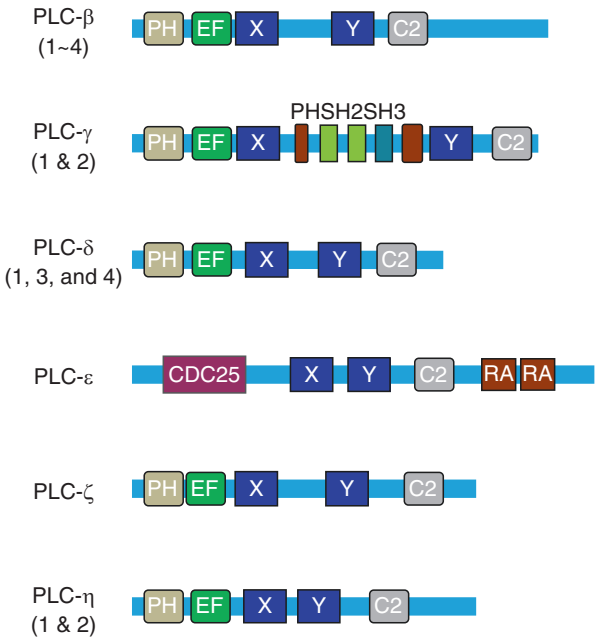

Fig. 1.2 Schematic structure of phospholipase C isozymes. Thirteen mammalian PLC isozymes are subdivided into six groups. All PLC isotypes have X and Y domains, which contain catalytic activity. Several isoforms have pleckstrin homology (PH) and a calcium-binding (C2) domain, which can regulate PLC activity. The EF-hand domain is responsible for forming a flexible tether to the PH domain. PLC-ε has an *Ras* guanine nucleotide exchange factor (GEF) domain for RAP1A122 and the RA2 domain mediates interaction with GTP-bound *Ras* and RAP1A. PLC-γ has SRC homology 2 (SH2) and SH3 domains, which interact with many proteins

overall PLC activity may be amplified and sustained by both intracellular calcium mobilization and extracellular calcium entry. Several studies have suggested positive feedback amplification of PLC signaling [5–8]. PLC-δ1 and PLC-η1 are activated via GPCR-mediated calcium mobilization and are involved in positive feedback signal amplification of PLC [9, 10]. By these mechanisms, it has been suggested that PLC-β, PLC-γ, and PLC-ε might be primarily activated by extracellular stimuli, and activation of PLC-δ1 and PLC-η1 might be secondarily enhanced by intracellular calcium mobilization to amplify PLCs activity. The activation mechanism for PLC-ζ remains to be revealed.

PC-PLD: Phosphatidylcholine-specific phospholipase D (PLD) hydrolyzes the phosphodiester bond of the glycerolipid phosphatidylcholine (PC) to produce phosphatidic acid (PA) and free choline (Fig. 1.1). PLD activity was first described in 1975 by Hannahan and Chaikoff in carrot extracts and demonstrated in rat brain by Saito and Kanfer in 1975 [11]. In mammals, PLD1 and PLD2 have been identified (Fig. 1.3). PLD has several conserved regions, including phox homology (PX) and PH domains, and two conserved catalytic domains (HKD), which are critical for enzymatic catalysis. PLD3, PLD4, and mitochondrial PLD also have

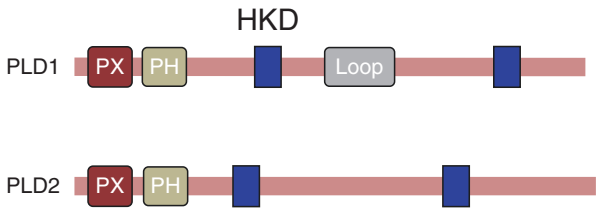

Fig. 1.3 Schematic structure of phospholipase D isozymes. Phospholipase D has PX, PH, and HKD motifs and a loop domain. HKD motifs mediate intra- and intermolecular interactions and the loop domain might be involved in the regulation of enzyme activity

an HKD domain, but little more is known [12–14]. PA, produced by PLD enzymatic activity, is involved in diverse cellular functions. PLD is activated in response to mitogenic signals, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [15–17]. PA activates MAPK signaling by recruiting RAF to the plasma membrane, regulating cell proliferation [18]. In addition, it activates mTOR, a key player in cell growth, differentiation and metabolism, by interaction with mTOR complexes [18]. Furthermore, PA also acts as an intermediate for the production of bioactive DAG or LPA (Fig. 1.4) [18, 19]. Aberrant expression or activation is closely linked to human diseases including cancer, diabetes, neurodegenerative disorders and myocardial disease.

PLA: PLA_1 and PLA_2 cleave acyl chains from the sn-1 and sn-2 position of glycerol moieties of phospholipids to produce free fatty acids and 2-acyl 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively (Fig. 1.1). PLA_1 can be divided into two groups according to cellular localization: intracellular and extracellular PLA_1. Three members of the mammalian intracellular phospholipase A1 subfamily have been identified: phosphatidic acid-preferring phospholipase A1, p125 and KIAA0725p. These enzymes commonly contain a lipase consensus sequence. There are ten mammalian extracellular phospholipase A1 enzymes: phosphatidylserine-selective phospholipase A1 (PS-PLA_1) (Fig. 1.5), membrane-associated phosphatidic acid-selective phospholipase A1α (mPA-PLA_1α), mPA-PLA1β, pancreatic lipase, lipoprotein lipase, hepatic lipase, endothelial lipase, and pancreatic lipase-related proteins-1, -2, and -3. These PLA_1s share multiple conserved motifs, including a lipase consensus sequence, a catalytic Ser-Asp-His triad, cysteine residues, and a lipid-binding surface loop [20]. These PLA_1s have multiple biological functions, including cell proliferation, apoptosis, blood coagulation, and smooth muscle contraction.

More than 30 enzymes that possess PLA_2 or related activity have been characterized in mammals (Fig. 1.5). The first PLA_2 was identified in snake venom and other enzymes were discovered in other organisms. PLA_2s are classified into several

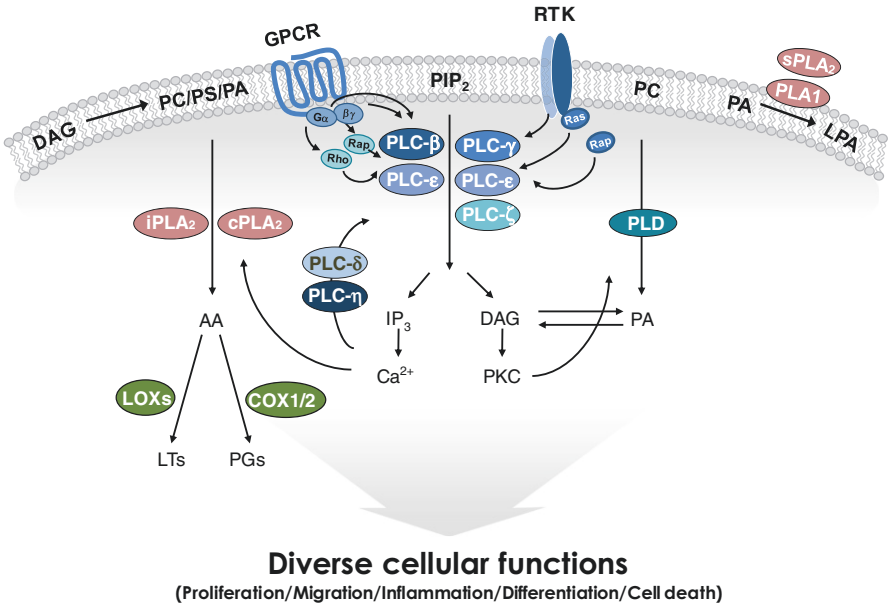

Fig. 1.4 Schematic illustration of the phospholipase signal network. Diverse extracellular ligands activate specific receptors, such as G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). Phospholipase C-β (PLC-β) is activated by the Gα or Gβγ subunit and PLC-ε is stimulated by a small GTPase (RAP2B or RHOA). PLC-δ and PLC-η are activated by calcium. In RTK signaling, RTKs directly recruit and activate PLC-γ. Activated PLCs hydrolyze phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to generate two second messengers, diacylglycerol (DAG), and inositol-1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC), which stimulates PLD activity, and IP_3 induces calcium release from the endoplasmic reticulum. PLD hydrolyzes phosphatidylcholine (PC) into phosphatidic acid (PA), which can recruit and activate various downstream molecules. $cPLA_2$ and $iPLA_2$ can hydrolyze a variety of phospholipids, including PC, phosphatidylserine (PS), and PA, into arachidonic acid (AA), which is further converted into prostaglandins (PGs) and leukotrienes (LTs). PGs and LTs are generated by the cyclooxygenase (COX) pathway and the lipoxygenase (LOX) pathway, respectively, and act as autocrine or paracrine mediators. Membrane-associated PA-selective$_{PLA1}$ (mPA-$_{PLA1}$) and secretory PLA_2 ($sPLA_2$) convert PA into lysophosphatidic acid (LPA), which acts as a ligand for LPA receptors

major types: secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), calcium-independent PLA_2 ($iPLA_2$), PLAtelet-activating factor acetylhydrolases (PAF-AHs), lysosomal PLA_2s and adipose-specific PLA. They differ from each other in terms of substrate specificity, calcium requirement and lipid modification [21, 22]. $cPLA_2$ is mainly involved in initiation of arachidonic acid generation. The $iPLA_2$ family is important for membrane homeostasis and energy metabolism and the $sPLA_2$ family modulates extracellular phospholipid environments (Fig. 1.4).

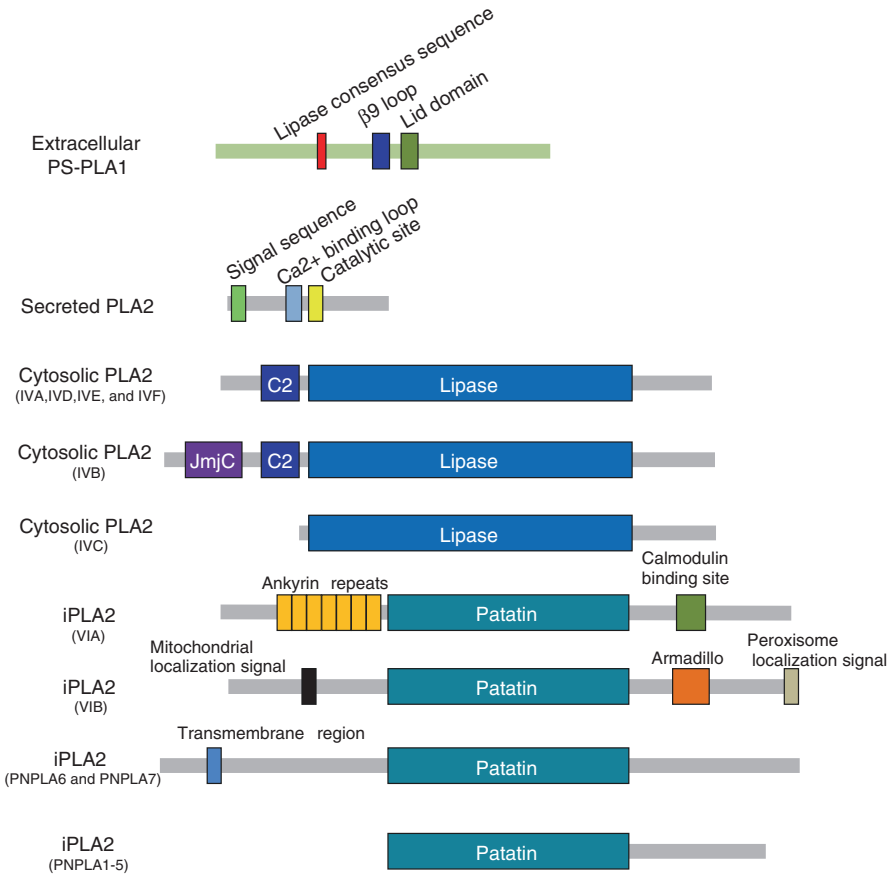

Fig. 1.5 Schematic structure of phospholipase A isozymes. Extracellular $_{PLA1}$ contains the lipase consensus sequence, β9 loop and lid domain. The β9 loop and lid domain play important roles in substrate selectivity. The three major types of PLA_2 include secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), and calcium-independent PLA_2 ($iPLA_2$). Eleven $sPLA_2$s, six $cPLA_2$s, and nine $iPLA_2$s have been found in mammals. Secreted PLA_2 has a signal sequence, calcium-binding loop, and catalytic site. $cPLA_2$-IVA, $cPLA_2$-IVD, $cPLA_2$-IVE, and $cPLA_2$-IVF have a C2 domain and a lipase domain. $cPLA_2$-IVB additionally contains a Jumonji C (Jmjc) domain. All $iPLA_2$ contain a Patatin domain, which contains a catalytic region. $iPLA_2$-VIA has an ankyrin repeat domain and calmodulin-binding site. $iPLA_2$-VIB has armadillo, mitochondrial and peroxisome localization signals

1.2 PI-PLC in Health and Disease

Each PLC subtype has a unique domain and PLC isozymes are differentially distributed in different tissues. The specific characteristics of PLC isozymes are reflected by their physiological and pathophysiological roles in diverse tissues. Each PLC isozyme is strongly linked to diverse human diseases (Table 1.1).

Table 1.1 Summary of PI-PLC roles in health and disease

PLC isozyme	Disease	Analysis system	Functional role	Reference
PLC-β1	Epilepsy	Knock-out mice	Regulate muscarinic acetylcholine receptor signaling	[26]
	Early-onset epileptic encephalopathy	Genetic studies		[29]
	Schizophrenia		Regulate neurotransmitter/ GPCR signaling	[30]
	Bipolar disorder			[31]
	Myelodysplastic syndromes		Regulate proliferation of myeloid cells	[71]
PLC-β2	Breast cancer	Expression level of patient sample	Up-regulated PLC-β2 may contribute to tumoregenesis	[46]
		Human breast cancer-derived cells	Promotes mitosis and migration	[47]
	Acute promyelocytic leukemia	Expression level of patient sample	Up-regulated in patients who were treated with drugs	[73]
PLC-β3	Myeloproliferative disease (lymphoma)	Knock-out mice	Acts as a tumor suppressor by modulating Stat5-suppressive mechanism	[68]
	Atherosclerosis	Knock-out mice	Promotes macrophage survival	[90]
PLC-β4	Ataxia	Knock-out mice	Regulates neurotransmitter/ GPCR signaling in cerebellum	[26]
	Visual-processing defect	Knock-out mice	Plays an important role in rod-mediated signaling in the retina	[105]
PLC-γ1	Epilepsy	Knock-in mice	Regulates TrkB receptor signaling	[38]
	Huntington's disease	R6/1 HD model mice	Regulates BDNF/TrkB signaling	[39–42]
	Depression	Antidepressant drug effect on cultured cortical cells		[43–45]
	Breast cancer	Expression level of patient sample	Up-regulated PLC-γ1 may contribute to tumorigenesis	[48]
	Colon cancer			[49]
	Breast cancer metastasis	Mice model of metastasis	Controls cell migration via Rac1 activation	[51]
	Autoimmune disease	T-cell-specific knock-out mice	Mediates T cell development	[81]
		LAT^{Y136F} knock-in mice	Regulates LAT-mediated T cell signaling	[80]
	Metabolic syndrome	Genetic studies	Contributes to development of metabolic disease	[95]
	Multicystic kidney	Chimeric knock-out mice	Regulates function and development of kidneys	[101]

(continued)

Table 1.1 (continued)

PLC isozyme	Disease	Analysis system	Functional role	Reference
PLC-γ2	Cold urticarial and immune dysregulation	Genetic studies	Constitutive PLC-γ2 activation causes dysfunction of immune system	[88]
	Arthritis	Knock-out mice	Regulates neutrophil activation and dendritic cell-mediated T cell priming	[91, 92]
PLC-ε1	Skin tumor	Knock-out mice	Activates *Ras* oncogene-induced carcinogenesis	[63, 64]
	Intestinal tumor	Knock-out mice with $APC^{min/+}$ background	Enhances inflammation and angiogenesis	[65]
	Esophageal squamous cell carcinoma	Genetic studies	Promotes tumorigenesis	[66]
	Gastric cancer			[67]
	Early-onset nephrotic syndrome	Genetic studies	Essential for glomerular development	[104]
PLC-δ1	Esophageal squamous cell carcinoma	Genetic studies	Acts as a tumor suppressor	[69]
	Skin tumor	Knock-out mice	Acts as a tumor suppressor	[70]
	Obesity		Negatively regulates thermogenesis and positively controls adipogenesis	[93]

1.3 Brain Disorders

In the synapse, diverse hormones and neurotransmitters activate PLC isozymes through GPCR and RTK, indicating that PLC isozymes are involved in diverse brain functions. Each PLC isozyme selectively couples to specific neurotransmitter receptors in different regions of the brain, contributing to specific functions. Many studies have implicated primary PLCs in brain disorders. PLC-β1 is abundant in the brain region, including the cerebral cortex, hippocampus, and amygdala [22, 23], and regulates cortical development and synaptic plasticity by modulating hippocampal muscarinic acetylcholine receptor signaling [24, 25]. Consistent with this, PLC-β1 knock-out mice exhibited epilepsy [26] and abnormal behaviors which are caused by excessive neurogenesis and aberrant migration of adult-born neurons [27, 28]. Interestingly, a PLC-β1 gene mutation in human patients has been observed, and genetic studies showed that the PLC-β1 mutation is associated with early-onset epileptic encephalopathy [29]. Furthermore, orbitofrontal cortex sample of patients with schizophrenia and bipolar disorder exhibited deletion of PLC-β1 gene [30, 31]. Unlike PLC-β1, PLC-β4 is expressed weakly in the cerebral cortex and

hippocampus and abundantly in the cerebellum [32] and regulates a long-term depression in rostral cerebellar purkinje cells [33]. In addition, mGluR1-mediated signals require PLC-β4 activation in the cerebellum. Both mGluR1 knock-out mice and PLC-β4 knock-out mice show ataxia [26, 34].

PLC-γ1 is highly expressed in a broad range of brain regions and regulates various neuronal functions, such as neurite outgrowth, neuronal cell migration, and synaptic plasticity. Neurotrophic factors activate PLC-γ1 through Trk receptors, which participate in diverse neuronal events [35, 36]. PLC-γ1 has been implicated in epilepsy, Huntington's disease (HD), depression, Alzheimer's disease (AD), and bipolar disorder [37]. Tyrosine phosphorylation of PLC-γ1 is elevated in pilocarpine-induced status epilepticus mouse model [38]. Consistent with this, epilepsy is markedly inhibited in $trkB^{PLC/PLC}$ knock-in mice lacking PLCγ-1docking sites in TrkB [38]. On the other hand, phosphorylation of PLC-γ1 is reduced in HD model mice [39]. Correlatively, the expression levels of BDNF and TrkB are decreased in humans and mice with HD [40–42]. Moreover, PLC-γ1-mediated signaling activates CREB, which elevates BDNF, for a long-term antidepressive effect in the hippocampus [43–45].

1.3.1 Cancer

Various extracellular ligands such as growth factors, hormones, cytokines, and lipids activate PLCs, which regulate cell growth, migration, inflammation, angiogenesis, and actin cytoskeleton reorganization. Thus, in cancer cells, activation of PLCs is involved in tumorigenesis and/or metastasis. Therefore, aberrant expression and activity of PLC isozymes is observed in a variety of human cancers and is related to tumor progression.

PLC-β2 is abnormally increased in breast tumors and correlates with poor clinical outcome, suggesting its role as a marker for breast cancer severity [46]. PLC-β2 is important for migration of breast cancer-derived cell lines and mitosis of breast-derived tumor cells [47]. In addition to PLC-β2, also PLC-γ1 level is aberrantly elevated in cancers [48, 49]. Many evidences have suggested that PLC-γ1 is required for cell migration and tumor cell invasiveness and metastasis, both in vitro and in vivo. Indeed, PLC-γ1 is required for cell spreading and migration mediated by integrins [50]. Correlatively, downregulation of PLC-γ1 expression blocked Rac1 activation and resulted in suppression of human breast cancer cell-derived lung metastasis in an in vivo mouse model [51]. In addition, PLC-γ1 has been shown to mediate the cell motility effects of growth factors including PDGF [52], EGF [53, 54], insulin-like growth factor (IGF) [55], and hepatocyte growth factor (HGF) [56, 57]. Phosphoinositide 3-kinase (PI3K)-mediated PLC-γ1 activation is required for EGF-induced migration of breast cancer cells [58, 59]. In fact, interactions between the SH3 domain of PLC-γ1 and Rac1 are important for EGF-induced F-actin formation and cell migration [60]. The critical role of PLC-γ1 in metastasis was demonstrated in mouse models. A fragment of

dominant-negative PLC-γ1 limited the metastatic potential of carcinomas in oncogene-induced mammary and prostate cancer tissues in mouse models [61]. This result suggests that PLC-γ1 is a potential therapeutic target for the clinical treatment of tumor metastasis. Similarly, involvement of PLC-ε in cancer development has been suggested. PLC-ε contains two *Ras*-associating (RA) domains (RA1 and RA2), which are essential for PLC-ε function. The RA domain binds to *Ras* and small GTPase, which are important in generation and progression of tumors [62]. 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation and tumorigenesis were suppressed in PLC-ε1 knock-out mice, suggesting the importance of PLC-ε1 in *Ras* oncogene-induced de novo carcinogenesis [63, 64]. In addition, $APC^{Min/+}$ mice lacking PLC-ε1 exhibited reduced intestinal tumorigenesis [65]. In addition, genome-wide association studies identified PLC-ε1 as a susceptibility locus in esophageal squamous cell carcinoma (ESCC) and gastric cancer [66, 67].

PLC-β3 and PLC-δ1 are suggested tumor suppressors. PLC-β3 knock-out mice showed development of myeloproliferative disease, lymphoma and other tumors, resulting from an impaired Stat5-suppressive mechanism. Further, PLC-β3 is down-regulated in leukocytes of patients with chronic lymphocytic leukemia [68]. PLC-δ1 was located within the 3p22 chromosomal region, which is frequently altered in many solid tumors, including ESCC. Interestingly, loss of the PLC-δ1 gene was frequently observed in ESCC. Consistent with this, PLC-δ1 acts as a tumor suppressor in ESCC cell lines [69]. Moreover, spontaneous skin tumors were detected in PLC-δ1-deficient mice [70].

1.3.2 Leukemia

PLC-β1 appears to regulate nuclear inositol lipid signaling in the nucleic compartment. It was suggested that dysfunction of nuclear PLC-β1 contributes to the development of myelodysplastic syndromes (MDS), which are a heterogeneous group of bone marrow disorders leading to progressive cytopenia. Interstitial PLC-β1 monoallelic gene deletion was observed in MDS patients whose disease rapidly evolved to acute myeloid leukemia (AML) [71]. Interestingly, Azacitidine, an anticancer drug (DNA methyltransferase inhibitor), targets PLC-β1. This drug increases the expression of PLC-β1 and decreases AKT activity, which plays important roles in MDS cell proliferation [72]. In addition to PLC-β1 abnormality, low levels of PLC-β2 were also observed in primary acute promyelocytic leukemia (APL) blasts isolated from patient bone marrow. APL is a subtype of AML and all-trans retinoic acid (ATRA), used for the treatment of APL by differentiating abnormal promyelocytes, strongly up-regulates PLC-β2 expression [73]. As_2O_3, a safe and effective agent for patients with APL, also produced a slight increase in PLC-β2. These observations suggest that PLC-β2 expression is closely correlated with the responsiveness of drugs in APL patients and is a specific marker to test the ability of differentiation agents for the treatment of APL [74].

1.3.3 Immune System Dysfunction

Interestingly, two PLC-γ isozymes show a distinct expression pattern in immune cells. PLC-γ1 is abundant in T-cells and PLC-γ2 is highly detected in B-cells. In view of their distinct expression pattern, PLC-γ1 and PLC-γ2 are essential for T- and B-cell development and immune responses, respectively. PLC-γ1 is critical for T-cell receptor-mediated signaling, which mediates activation of NF-κB, *Ras*-ERK, and NFAT signaling [75–77]. Linker for activation of T-cells (LAT), a scaffold adaptor protein, regulates T-cell signaling and development [78]. Mutation of Y136 site (a binding site for PLC-γ1) impaired T-cell development, with a polyclonal lymphoproliferative disorder and signs of autoimmune disease [79]. In addition, a severe defect in positive and negative thymocyte selection was observed in LAT^{Y136F} knock-in mice, suggesting that aberrant negative selection might contribute to the proliferation of autoreactive T-cells due to a skewed TCR repertoire [80]. Moreover, deletion of T-cell-specific PLC-γ1 impaired T-cell development and function and developed inflammatory/autoimmune disease in mice model [81]. Also, PLC-γ2 is highly expressed in hematopoietic lineage cells and plays a crucial role in immune responses [82–84]. As expected, PLC-γ2 knock-out mice exhibited defects in B-cell functions and Fc receptor-mediated signaling [85, 86]. Significantly, whole-exome sequencing of a family affected by dominantly inherited inflammatory disease identified p.Ser707Tyr substitution in the PLC-γ2 SH2 domain, which is essential for PLC-γ2 activation. Consistent with these data, overexpression of the p.Ser707Tyr mutant, PLC-γ2, in leukocytes resulted in elevated PLC-γ2 activity [87]. Additionally, genetic studies reported that the in-frame deletion of PLC-γ2 resulted in constitutive forms of PLC-γ2 in individuals with cold urticarial and immune dysregulation [88].

1.3.4 Atherosclerosis

The accumulation of leukocytes (particularly monocytes/macrophages) in an arterial lesion leads to atherosclerosis. Thus, numerous abnormalities in leukocytes are closely linked to atherosclerosis [89]. PLC-β3 deficiency elevated sensitivity of macrophages to apoptosis induction in vitro and led to reduction in the number of macrophages in the apoE-deficient mouse model of atherosclerosis [90]. These results indicate that PLC-β3 activation promotes macrophage survival in atherosclerotic plagues, suggesting PLC-β3 as a potential target for the treatment of atherosclerosis.

1.3.5 Arthritis

During rheumatoid arthritis, which is characterized by proliferation of synovial tissues and associated joint destruction, many immune cells are involved in autoimmunity. PLC-γ2 is highly expressed in hematopoietic lineage cells and plays a

crucial role in immune responses as described above. PLC-γ2 knock-out mice were protected in both the serum transfer arthritis model and methylated BSA-induced arthritis model. These reports suggest that PLC-γ2 is required for neutrophil activation, dendritic cells (DCs)-mediated T cell priming, and focal osteolysis in progression of arthritis [91, 92].

1.3.6 Metabolic Diseases

PLC isozymes are expressed in metabolic tissues and respond to extracellular signals linked to metabolic regulation. PLC-δ1 knock-out mice demonstrated that PLC-δ1 negatively regulates thermogenesis and positively controls adipogenesis. Because of increased oxygen consumption and heat production, PLC-δ1 knock-out mice showed decreases in weight gain and lipid droplets on a high-fat diet [93].

The metabolic syndrome represents a combination of metabolic phenotypes, including high blood pressure, obesity, cholesterol levels, and insulin resistance [94]. A phenomics-based strategy found that PLC-γ1 missense mutation was associated with metabolic syndrome in the European American and African American populations [95]. This result suggested that PLC-γ1 may contribute to the development of the metabolic syndrome. Although the early death of PLC-γ1 knock-out mice limits in vivo studies, studies using conditional knock-out mice will increase our understanding of PLC-γ1 function in metabolic disease.

1.3.7 Kidney Dysfunction

The kidneys play a fundamental role in the regulation of arterial blood pressure and fluid/electrolyte homeostasis. Many RTKs and their respective ligands have been implicated in the control of metanephric kidney and urinary tract development. Many genetic mouse models have demonstrated the role of RTKs in renal development [96–99]. The important functions of PLC-γ1 in RTK signaling have also been extensively studied [100]. Chimeric PLC-γ1 knock-out mice display multicystic kidneys due to severe renal dysplasia and renal tube dilation [101]. Recent studies have suggested that PLC-γ1 contributes to the response to hypertonic stress by regulating tonicity-responsive enhancer-binding protein (TonEBP), a transcription factor that is essential in the function and development of the renal medulla [102, 103].

PLC-ε1 is abundant in podocytes of mature renal glomeruli, implicating it in kidney function. Using positional cloning, a PLC-ε1 mutation was identified in patients with early-onset nephrotic syndrome, a malfunction of the kidney glomerular filter. Patients with PLC-ε1 mutation showed defects in glomerular development. Consistent with this, PLC-ε1 knockdown in zebrafish leads to nephrotic syndrome [104, 105].

1.4 PC-PLD in Health and Disease

PLD and its product PA are involved in a variety of cellular processes. The PLD functions in cellular signaling, vesicle transport, endocytosis, exocytosis, and cytoskeletal rearrangement have been largely defined and are implicated in a diverse range of pathophysiological processes and diseases, such as neuronal, cardiac, and vascular diseases, as well as oncogenesis and metastasis [106] (Table 1.2).

Table 1.2 Summary of PC-PLD roles in health and disease

PLD isozymes	Disease	Analysis system	Functional role	Reference
PLD1	Brain ischemia	Ischemia-reperfusion model	Protects neuronal calls from apoptotic condition	[113]
	Alzheimer's disease	Expression level of patient sample	Up-regulated expression and activity of PLD	[118]
		Blastocyst-derived wt and PS1$^{-/-}$/PS2$^{-/-}$ cells	Disrupts the association of γ-secretase components	[125]
	Bleeding disorder	Knock-out mice	Regulates integrin αIIbβ3 activation and aggregate formation	[129]
		Histamine-induced secretion of von Willebrand factor model	Regulates secretion of Weibel–Palade bodies	[130]
	Breast cancer	Expression level of patient sample	Up-regulated PLD1 may contribute to tumorigenesis	[132, 133]
			Over-expressed PLD1 associates with poor prognosis	[134]
	Melanoma, lung carcinoma, breast cancer	Knock-out mice	Promotes tumor growth and metastasis in the tumor environment	[139]
PLD2	Colorectal cancer	Genetic studies	Polymorphism of PLD2 is associated with colorectal cancer	[135]
		Expression level of patient sample	Up-regulated PLD2 may contribute to tumor size and survival	[136]
	Brain ischemia	Ischemia-reperfusion model	Protects neurons from ischemia	[117]
	Alzheimer's disease	Transgenic mouse model of AD (SwAPP)	Deletion of Pld2 rescues deficits of SwAPP mouse	[122]
	Renal cancer	Genetic studies	Up-regulated PLD2 may contribute to tumorigenesis	[138]

1.4.1 Brain Disorder

Brain-associated PLD activity was first reported in 1973. Indeed, both PLD1 and PLD2 are expressed throughout the brain during development and postnatal life. In the rat, PLD1 mRNA levels and activity are increased from embryonic day 19 to postnatal day 14 and remain constant thereafter [107], and PLD2 expression increases postnatally [108]. In addition to neurons, PLDs are also highly expressed in oligodendrocytes (PLD1), astrocytes (PLD2), and ependymal cells [109]. PLDs regulate various neuronal activities. For instance, PLD2 mediates the constitutive internalization of metabotropic glutamate receptors, mGluR1a and mGluR5a, and the trafficking of opioid receptors [110, 111]. In addition, PLDs regulate neurite outgrowth through Src and *Ras*, Erk1/2, and the CREB signal pathway in response to NGF and neuronal cell adhesion molecule L1, lysophosphatidylcholine (LPC), and bFGF [112]. PLDs are also involved in survival of neuronal cells in ischemia. Forebrain ischemia increases the expression of PLD1 and increased PLD activity was detected in reactive astrocytes in the rat [113]. Recent studies have shown that overexpression of PLD2 protects neurons exposed to apoptotic conditions [114–117].

In 1986, the relationship between PLDs and Alzheimer's disease (AD) was first described. The activity of PLDs in AD-affected brain was reduced by 63 % in comparison with controls. In recent studies, however, increased protein levels and activity of PLDs in AD patients and increased activation of PLDs by amyloid β-peptide (Aβ) have been reported [118–121]. Furthermore, Aβ1-42 increases PLD activation in neurons, astrocytes, and microglia. Consistent with this, the deletion of PLD2 prevents toxicity and synaptic dysfunction induced by Aβ1-42 despite a significant amyloid β load [122]. Conversely, PLD1 is also involved in the generation and secretion of Aβ. PLD1 accelerates the formation of amyloid precursor protein (APP)-containing vesicles from the trans-Golgi network and the cell surface accumulation of APP and presenilin 1 (catalytic component of γ-secretase complex) [123, 124]. In contrast to its positive role in trafficking, PLD1 also functions as a negative regulator of Aβ generation. PLD1 physically interacts with the cytoplasmic loop of presenilin 1. This interaction recruits PLD1 to the Golgi and inhibits cleavage of βCTF to Aβ by disrupting association of γ-secretase [125].

1.4.2 Bleeding Disorder

PLDs are present in platelets and PLDs rapidly localize to the plasma membrane in response to platelet activation [126–128]. Platelets from *Pld1* knock-out mice exhibit impaired integrin αIIbβ3 activation and abnormal aggregate formation in vitro under high shear flow conditions [129]. Additionally, PLD1 is implicated in the histamine-induced secretion of von Willebrand factor (vWF) from endothelial cells [130]. The vWF is a major clotting factor and its deficiency results in the most

common inherited bleeding disorder, von Willebrand disease. Knockdown of PLD1 dramatically decreased histamine-induced secretion of vWF, whereas knockdown of PLD2 had no effect [131]. These results suggest that PLD1 may be a critical regulator of thrombosis in endothelial cells and platelets.

1.4.3 Cancer

Elevated PLD activity and a driver mutation in PLDs have been reported in various cancers (breast, gastric, renal, and colorectal cancer). In malignant breast cancer, PLD activity is increased, as is the expression of PLD1/2 [132, 133]. PLD1 tends to be overexpressed in tumors that show high expression of cytokeratins 5/17, which are frequently associated with poor prognosis [134]. Polymorphism in *Pld2* was reported and was significantly associated with the prevalence of colorectal cancer [135]. Moreover the expression level of PLD2 is also elevated in colorectal carcinoma and the ratio is proportional to tumor size and survival [136]. Additionally, increased activity of PLD is found in gastric carcinomas [137] and PLD2 protein levels and activity are increased in renal cancers [138]. Moreover, PLD1 has a critical function not only in the cancer cell itself but also in the tumor microenvironment. PLD1-deficient mice showed that PLD1 promotes tumor growth and metastasis through enhanced angiogenesis and decreased tumor cell–platelet interactions [139].

Although the molecular mechanism through which PLDs contribute to the occurrence and progression of cancer remains unclear, PLDs contribute to key events in the oncogenic process, including growth signaling, overriding gatekeeper, and suppression of apoptosis and metastasis. PLDs have been involved in oncogenic signaling. The oncogenic signaling network is mediated by the interaction between PLDs and *Ras* and facilitates the activation of MAPK [140, 141]. PLD and its product PA suppress cancer cell apoptosis through activation of mTOR [18, 142, 143]. PLD and PA also act to suppress the expression of p53 by stabilizing the MDM2-p53 complex [144, 145]. PLD1 was reported to be required for secretion of matrix metalloproteinase (MMP)-9 by colorectal cancer cells [146] and MMP-2 by glioma cells [147]. PLD2 activation increases phosphorylation of focal adhesion kinase and Akt and these enhance the invasion activity of EL4 lymphoma cells, whereas inactive PLD inhibits metastasis by disrupting actin cytoskeletal reorganization, cell spreading, and chemotaxis [148, 149].

1.5 PLA in Health and Disease

PLAs are divided into two subtypes, type 1 and type 2. In contrast to other phospholipases, the physiological functions of PLA_1 remain largely unknown [20]. Each subtype of PLA_2 has different structures and regulatory mechanisms, distribution, and cellular localization [21]. In particular, $sPLA_2$s are secreted and not limited to

intracellular functions, acting also in extracellular regions. Through this diversity, PLA_2s are involved in various biological processes. Each isotype of PLA_2 has specific roles and is implicated in various human diseases (Table 1.3).

1.5.1 Brain Disorders

$sPLA_2$-IIA and $sPLA_2$-IIC are ubiquitously expressed in the rat brain. $sPLA_2$-V is highly expressed in the hippocampus. Among the various $sPLA_2$s, $sPLA_2$-IIE, $sPLA_2$-V, and $sPLA_2$-X are expressed in the human brain and the expression of $sPLA_2$-IIA is induced under inflammatory conditions [150]. $sPLA_2$s released from neuronal cells regulate neurite outgrowth [151] and neurotransmitter release [152].

$cPLA_2$s are expressed in the gray matter of many regions, including the olfactory cortex, hippocampus, amygdala, thalamus, hypothalamus, and cerebellum, and the expression is confined to astrocytes [153, 154]. Compared with $iPLA_2$s, $sPLA_2$-IIA and $sPLA_2$-V, the expression levels of $cPLA_2$ are lower in the brain [155]. However, $cPLA_2$s have crucial function in brain. $cPLA_2$s cleave membrane phospholipids at the sn-2 position and preferentially release arachidonic and docosahexaenoic acids. The arachidonic and docosahexaenoic acids regulate the release, uptake, and transport of neurotransmitters [156–158]. Additionally, administration of arachidonic acid and docosahexaenoic acid into hippocampus induces a long-term potentiation of synaptic transmission [159, 160]. $cPLA_2$ levels in occipital cortex and cerebellum of Alzheimer's patients were elevated above those in normal persons [161]. In the cerebral cortex, the increased expression of $cPLA_2$ was detected on astrocytes in Aβ amyloid accumulated regions. This elevated $cPLA_2$ is associated with active inflammatory response in AD. Contrary to the occipital cortex, in the parietal region of AD brains, $cPLA_2$ was significantly decreased. Moreover, lower PLA_2 activity was significantly correlated with earlier onset of the disease and with higher mortality, higher neurofibrillary tangle counts, and senile plaques [162].

The pathological relevance of $cPLA_2$-IVA has been suggested in Parkinson's disease. Administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) led to less dopamine depletion and neurotoxicity in *Pla2g4a* knock-out mice as compared with their normal littermates [163]. The reduced MPTP-induced neurotoxicity is explained as a consequence of reduced excitotoxicity and mitochondrial injury by decreases in free fatty acids, lysophospholipids, and reactive oxygen metabolites. This evidence suggests that $cPLA_2$-IVA plays a role in the development of Parkinson's disease.

The main cytosolic PLA_2 activity comprises $iPLA_2$-VI in adult rat brains and the activity is highest in the hippocampus and striatum [155, 164, 165]. Mutations in the *PLA2G6* gene have been identified on chromosome 22q12-q13 and this locus is associated with infantile neuroaxonal dystrophy, neurodegeneration with brain iron accumulation and the related Karak syndrome [166]. Consistent with this, *Pla2g6* knock-out mice show severe motor dysfunction accompanied by numerous spheroids and vacuoles and widespread degeneration of axons and synapses after

Table 1.3 Summary of PLA roles in health and disease

PLA isozymes	Disease	Analysis system	Functional role	Reference
sPLA2-IB	Obesity	Knock-out mice	Prevent metabolically beneficial adaptation	[198, 199]
		Genetic study	Associates with a locus for obesity susceptibility	[120]
sPLA2-IIA	Colorectal cancer	Knock-out mice	Inhibits colon tumorigenesis	[216]
		Transgenic mice		[217]
		Genetic study	Resists carcinogens	[215, 218]
	Prostate cancer	Clinical studies	Contributes to the pathogenesis of prostate cancer	[219, 221]
	Skin cancer	Transgenic mice	Sensitizes to a two-stage chemical carcinogenesis	[220]
	Gastric cancer	Clinical studies	Associates with patient survival and less frequent metastasis	[218]
	Arthritis	Knock-out mice	Involved in joint inflammation response	[177]
		Transgenic mice		[178]
		Clinical studies	Associates with prevalence of rheumatoid arthritis	[176]
	Atherosclerosis	Clinical studies	Promotes aggregation and fusion of the LDL	[186]
		Transgenic mice		[187]
sPLA2-III	Atherosclerosis	Transgenic mice	Regulates plasma lipoprotein modification and macrophage foam cell formation	[191]
	Colorectal cancer	Genetic study	Polymorphisms of PLA2G3 May contribute to cancer	[223]
sPLA2-V	Atherosclerosis	Clinical studies	Contributes to development of atherosclerosis through macrophages	[188]
		Transgenic mice	Increases collagen deposition	[189]
	Arthritis	Knock-out mice	Anti-inflammatory role and promotes immune complex clearance	[177]
		Clinical studies	Associated with prevalence of asthma	[181]
	Acute respiratory distress syndrome	Knock-out mice	Regulates acute lung injury and neutrophilic inflammation	[183]
	Asthma	Knock-out mice	Regulation of leukocyte migration during immunosensitization	[182]
sPLA2-X	Asthma	Knock-out mice	Regulates allergen-induced airway inflammation	[185]
	Atherosclerosis	Knock-out mice	Regulates macrophages and increases atherosclerotic lipid accumulation	[190]

(continued)

Table 1.3 (continued)

PLA isozymes	Disease	Analysis system	Functional role	Reference
cPLA2-IVA	Parkinson's disease	Knock-Out mice	Involved in MPTP-induced dopamine depletion	[163]
	Asthma	Knock-Out mice	Contribute to development of asthma	[184]
	Arthritis	Knock-Out mice	Contributes collagen-induced arthritis	[179]
	Intestinal polyposis	Genetic study/ knock-out mice	Regulates expansion of polyps	[224, 225]
	Colorectal cancer	Knock-out mice	Regulates pro-apoptosis signal	[226]
	Platelets dysfunction	Genetic studies	Regulates production of TXA2 and 12-hydroxyeicosatetraenoic acid, and platelet aggregation	[196]
		Knock-out mice		[197]
iPLA2-VIA	Islet dysfunction	Knock-out mice	Regulates insulin secretion	[201, 202]
	Diabetes-associated vascular complications	Knock-out mice	Regulates vascular contraction	[222]
	Colorectal cancer	Genetic study	Associated with generation of colorectal cancer	[223]
	Ovarian cancer	Knock-out mice	Regulates a tumorigenesis and invasion	[227]
	INAD and NBIA, related Karak syndrome	Genetic study	Protects against neuroaxonal dystrophy	[166]
		Knock-out mice		[167, 168]
iPLA2-VIB	Obesity	Knock-out mice	Resistant to high-fat diet-induced dysfunction	[204, 205]
	Cognitive dysfunction	Knock-out mice	Regulates hippocampus function through mitochondrial phospholipid composition	[171]
PNPLA2	Chanarin–Dorfman syndrome	Genetic studies	Regulates neutral lipid storage	[206]
	Neutral lipid storage disease	Genetic studies		[207, 208]
PNPLA3	Obesity	Genetic studies	Involved in insulin secretion	[212]
	NAFLD	Genetic studies	Regulates triglyceride hydrolysis	[213]
PNPLA6	Motor neuron disease	Knock-out mice	Influences sensory and motor neurons	[169]
		Genetic studies		[170]
PAF-AH VIIA	Atherosclerosis	Genetic studies	Produces pro-inflammatory mediators, LPC and oxidized non-esterified fatty acids	[194, 195]
AdPLA-XVI	Obesity	Knock-out mice	Regulates adipocyte lipolysis	[214]

1–2 years of age [167, 168]. Brain-specific *Pnpla6* knock-out mice also exhibit a progressive neuronal degeneration in the hippocampus, thalamus, and cerebellum [169]. The deletion of PNPLA6 leads to disruption of the ER and induces degeneration and massive swelling of the axons of sensory and motor neurons. Moreover, mutations of *PNPLA6* are reported as the cause of severe motor neuron diseases in humans [170]. *Pnpla8* knock-out mice exhibit cognitive dysfunction accompanied by enlarged and degenerate hippocampal mitochondria [171]. The absence of iPLA$_2$-VIB induced the elevation of mitochondrial cardiolipin composed of long chain length species and alterations in mitochondrial phospholipid composition. These changes result in increased reactive oxygen species and neuronal cell death with deficits in spatial learning and memory.

1.5.2 Arthritis

The local and systemic expression level of sPLA$_2$-II is elevated in inflammation and sPLA$_2$-II has been considered as a key enzyme in the pathogenesis of inflammatory diseases [172, 173]. Some inbred mouse strains (199/SV; BALB/c) have a natural mutation in the sPLA$_2$ gene and exhibit higher susceptibility to arthritis than sPLA$_2$-IIA expressing mouse strains [174, 175]. In addition, the synovial cells and chondrocytes in the joints of rheumatoid arthritis patients strongly express sPLA$_2$-IIA [176]. Consistent with these clinical genetic reports, the inflammation response is noticeably attenuated in the joints of sPLA$_2$-IIA-deficient BALB/c mice under antibody-induced arthritis compared with wild-type BALB/c mice [177]. Furthermore, transgenic mice overexpressing human sPLA$_2$-IIA show exacerbated arthritis [178]. *Pla2g4a* knock-out mice also show markedly reduced severity and incidence of rheumatoid arthritis compared with control mice [179]. These findings strongly support that sPLA$_2$-IIA and cPLA$_2$-IVA have pro-inflammatory roles in inflammatory arthritis. However sPLA$_2$-V exerts opposite effects to sPLA$_2$-IIA and cPLA$_2$-IVA in inflammatory arthritis. *Pla2g5* knock-out mice show exacerbation of arthritis. This deterioration arises from the attenuated immune complex clearing by macrophages of *Pla2g5* knock-out mice [177].

1.5.3 Asthma

sPLA$_2$-V and sPLA$_2$-X are widely expressed in airway epithelia [180]. The expression of sPLA$_2$-V and sPLA$_2$-X is markedly elevated in asthmatic mouse model. Moreover, increased expression of sPLA$_2$ is also detected in patients with asthma [181, 182]. Consistent with this, methacholine-induced airway hyperresponsiveness is markedly attenuated in *Pla2g5* knock-out mice [182]. And lipopolysaccharides-induced acute lung injury is also attenuated in *Pla2g5* knock-out mice [183]. sPLA$_2$-V is involved in airway disorders by regulating antigen processing, maturation of dendrite cells and following Th2 immune response, and sPLA$_2$-V facilitates the

subsequent propagation of pulmonary inflammation in resident airway cells [184]. In the ovalbumin-induced asthma model, the lungs of *Pla2g10* knock-out mice also show marked attenuations. $sPLA_2$-X-deficient mice exhibit lower infiltration by CD^{4+} and CD^{8+} T cells and eosinophils than wild-type littermates. Metaplasia of goblet cell and smooth muscle cell layer thickening, subepithelial fibrosis, and levels of Th2 cytokines and eicosanoids are also reduced in $sPLA_2$-X-deficient mice [185]. Like $sPLA_2$, the airway anaphylactic response in $cPLA_2$-IVA-deficient mice is also markedly reduced compared with wild-type littermates [184]. These findings suggest that PLA_2s are involved in antigen-induced bronchial hyperreactivity and asthma.

1.5.4 Atherosclerosis

Hydrolysis of PC by $sPLA_2$ produces non-esterified fatty acids and LPC. These products trigger chemotactic and vasoactive proinflammatory events, which facilitate atherosclerosis. Hydrolysis of low-density lipoprotein (LDL) by $sPLA_2$s leads to an alteration of phospholipid-degraded particles and promotes aggregation. In human atherosclerotic plaques, the expression of $sPLA_2$-IIA is markedly increased in macrophage-rich regions [186]. Consistent with the expression pattern, *PLA2G2A*-transgenic mice exhibit increased incidence of atherosclerotic lesions with a high-cholesterol diet [187]. In addition, $sPLA_2$-V is also enriched in atherosclerotic lesions in humans. Recent genetic studies reported that $sPLA_2$-V induces the formation of foam cells and regulates the development of atherosclerosis [188, 189]. A study using *Pla2g10* knock-out mice provided evidence that $sPLA_2$-X negatively regulates efflux of cholesterol in macrophages and contributes to lipid accumulation [190]. Additionally, $sPLA_2$-III is also linked to atherosclerosis. $sPLA_2$-III is accumulated in the atherosclerotic lesion of human. And the aortic atherosclerotic lesions in *PLA2G3*-Tg mice are more severe than in control mice on the apoE-null background after intake of an atherogenic diet [191].

Unlike other PLA_2s, plasma-type PAF-AH has been identified as a protective factor against the development of atherosclerosis by removing oxidized LDL [192]. However, recently studied data suggest that PAF-AH has an active role in the development and progression of atherosclerotic [193]. Additionally, it has been revealed that A379V polymorphism of PAF-AH correlates with coronary artery disease as well as heart attacks by epidemiological studies [194, 195]. The active role of PAF-AH is explained by its ability to generate two key pro-inflammatory mediators, oxidized non-esterified fatty acids and LPC, by cleaving oxidized phospholipids from LDL.

1.5.5 Platelet Dysfunction

The mutation in *PLA2G4A* gene is associated with platelet dysfunction in human [196]. The production of thromboxane (TX)B2 and 12-hydroxyeicosatetraenoic acid from platelets of patients who has heterozygous mutations of *PLA2G4A* was

markedly reduced and platelet aggregation and degranulation, induced by adenosine diphosphate (ADP) or collagen, were diminished. Consistent with human, the production of pro-thrombotic TXA2 by collagen-stimulated platelets was decreased in cPLA$_2$-IVA-deficient mice; however, cPLA$_2$-IVA does not influence the ADP-stimulated production of TXA2 [197]. The platelet aggregation of *Pla2g4a* knock-out mice is slightly decreased. In mice, the TXA2, regulated by cPLA$_2$-IVA, may mainly act as a vasoconstriction regulator. Collectively, these findings indicate that cPLA$_2$-IVA is involved in platelet function and hemostasis.

1.5.6 Metabolic Disease

Pla2g1b knock-out mice exhibit resistance to high fat diets, which induce obesity [198], with lower plasma insulin and leptin levels and improvement in insulin resistance. The reduced production and absorption of LPC in the lumen of the small intestine by the absence of sPLA$_2$-IB mainly contributes to these phenotypes. *Pla2g1b* knock-out mice also display increased postprandial hepatic fat utilization and energy expenditure because of increased expression of the peroxisome proliferator-activated receptors, CD36/Fat and UCP2, coincided with reduced postprandial plasma lysophospholipid levels [199]. Moreover, a recent genome-wide linkage scan study identified that the human *PLA2G1B* gene resides within a locus for obesity susceptibility [200]. These data suggest that sPLA$_2$-IB and its product, lysophospholipid, suppress hepatic fat utilization and energy metabolism in diet-induced obesity.

Pancreatic islets of *Pla2g6* knock-out mice exhibit abnormal insulin secretion patterns based on glucose level [201]. *Pla2g6* knock-out mice have normal blood glucose concentrations on normal diets, but with high fat diets they show more severe glucose intolerance than wild-type mice, with a highly sensitive response to exogenous insulin. Conversely, iPLA$_2$-VIA transgenic mice have low blood glucose levels and high insulin levels [202]. This implies that iPLA$_2$-VIA regulates glucose-stimulated insulin secretion. iPLA$_2$-VIA is also involved in diabetes-associated vascular complications [203]. iPLA$_2$-VIA is increased in diabetic animals and the lack of iPLA$_2$-VIA diminishes diabetes-associated vascular hypercontractility. In contrast, mice lacking iPLA$_2$-VIB exhibit resistance to obesity and subsequent complications with increase in fatty acids oxidation and mitochondrial uncoupling after high fat feeding. Adipocytes of *Pnpla8* knock-out mice appear to have increased oxidation rates and their skeletal muscles exhibit impaired mitochondrial β-oxidation of fatty acids, accompanied by accumulation of long-chain acylcarnitine in the muscle and urine. [204, 205]. This implies that iPLA$_2$-VIB is a critical enzyme for efficient electron transport chain coupling and energy production.

Mutations in the *PNPLA2* gene are implicated in the pathogenesis of Chanarin–Dorfman syndrome [206] and neutral lipid storage disease [207, 208]. PNPLA$_2$ regulates lipid droplet association through its C-terminal domain [209, 210]. Consistent with this, *Pnpla2* knock-out mice have increased lipid deposition in adipose tissues and many non-adipose tissues with severe triglyceride (TG) hydrolysis defects [211].

There is a strong association between polymorphisms of the *PNPLA3* gene and ancestry-related predisposition to both nonalcoholic and alcoholic fatty liver and PNPLA3 is also associated with insulin secretion and obesity [212]. The I148M point mutation in *PNPLA3* is associated with nonalcoholic fatty liver disease. This mutation disrupts TG-hydrolytic activity [213]. Adenoviral transfer of the I148M mutant *PNPLA3* into mouse liver causes TG accumulation like human fatty liver disease. In addition, another catalytically dead S47A mutant also induces TG accumulation. Collectively, the relation of PNPLA3 and lipid droplets suggests that PNPLA3 serves to hydrolyze TG.

Pla2g16 knock-out mice have markedly reduced white adipose tissue mass and TG content but normal adipogenesis [214]. They exhibit the high energy expenditure of adipocytes with increased fatty acid oxidation. *Pla2g16* knock-out mice also show a markedly higher rate of lipolysis because of increased levels of cAMP arising from a marked reduction in the amount of adipose prostaglandin E2 (PGE2). Moreover, AdPLA-deficient ob/ob mice are hyperphagic but lean and have increased energy expenditures with ectopic TG storage and insulin resistance. This implies that AdPLA is a major regulator of adipocyte lipolysis and is crucial for the development of obesity.

1.5.7 Cancer

$sPLA_2$-IIA has an antitumor function in colorectal cancer. Expression levels of mouse $sPLA_2$-IIA correlates with the resistance of different mouse strains to the carcinogen azoxymethane [215] and overexpression of $sPLA_2$-IIA strongly inhibits azoxymethane-induced colon tumorigenesis in C57BL/6 mice [216]. Consistent with the *Pla*$_2$*g2a* transgenic mouse, *Pla2g2a* knock-out mice are susceptible to colorectal tumorigenesis [217]. $sPLA_2$-IIA expression in human gastric cancer is associated with patient survival and less frequent metastasis [218]. In contrast to colorectal and gastric cancer, $sPLA_2$-IIA has a pro-tumorigenic effect in prostate cancer and skin cancer, increasing sensitivity to chemical carcinogenesis [219, 220]. The expression of $sPLA_2$-IIA has a strong correlation with prostate cancer progression and mortality [221]. In several types of human cancers, $sPLA_2$-III is detected in microvascular endothelial cells, as well as in tumor cells [222]. These reports show that $sPLA_2$-III has a crucial role in cancer development by stimulating tumor cell growth and angiogenesis. Especially, in human colorectal cancer the polymorphisms of *PLA2G3* are significantly associated with a higher risk of cancer [223]. Consistent with these reports, the growth of $sPLA_2$-III-transfected colorectal cancer cells is promoted in xenograft nude mice model through the PGE2-dependent pathway.

$cPLA_2$-IVA expression is markedly elevated in polyps in the small intestine of APCΔ716 knock-out mice [224, 225]. Mutation in the *Pla2g4a* gene reduces polyp size, but there is no difference in number. This implies that $cPLA_2$-IVA plays a key role in the expansion of polyps rather than initiation in the intestine. However, in an azoxymethane-induced colon tumorigenesis model, deletion of $cPLA_2$-IVA

exacerbates colorectal cancer [226]. This exacerbation may be the result of the attenuated apoptosis of the colonic epithelium by $cPLA_2$-IVA deficiency.

$iPLA_2$-VIA is also involved in tumorigenesis. The *Pla2g6* haplotypes are strongly associated with colorectal cancer [223]. In the case of ovarian cancer, the genetic deletion and siRNA-mediated suppression of the *Pla2g6* gene reduced tumorigenesis and invasion of ovarian cancer cells [227].

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Chapter 2
Role of Phospholipases in Regulation of Cardiolipin Biosynthesis and Remodeling in the Heart and Mammalian Cells

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Abstract Cardiolipin is a key mitochondrial membrane phospholipid involved in the regulation of generation of ATP. Cardiolipin synthesis and remodeling are tightly regulated processes in eukaryotic cells. The role of phospholipases in the regulation of cardiolipin metabolism is becoming much clearer. Cardiolipin is hydrolysed by several classes of phospholipases including calcium-independent phospholipase A_2, secretory phospholipase A_2, and cytosolic phospholipase A_2. Mitochondrial calcium-independent phospholipase A_2 gamma has emerged as a key player not only in the regulated hydrolysis of cardiolipin to monolysocardiolipin, but also in the overall regulation of mitochondrial function and energy production. The purpose of this chapter is to summarize some of the more current findings on the role of phospholipases in the regulation of cardiolipin metabolism in the heart and mammalian tissues. In addition, a brief discussion on the role of exogenous phospholipase-treatment of cells on cardiolipin metabolism is presented.

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_2,

Keywords Cardiolipin • Calcium-independent phospholipase A_2 γ • Heart • Acyltransferase • Trifunctional protein • Mono lysocardiolipin • Barth syndrome • Human • Mammalian

2.1 Introduction

Phospholipids are important structural and functional components of the cell membrane and alterations in the composition of phospholipids within the heart are linked to alterations in myocardial electrical rhythm [1, 2]. Bis-(1,2-diacyl-*sn*-glycero-3-phospho)-1′,3′-*sn*-glycerol or cardiolipin (CL) is the principal polyglycerophospholipid found in the heart and mammalian tissues [3]. CL was initially discovered in beef heart by Mary Pangborn in 1942 and was subsequently shown to comprise approx. 15–20 % of the entire phospholipid phosphorus mass of the heart [3–6]. The heart contains the highest concentration of CL found in any mammalian tissue due to its vast abundance of mitochondria. CL is found within both inner and outer mitochondrial membranes and within their contact sites [7–9]. Both the appropriate content and the fatty acyl molecular composition of CL are critical for the ability to modulate the activity of mitochondrial enzymes involved in the generation of ATP (reviewed in [6, 10]). In fact, CL is the "glue" that holds the mitochondrial respiratory complex together [11]. Hence, maintenance of the appropriate content and fatty acid composition of CL in mitochondria is essential for mammalian cell function.

2.2 Cardiolipin: Its Role in Apoptosis, General Mitochondrial Function, and Genetic Disease

CL has been implicated in the intrinsic pathway of apoptosis [12] and is required for caspase-8 cleavage of Bid at the mitochondrial outer membrane [13]. Stomatin like-2 (SLP-2), a widely expressed mitochondrial inner membrane protein of previously unknown function, expression in T lymphocytes resulted in increased CL content and resistance to apoptosis mediated through the intrinsic pathway [14]. Alteration in the content of CL has been shown to alter oxygen consumption in mitochondria [15, 16]. In rat heart subjected to ischemia and reperfusion the reduction in electron transport chain activity was coupled with reduction in CL [17]. When CL is removed or digested away from mitochondrial respiratory chain proteins by phospholipases, denaturation and complete loss in activity occur (reviewed in [18]). The prohibitins (PHB-1 and PHB-2) are an evolutionarily conserved and ubiquitously expressed family of membrane proteins that are essential for cell proliferation and development in higher eukaryotes [19, 20]. PHB complexes function as protein and lipid scaffolds that ensure the integrity and functionality of the mitochondrial inner membrane and they associate with CL. CL is important for formation of the prohibitin-m-AAA protease complex, the alpha-ketoglutarate

dehydrogenase complex, and mitochondrial respiratory chain supercomplexes [21]. SLP-2 interacts with PHB-1 and -2 and binds to CL to facilitate formation of metabolically active mitochondrial membranes [14]. In T cell-specific SLP-2-deficient mice impaired CL compartmentalization in mitochondrial membranes results in decreased protein and activity of complex I of the mitochondrial respiratory chain [22]. Hence, the function of SLP-2 is to recruit PHBs to CL to form CL-enriched microdomains in which electron transport complexes are optimally assembled. In addition, reduced expression of mitochondrial respiratory complex proteins in right ventricle (RV) of persistent pulmonary hypertension of the newborn (PPHN) piglets provided evidence that PHB complexes may be disrupted in RV cardiac mitochondria of these animals [23].

Barth syndrome (BTHS) is a rare X-linked genetic disorder in young boys characterized by the triad of cardiomyopathy, cyclic neutropenia, and a 3-methyglucaconic aciduria [24–26]. In 50 % of the cases a mild hypocholesterolemia is also observed. The documented hypocholesterolemia observed in at least one BTHS patient may be due to a reduced ability to upregulate mRNA expression and enzyme activity of hydroxymethylglutaryl-Coenzyme A reductase, the rate-limiting enzyme of de novo cholesterol biosynthesis [27]. BTHS is caused by mutations in the tafazzin gene, *TAZ*, localized to chromosome Xq28.12. There are over 100 mutations in *TAZ* identified. However, to date there has been no correlation between genotype and severity of the disease. A reduced ability to resynthesize CL from monolysocardiolipin (MLCL) is the underlying molecular mechanism responsible for BTHS (reviewed in [24, 25]). Hence, BTHS is the only genetic disease identified to date in which the specific biochemical defect is a reduction in mitochondrial CL and accumulation of MLCL. Four *TAZ* mRNA transcripts were shown to be generated in human cells [28]. *Taz* knockdown mice exhibited a dramatic decrease of tetralinoleoyl-CL (L_4-CL) in cardiac and skeletal muscles, accumulation of MLCL, and pathological changes in mitochondria [29, 30]. Moreover, disruption of TAZ alters both assembly and stability of the respiratory chain supercomplexes in the mitochondrial inner membrane [31]. Interestingly, decreased levels of PHB complexes in TAZ-deficient mitochondria were shown to be due to a decreased content of CL [21]. Introduction of TAZ into yeast with defective TAZ or into TAZ knockout zebrafish or onto TAZ knock out drosophila restored CL levels and mitochondrial function to that of near normal levels [32–34].

2.3 Cardiolipin Biosynthesis and Remodeling

The de novo biosynthesis of CL in the heart occurs via the cytidine-5′-diphosphate-1,2-diacylglycerol (CDP-DG) pathway [35] (Fig. 2.1). Initially, phosphatidic acid (PA) is converted to CDP-DG by CDP-DG synthetase (CDS). The human CDS has been cloned and CDS-2 is the major isoform expressed in mammalian heart [36]. CDS-2 mRNA expression is reduced in AMP-activated protein kinase α2 null mice and this accounted for the reduction in cardiac CL seen in these animals [37]. Clofibrate-mediated activation of peroxisome proliferator-activated receptor

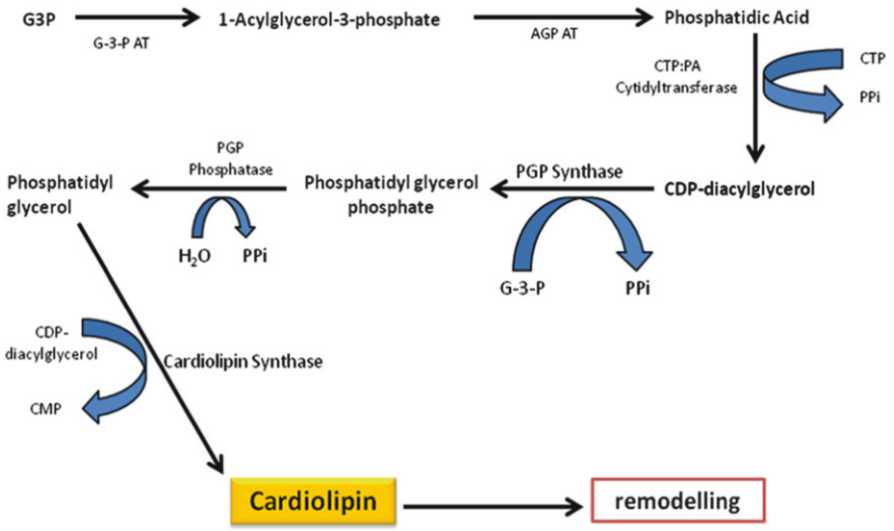

Fig. 2.1 Cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol pathway (CDP-DAG). The de novo biosynthesis pathway of Cardiolipin begins with the formation of PA from G-3-P. PA then reacts with CTP to eventually produce CDP-diacylglycerol. Another G-3-P then interacts with CDP-diacylglycerol to produce phosphatidyl glycerol phosphate which is then hydrolysed to yield a Phosphatidyl Glycerol. From this step, the formation of de novo Cardiolipin is catalyzed by an enzyme called cardiolipin synthase. The newly formed cardiolipin is quickly remodeled with specific acyl groups with the help of remodeling enzymes (includes Tafazzin, MLCL AT-1, and/or ALCAT-1). G-3-P, glycerol-3-phosphate; G-3-P AT, glycerol-3-phosphate acyl transferase; AGP-AT, 1-Acylglycerol-3-phosphate acyl transferase; *CTP* cytidine triphosphate, *PA* Phosphatidic acid, *PPi* pyrophosphate, *CDP* cytidine diphosphate, *PGP* phosphatidyl glycerol phosphate, *CMP* cytidine monophosphate, *MLCL AT-1* monolysocardiolipin acyl transferase 1, *ALCAT-1* Acyl-CoA:Lysocardiolipin acyltransferase-1

α (PPARα) in murine heart stimulated CL biosynthesis via an increase in mRNA expression of the CDS-2 isoform of CDS and such an activation was not observed in clofibrate-treated PPARα knockout mice [38]. In the second step of the pathway, CDP-DG condenses with *sn*-glycerol-3-phosphate to form phosphatidylglycerol (PG) catalyzed by phosphatidylglycerolphosphate (PGP) synthase (PGPS) and PGP phosphatase. The G protein RhoGap plays a key role in controlling PGPS activation and CL synthesis at the transcriptional level [39]. In addition, it is well documented that expression of mitochondrial fusion proteins is altered in heart failure (HF) and expression of the mitochondrial fusion protein, mitofusion-2, may be involved in the regulation of CL de novo biosynthesis through PGPS [40, 41]. In the third step of the pathway PGP is rapidly dephosphorylated by PGP phosphatase [3]. PGP phosphatase was recently identified in yeast and in mammalian cells PGP phosphatase is known as protein tyrosine phosphate localized to mitochondrion-1 (PTPMT-1) and is a member of the protein tyrosine phosphatase superfamily [20, 42]. Fibroblasts from Ptpmt1-deficient mice accumulate PGP and exhibit a decrease in phosphatidylglycerol (PG) and CL [43]. In the last step of the pathway, PG is converted to CL

in the heart by condensation with CDP-DG catalyzed by CL synthase (CLS) [35, 44]. CLS is localized exclusively to the inner mitochondrial membrane [44, 45] and was purified to homogeneity from rat liver [46]. The genes encoding human (hCLS1) and murine CLS (mCLS1) have been identified and the enzyme is highly expressed in heart [47–49]. Loss of CLS mRNA in tissues of lipopolysaccharide-treated mice did not result in loss in CLS activity indicating that the rate of CLS enzyme turnover may be slow in mammalian cells [50].

Subsequent to its biosynthesis, CL is rapidly remodeled to yield molecular species of CL found in the mitochondrial membrane [24]. In mammalian heart, linoleic acid (18:2) comprises 80–90 % of the acyl chains in CL [51]. The major tetra-acyl molecular species in human heart (approximately 80 % of total) are (18:2-18:2)-(18:2-18:2)-CL or L_4-CL. Remodeling may occur through the concerted deacylation followed by reacylation (resynthesis) [52]. CL may be hydrolyzed by many different phospholipases A_2 [53] including calcium-independent PLA_2 ($iPLA_2$-VIA) [54, 55], secretory PLA_2 [56], and cytosolic PLA_2 [57]. Resynthesis of cardiac CL from MLCL and linoleate is required to achieve the enrichment in 18:2. CL resynthesis from MLCL occurs via at least three enzymes. A mitochondrial-associated membrane acyllysocardiolipin acyltransferase-1 (ALCAT-1) with specificity for multiple anionic lysophospholipid substrates has been identified [58, 59]. Upregulation of ALCAT-1 by oxidative stress or diet-induced obesity in mice resulted in mitochondrial dysfunction, reactive oxygen species production, and insulin resistance [60]. ALCAT-1 null mice have resistance to diet-induced obesity indicating that this enzyme may be a stress-response enzyme. A decrease in ALCAT-1 mRNA expression was associated with a decrease in CL in AMP-activated protein kinase null mice [37]. However, no alterations in ALCAT-1 mRNA expression were observed in heart explants from humans or spontaneous hypertensive heart failure prone (SHHF) rats in heart failure (HF) in which CL was decreased [51, 61]. A mitochondrial deacylation–reacylation cycle was identified in which newly synthesized CL was rapidly deacylated to MLCL and then reacylated back to CL with linoleoyl-CoA [62]. The mitochondrial activity was characterized and the enzyme purified from pig liver [63, 64] and was shown to be a previously unidentified human protein [65]. An in vitro CL transacylase activity that remodels CL was reported in crude mitochondrial fractions from rat liver [66]. This CL transacylase is the BTHS gene product TAZ described above in Sect. 2 [66, 67]. A novel mitochondrial protein, Them5, which exhibits thioesterase activity with long-chain acyl-CoAs and a strong substrate preference for C18 polyunsaturated fatty acids was recently identified [68]. *Them5*$^{-/-}$mice exhibit an increase in MLCL implicating thioesterase activity in the regulation of CL remodeling.

Although evidence indicates that the BTHS gene product *TAZ* clearly and specifically remodels mitochondrial CL with linoleic acid, the idea that *TAZ* alone determines the fatty acid profile of CL contradicts experimental evidence. For example, in hearts of AMP-activated protein kinase null mice, cytidine-diphosphate diacyl-*sn*-glycerol synthetase-2, a rate-limiting enzyme of de novo CL biosynthesis, and ALCAT-1 mRNA expression were reduced compared to controls and this accompanied reduced levels of CL and linoleic acid in phospholipids within cardiac

mitochondria [37]. *Taz* mRNA expression was unaltered in the hearts of these mice. Moreover, the presence of at least two patients with BTHS and an exon 5 mutation in *TAZ* but with normal CL levels highlight the fact that *TAZ* alone may not be responsible for all mitochondrial CL remodeling (Michael Schlame, personal communication). These data suggest that in addition to TAZ, other enzymes may play a key role in mammalian and human mitochondrial CL remodeling [69]. In Epstein–Barr virus-transformed human BTHS lymphoblasts, a 60–80 % reduction in CL levels were observed and transfection of these cells with the CL remodeling enzyme monolysocardiolipin acyltransferase-1 (MLCL AT-1) or the alpha subunit of trifunctional protein restored CL levels to that of control lymphoblasts [65, 70, 71].

2.4 Role of Phospholipases in the Regulation of Cardiolipin Metabolism

The observation that elevated CL remodeling occurs as a compensatory mechanism for increased hydrolysis of CL mediated by phospholipase activation is supported by several studies. Elevated PLA_2 activity has been seen in various models of stimuli-induced apoptosis. Addition of the proapoptotic factor TNF-α to H9c2 cardiac myoblast cells stimulated mitochondrial PLA_2 activity towards mitochondrial phospholipids [72]. In addition, MLCL accumulates during Fas-mediated apoptosis as a by-product of CL degradation by mitochondrial PLA_2 [73]. Furthermore, MLCL generated by PLA_2 hydrolysis of CL during induction of apoptosis was shown to enhance t-Bid binding to membranes [73–75]. 2-Deoxyglucose (2-DG) has been shown to induce apoptosis by stimulating intracellular reactive oxygen species production, CL oxidation, and the release of cytochrome *c* from mitochondria in several cell lines. The effect of apoptosis mediated by metabolic hypoxia on phospholipase A_2 activity and CL metabolism was examined in the surviving population of H9c2 cells exposed to 2-DG [76]. Treatment of these cells with 100 mmol/L 2-DG for 16 h stimulated caspase-3 and PARP cleavage, indicating that apoptosis occurred in this cell population. Mitochondrial PLA_2 activity towards mitochondrial phospholipids was elevated indicating the potential for enhanced CL hydrolysis in these cells. However, the pool size of CL and incorporation of [1-^{14}C]linoleic acid as a precursor into CL was unaltered due to an increase in expression and activity of mitochondrial MLCL AT activity. These results indicated that there was an elevation in the resynthesis of CL from MLCL in the surviving population of H9c2 cells treated with 2-DG likely as a compensatory mechanism for elevated mitochondrial PLA_2 activity. Interestingly, the activity of ALCAT-1, the mitochondrial-associated membrane protein capable of resynthesizing CL from MLCL and unsaturated fatty acid, was reduced in 2-DG-treated cells supporting the observation that ALCAT-1 and MLCL AT-1 are reciprocally regulated [60]. If the accumulation of MLCL indeed plays a role in mitochondria-mediated apoptosis, it is possible that rapid CL resynthesis from MLCL is required in response to proapoptotic stimuli-mediated CL degradation to restore cellular homoeostasis and thus prevent the

apoptotic cascade. The expression of group VIA calcium-independent PLA_2 has been shown to play a role in the protection of mitochondrial function from damage caused by mitochondria-generated reactive oxygen species during apoptotic induction by staurosporine [55]. 2-DG addition to cells was shown to result in the generation of reactive oxygen species [77, 78]. Since cell viability of the 45 % surviving population of H9c2 cells exposed to 2-DG was greater than 95 %, as assessed by Trypan blue exclusion, it is possible that increased mitochondrial MLCL AT activity and its expression, and hence, elevated CL resynthesis, may work in concert with elevation in mitochondrial PLA_2 activity to be a protective mechanism against MLCL-mediated apoptosis [76].

Mitochondrial PLA_2 activity towards CL may also be regulated by an intracellular ceramide-regulated process not directly related to cell killing [39]. Mitochondrial PLA_2 activity was examined in a novel Chinese hamster ovary (CHO) cell line resistant to ceramide-induced apoptosis. A promoter trap mutagenesis approach was used to isolate this etoposide-resistant CHO cell line. The resistant cell line, named E91, showed cross-resistance to *N*-acetylsphingosine. The promoter trap retrovirus was found integrated into intron 1-2 of the Dlc-2 (Stard13) RhoGap gene. The E91 cells showed elevated guanosine triphosphate (GTP)-bound RhoA levels compared to parental cells, suggesting that the retrovirus integration had inactivated one of the Dlc-2 RhoGap alleles. The parental cells showed elevated PLA_2 activity after treatment with *N*-acetylsphingosine. Intracellular ceramide-signaling was defective in the E91 cells due to increased levels of active GTP-bound RhoA. This study was the first report for the regulation of a mammalian PLA_2 through RhoGap expression [39].

Chlamydia trachomatis is a prevalent sexually transmitted bacterial disease and is the leading cause of infectious blindness in developing nations [79]. *C. trachomatis* is an intracellular parasite and obtains its phospholipids from the host cell. However, no PLA_2 homologues have been identified in chlamydial genomes. It was previously demonstrated that endogenous host cell-derived phospholipids are trafficked to *C. trachomatis* and that the phospholipid composition of *C. trachomatis* mimics that of the eukaryotic host cell in which it was grown [18, 80–82]. In these studies, *C. trachomatis* infection of each mammalian cell type investigated resulted in an increase in host cell PLA_2 activity resulting in hydrolysis of host cell phospholipids, including CL, to their respective lysophospholipid. This was followed by trafficking of the lysophospholipid to the intracellular chlamydial inclusion where it was rapidly remodeled with a bacterial-specific branched chain fatty acid to form the chlamydial-specific parent phospholipid. Activation of the host Raf-MEK-ERK-cPLA2 signaling cascade was required for this chlamydial uptake of host glycerophospholipids [57]. Both the MAP kinase pathway (Ras/Raf/MEK/ERK) and calcium-dependent cytosolic PLA_2 ($cPLA_2$) are activated in chlamydia-infected cells. Inhibition of $cPLA_2$ activity blocked chlamydial uptake of host glycerophospholipids and resulted in impairment in chlamydial growth. In addition, attenuation of either c-Raf-1 or MEK1/2 activity prevented the chlamydial activation of ERK1/2, leading to the suppression of both chlamydial activation of the host $cPLA_2$ and the uptake of glycerophospholipids from the host cells.

The role of PPARα-stimulated PLA_2 in cardiac mitochondrial CL biosynthesis was examined in both in vivo and in vitro models [38]. Treatment of rat heart H9c2 cells with clofibrate increased the expression and activity of 14 kDa mitochondrial PLA_2, but did not affect the pool size of CL. Clofibrate treatment stimulated de novo CL biosynthesis via an increase in PGPS activity, accounting for the unaltered CL content. Cardiac PLA_2, PGPS, and CDS-2 activities and CDS-2 mRNA levels were elevated in mice-fed clofibrate for 14 days compared with controls. In PPARα-null mice, clofibrate feeding did not alter cardiac PLA_2, PGPS activities, or CDS-2 activity and mRNA level, confirming that these enzymes are regulated by PPARα activation. This study was the first to demonstrate that CL de novo biosynthesis is regulated by PPARα activation through PLA_2 activation.

Eukaryotic cell reproduction involves duplication of cellular components, including biological membranes and DNA content, resulting in a doubling in size and then division into two components. In the absence of growth factors (e.g. serum starvation) cells will not divide, but enter into a quiescent state known as G_0. Cells depleted of serum in G_0 may be triggered to enter into the S-phase by the addition of serum. Since CL plays an important role in generation of ATP required for the human cell cycle, the role of PLA_2 in CL metabolism was investigated in quiescent HeLa cells induced to enter into the S-phase of the cell cycle [70]. Hela cells were serum starved for 24 h, then incubated for up to 24 h in the absence or presence of serum. CL mass was doubled by 16 h of incubation and this was accompanied by dramatic increases in the expression and activities of the CL de novo biosynthetic enzymes. In addition, an increase in mitochondrial PLA_2, MLCL AT-1, and ALCAT-1 activities were observed. It was suggested that the elevated activities of the CL remodeling enzymes PLA_2, MLCL AT-1, and ALCAT-1 were required to support remodeling of the increased newly synthesized CL required during S-phase of the human cell cycle.

In models of cerebral stroke, the activity, mRNA expression, and immunoreactivity of $cPLA_2$ and the activity and mRNA expression of secretory PLA_2 ($sPLA_2$) were shown to be elevated and may be involved in CL degradation leading to mitochondrial dysfunction and subsequent reactive oxygen species generation [56]. MLCL was shown to be generated through cleavage of mycobacterial CL by a lysosomal type calcium-independent PLA_2 present in macrophage lysosomes [83]. Finally, group VIA calcium-independent PLA_2 beta ($iPLA_2\beta$) localizes in and protects beta-cell mitochondria from oxidative damage during staurosporine-induced apoptosis [84]. In that study, islets isolated from $iPLA_2\beta$ null mice are more sensitive to staurosporine-induced apoptosis than those from wild-type littermates and that 2 weeks of daily intraperitoneal administration of staurosporine to $iPLA_2\beta$ null mice impairs both the animals' glucose tolerance and glucose-stimulated insulin secretion by their pancreatic islets. $iPLA_2\beta$ was expressed only at low levels in islet beta-cells from obesity- and diabetes-prone *db*/*db* mice. Hence, the low $iPLA_2\beta$ expression level observed in *db*/*db* mouse beta-cells may render them vulnerable to injury by reactive oxygen species.

2.5 Role of Calcium-Independent PLA_2 in CL Metabolism in Mammalian Models

Alterations in calcium-independent PLA_2 ($iPLA_2$) have been shown to contribute to diminished cardiac function in failing hearts due to myocardial infarction [85]. In cardiac myocytes prepared from normal rats and rat with SHHF, it was demonstrated that CL remodeling was performed singly with respect to each fatty acyl moiety, was attenuated in heart failure (HF) relative to non-HF, and was partially sensitive to $iPLA_2$ inhibition suggesting that CL remodeling occurs in a step-wise manner, that compromised 18:2 incorporation contributes to a reduction in L_4-CL in the failing rat heart, and that mitochondrial $iPLA_2$ plays a role in the remodeling of CL acyl composition in the heart [86]. Genetic ablation of $iPLA_2$ gamma ($iPLA_2\gamma$) in mice resulted in decreased L_4-CL and abnormal mitochondrial function and a deficient mitochondrial bioenergetic phenotype including a mitochondrial neurodegenerative disorder characterized by degenerating mitochondria, autophagy, and cognitive dysfunction in mice [54, 87]. In $iPLA_2$ null mice impairment of $iPLA_2\gamma$ caused mitochondrial dysfunction and increased oxidative stress, leading to the loss of skeletal muscle structure and function [88]. These authors found that the composition of CL and other phospholipid classes were altered and that the levels of myoprotective prostanoids were reduced in skeletal muscle of $iPLA_2\gamma$ null mice. Thus, in addition to maintenance of homeostasis of the CL within the mitochondrial membrane, $iPLA_2\gamma$ may contribute to modulation of lipid mediator production in vivo.

Mice null for $iPLA_2\gamma$ are also completely resistant to high fat diet-induced weight gain, adipocyte hypertrophy, hyperinsulinemia, and insulin resistance, which occur in wild-type mice after high fat feeding [89]. Notably, $iPLA_2\gamma$ null mice were lean, demonstrated abdominal lipodystrophy, and remained insulin-sensitive despite having a marked impairment in glucose-stimulated insulin secretion after high fat feeding. Respirometry of skeletal muscle mitochondria from $iPLA_2\gamma$ null mice demonstrated marked decreases in state 3 respiration using multiple substrates whose metabolism was uncoupled from ATP production. Shotgun lipidomics of skeletal muscle revealed a decreased content of CL with an altered molecular species composition, thereby identifying the mechanism underlying mitochondrial uncoupling in the $iPLA_2\gamma$ null mice. Collectively, these results identify $iPLA_2\gamma$ as an obligatory upstream enzyme that is necessary for efficient electron transport chain coupling and energy production through its participation in the alterations of cellular bioenergetics that promote the development of the metabolic syndrome.

Reductions in L_4-CL and alterations in CL biosynthetic and remodeling processes have been observed in left ventricular (LV) hypertrophy and subsequent HF in SHHF rats and in LV human heart explants isolated from HF patients [61]. PPHN results in right ventricular (RV) hypertrophy followed by right heart failure and an associated mitochondrial dysfunction [90, 91]. $iPLA_2\gamma$ mRNA expression was decreased in the LV and RV of PPHN piglets compared with control animals [23].

In addition, a decrease in [1-^{14}C]linoleoyl-CoA incorporated into MLCL in the LV and RV of PPHN piglets was observed indicating that iPLA$_2$γ may be reduced in PPHN. This was confirmed by the decreased mRNA expression of iPLA$_2$γ observed in the LV and RV of these PPHN animals. The above data clearly support iPLA$_2$γ as the enzyme in the remodeling and the metabolism of CL.

2.6 Role of Exogenous Phospholipase-Treatment of Cells on CL Metabolism

Controlled and limited treatment of H9c2 cardiac myoblast cells with *Naja mocambique mocambique* PLA$_2$ reduced the pool sizes of PC and PE and resulted in elevation of LPC and LPE, whereas the pool size of CL and other phospholipids were unaltered [92]. Pulse radiolabeling and pulse-chase radiolabeling experiments with [1,3-^{3}H]glycerol in cells incubated or preincubated in the absence or presence of PLA$_2$ resulted in reduced radioactivity incorporated into CL indicating attenuated de novo biosynthesis of CL. The mechanism for the reduction in CL appeared to be a decrease in the activity of phosphatidic acid:cytidine-5′-triphosphate cytidylyltransferase, a rate-limiting enzyme of de novo CL biosynthesis in H9c2 cells, mediated by elevated cellular LPC levels. The results indicated that de novo CL biosynthesis in H9c2 cells may be regulated by the cellular level of the PLA$_2$ product LPC.

Treatment of H9c2 cardiac myoblast cells with PC-specific *Clostridium welchii* phospholipase C (PLC) was shown to reduce the cellular pool size of PC without altering cellular CL levels [93]. Pulse radiolabeling and pulse-chase radiolabeling experiments with [1,3-^{3}H]glycerol demonstrated that radioactivity incorporated into CL was reduced in PLC-treated cells with time compared with controls indicating attenuated de novo biosynthesis of CL. Addition of 1,2-dioctanoyl-*sn*-glycerol, a cell permeable 1,2-diacyl-*sn*-glycerol analog, to cells mimicked the inhibitory effect of PLC on CL biosynthesis indicating the involvement of 1,2-diacyl-*sn*-glycerol. The mechanism for the reduction in CL biosynthesis in PLC-treated cells appeared to be a decrease in the activities of phosphatidic acid:cytidine-5′-triphosphate cytidylyltransferase and PGPS, mediated by elevated 1,2-diacyl-*sn*-glycerol levels. These data indicated that de novo CL synthesis may be regulated by 1,2-diacyl-*sn-glycerol* and may be coordinated with PC biosynthesis in H9c2 cardiac myoblast cells.

2.7 Conclusions

It is clear that CL may be hydrolyzed by several different classes of PLA$_2$ including iPLA$_2$, sPLA$_2$, and cPLA$_2$. Important questions remain to be addressed including whether there is indeed a coordination between CL de novo synthesis and the remodeling of CL mediated by the hydrolysis of these PLA$_2$'s and subsequent resynthesis in vivo and whether the by-products of CL degradation themselves play

a role in cellular metabolism. The generation of the TAZ knock down mouse is likely to provide more concrete evidence surrounding the role that the iPLA$_2\gamma$ plays in the regulation of CL metabolism in mammalian tissues.

Acknowledgements This work was supported by HSFC and CIHR operating grants (Grant M. Hatch) and the DREAM Theme (Vernon W. Dolinsky and Grant M. Hatch). Edgard M. Mejia is the recipient of a University of Manitoba Tricouncil GETS graduate studentship. Grant M. Hatch is a Canada Research Chair in Molecular Cardiolipin Metabolism.

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Chapter 3
Role of Phospholipases and Oxidized Phospholipids in Inflammation

Devin Hasanally, Rakesh Chaudhary, and Amir Ravandi

Abstract Long thought of as a bystander in pathophysiological processes, lipid molecules have emerged as bioactive mediators of cellular activity. Oxidized phospholipids (OxPLs), generated during enzymatic and non-enzymatic processes, modulate cellular processes through receptor-mediated pathways that can effect a whole host of activities including apoptosis, monocyte adhesion, platelet aggregation, and regulation of immune responses. Initially discovered as platelet activating factor analogs, there have been close to 50 distinct OxPL molecules that have been identified within biological tissues. With the advent of robust analytical systems, we are better able to identify and quantitate these molecules in an ever growing list of different biological tissues which has allowed for the generation of a comprehensive oxolipid profiles in both normal and disease states. Given the increased affinity of phospholipases towards OxPLs we are in the early stages of understanding of the complex interplay between the modification of OxPL through phospholipase activity and the cellular responses to the released hydrolyzed products. In this review we will summarize the role of OxPL in different pathological states and the specific phospholipases that have been shown to interact with OxPLs.

Keywords Oxidized phospholipids • Phospholipases • Oxidative stress • Mass spectrometry • Lipidomics

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_3,

3.1 Introduction

For many years phospholipids (PLs) were considered to be only cellular building blocks with very little biological activity. Due to their susceptibility to oxidation, they are modified in the presence of reactive oxygen species (ROS). Apart from impairment of their structural function, oxidation makes oxidized phospholipids (OxPLs) acquire novel biological activities not characteristic of their unoxidized precursors (Fig. 3.1). The effects of OxPLs described in vitro and in vivo suggest their potential relevance in different pathologies including atherosclerosis, acute inflammation, lung injury, and many other disease conditions [1, 2]. The actions of OxPL can vary depending upon the specific species of phospholipid being oxidized. Recently, oxidized phosphatidylcholines (OxPC) have been recognized as not only products of oxidative damage but also mediators of its progression. These compounds exert their biological activity through multiple pathways. They have been shown to be potent stimulators of platelet-activating factor (PAF) receptor, prostaglandin receptors, and PPARγ receptors resulting in platelet aggregation, induction of the coagulation cascade, and apoptosis and cell death [3, 4]. Recent advancements in softer methods of ionization, such as electrospray mass spectrometry, have allowed us to identify and quantitate OxPLs in biological tissues. With the better understanding of the OxPL structure, we are also identifying the specific role phospholipases play in modulating the effects of OxPL on cellular signaling. As we move forward in trying to better understand the role of OxPL in pathology, it necessitates a detailed understanding of the oxidized lipidome and the specific phospholipase that act as a defensive mechanism to protect the cell from their deleterious effects.

3.2 Generation of Oxidized Phospholipids

Phospholipids represent the major component of lipid bilayers due to their amphipathic structure. Polar head groups interact with the aqueous environment and cytoplasm, and the fatty acid chains sequester to form the lipid core of the membrane acting as a semi-permeable barrier. During disease processes, not only does the structural integrity of the phospholipid bilayer become compromised but also chemical modification of the phospholipids through enzymatic and non-enzymatic pathways alters their function. One such disease process is the inflammatory cascade that is a unifying mechanism in many pathological processes. A hallmark of inflammation is the increased generation of ROS which can occur in multiple pathways [5–7] and results in the generation of superoxide radicals, $OONO^{\bullet}$ and $O^{\bullet}$. This process is well described, for example, during ischemia-reperfusion injury within cardiomyocytes. The rapid correction of acidosis through the Na^{+}/H^{+} exchanger, the Na^{+}/HCO^{3-} cotransporter [8], and the washout of lactate causes secondary activation of the Na^{+}/Ca^{2+} exchanger in the reverse direction aggravating the cytosolic Ca^{2+} balance [9]. Abrupt re-exposure to oxygen of the ischemia-inhibited respiratory chain generates a mitochondrial membrane potential to drive ATP synthesis, which leads to a rapid cytosolic Ca^{2+} overload and consequently a Ca^{2+} accumulation in the matrix [10].

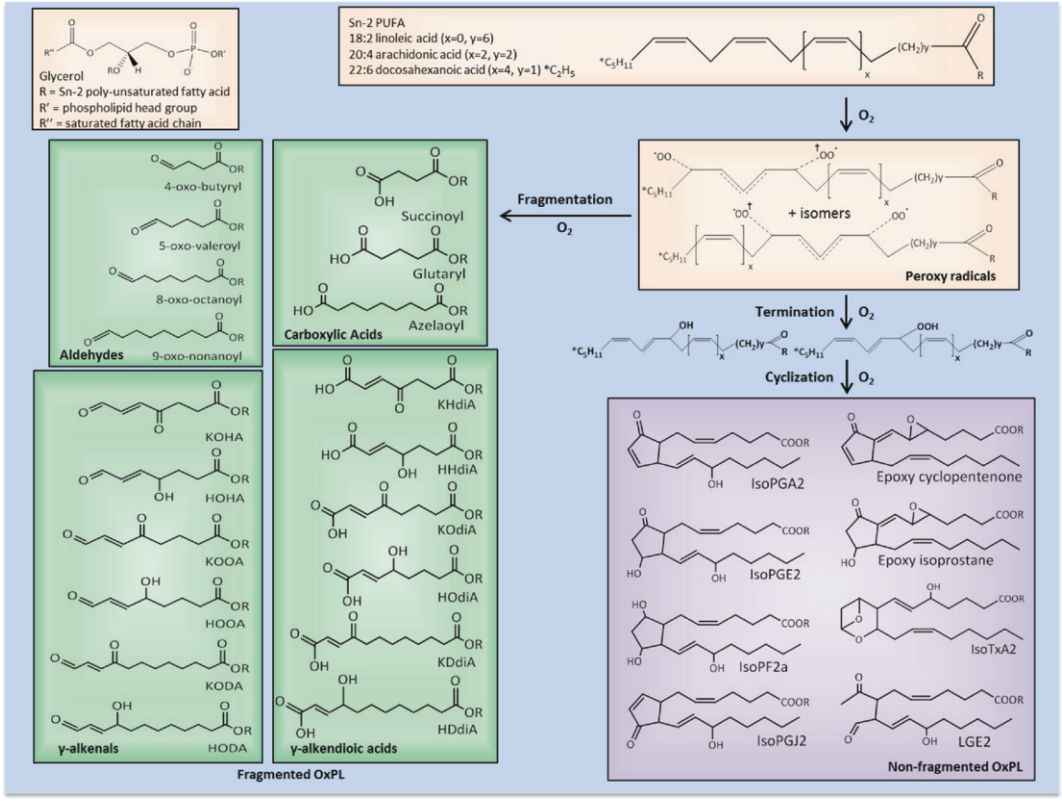

Fig. 3.1 Peroxidation of unsaturated phospholipids generates multiple esterified oxidation products. Reactive oxygen species (ROS) generation forms peroxy radicals which may cause the unsaturated fatty acid to undergo further fragmentation or cyclization. † Denotes more favored oxidation location. *OxPL* (oxidized phospholipids), *PUFA* (polyunsaturated fatty acid)

Moreover, reactivation of the energy metabolism induces a large production of ROS. This localized oxidative burst and regional inflammatory response results in non-enzymatic oxidation of cellular proteins, DNA, and lipids resulting in generation of molecules that have a powerful biological activity [11, 12]. Dysfunctional and dying cells themselves can generate large amounts of mitochondrially derived ROS [13]. The targets of ROS include critical proteins and enzymes, lipids, nucleic acids, and nitric oxide (NO), among others. Certain cells, like active neutrophils, can release large amounts of enzymatically produced superoxide anions and hypochlorous acid from their nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase systems [14, 15]. Many PLs contain poly-unsaturated fatty acid chains which make them susceptible to oxidative modification. The location and number of double bonds in addition to the formation of stabilized intermediates by hydrogen transfer to neighboring carbon molecules determine the final structure. The initial oxidation of a conjugated diene allows for the cleavage of carbon–carbon bonds after hydrogen removal that produces shorter chain, lower mass, fragmented species [16]. If the conjugated diene becomes stabilized and remains intact, further oxidation yields longer chain, higher mass, non-fragmented oxidized species. The ROS-based oxidation of PL forms a heterogeneous pool of OxPL in which the oxidized fatty acid remains esterified to the glycerol backbone (Fig. 3.1) [17]. The OxPLs can be broadly categorized into two groups: the fragmented OxPLs and the non-fragmented OxPLs. Fragmented OxPLs generally comprise of terminal aldehyde or carboxylic acid species. Non-fragmented species have hydroxide and/or peroxide additions and rearrangement by cyclization generate other end-products like the eicosanoids.

OxPLs represent a heterogeneous group of oxidized lipids with multiple functional groups present at the *sn*-2 position. The generation of specific OxPLs and their physiological effects are tissue specific. For instance, in the setting of rat lung oxidative injury, the most abundant OxPC is an isoprostane containing PC [18] whereas in human atherosclerotic tissue, the fragmented OxPC molecule, POVPC (1-palmitoyl-2-5′-oxo-valeroyl-*sn*-glycero-3-phosphocholine) is the most abundant [19]. Not only is the structure of OxPC tissue specific but its biological roles are also cell and tissue specific. For example, POVPC acts as an anti-inflammatory molecule by inhibiting LPS-induced intracellular signaling and the expression of adhesion molecules in human umbilical vein endothelial cells (HUVECs) [20], while in mouse lung macrophages POVPC induce IL-6 production resulting in a pro-inflammatory effect [21]. OxPL have been shown to play a role in multiple disease processes where oxidative stress and inflammation are known mechanisms. These include atherosclerosis [17], diabetes [22], malignancy [23], chronic heart failure [24], cystic fibrosis, [25] and neurodegenerative diseases [26] like Parkinson's disease.

3.3 Detection of Oxidized Phospholipids

Over the last 20 years there has been a revolution in the understanding of lipids and their biological activity [27]. This has been driven by the advent of new mass spectrometric tools that allow us to identify and quantitate complex lipid mixtures [28].

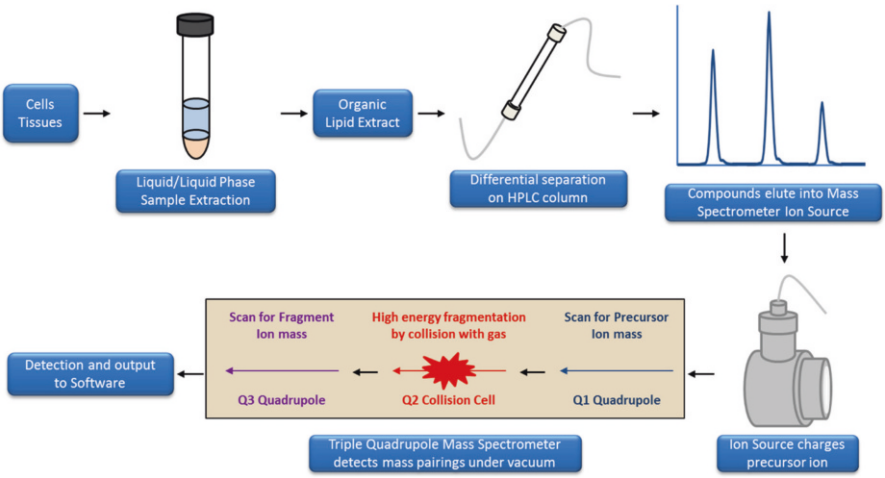

Fig. 3.2 Phospholipid extraction workflow. Procedure from sample to data output established for phospholipid extraction, separation, and detection with a HPLC column linked to an electrospray ionization triple quadrupole tandem mass spectrometer [30]. *HPLC* (high performance liquid chromatography)

With the softer methods of ionization, we can identify phospholipid molecules as whole structures and this allows us to follow their chemical modifications through pathological processes.

Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry allow us to ionize PL molecules without causing fragmentation permitting for identification of whole molecules within heterogeneous samples [27]. Mass spectrometry is being used to determine comprehensive lipid profiles in cells, tissues, and pathological samples. These lipidomic analyses usually follow the same workflow and employ extraction, separation, and detection methodology to establish the lipid profile (Fig. 3.2). There has been great progress in applying this methodology to understand the oxidative changes that occur within the phospholipidome [29, 30]; not only of OxPC which are the most abundant but also other OxPL species generated from phosphatidylserine (PS), phosphatidyl ethanolamine (PE), cardiolipin (CL), and phosphatidylinositol (PI). With these novel techniques, both with a targeted approach or a wide spectrum approach, such as a gunshot lipidomic analysis, we can follow the changes that occur within a specific phospholipid class during disease processes [27]. Given that PC represents the largest phospholipid group in mammalian cells, the majority of our understanding of oxidative modification comes from studies on OxPC molecules.

Through a joint research study conducted by the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Standards, and the LIPID MAPS Consortium, a comprehensive profile of the human plasma lipidome encompassing all of the major lipid classes has been reported [31]. The study was able to identify over 500 individual lipid species from a pooled reference plasma sample. Recent follow-up studies correlated sex, smoking status, body mass index

(BMI), and age with changes in the lipid classes in plasma, with BMI and age showing significant changes in PL amounts [32]. Even though phospholipid represented 43 % of the plasma lipidome by mass, the report did not address the identity of OxPL within plasma. Lipidomics has also made strides in identifying phospholipids from cell-specific samples such as macrophages, which have been shown to play a major role in the inflammatory cascade. Macrophage activation by Toll-like receptor 4 (TLR-4) agonists led to changes within the lipid profiles identified by mass spectrometry at the cellular and subcellular levels [33, 34]. Therefore, PLs are important for a rapid inflammatory response before and after activation of macrophages.

There are fewer studies that have looked at the OxPL profile within tissues since they represent only 1 % of the total phospholipid pool. The majority of the studies investigating the role of OxPL have been related to vascular pathology and atherosclerosis in particular since there is a larger body of research correlating oxidized LDL (OxLDL) with initiation and progression of atherosclerotic plaques. Lipidomic profile of atherosclerotic plaques at different stages of development has shown the presence of both fragmented and non-fragmented OxPCs within carotid endarterectomy plaque material [19]. The PCs represented the largest class of phospholipids within plaques with PC aldehydes, being the largest OxPC fraction. Both fragmented and non-fragmented OxPCs were present through all stages of plaque progression which indicated continual generation and catabolism of these bioactive molecules within atherosclerotic plaques.

In other inflammatory states, OxPCs have been shown to play a role in mediating pathological response. Recently in the setting of myocardial ischemia and reperfusion an oxolipidomics analysis of myocardial tissue demonstrated a significant increase in OxPC species within the myocardial tissue during ex vivo model of ischemia and reperfusion [35]. In this experimental model, there was a correlation between ventricular function and OxPL levels in response to ischemia and reperfusion.

3.4 Biological Activity of Oxidized Phospholipids

Due to their fatty acid's susceptibility to oxidation, phospholipids can be modified in the presence of ROS. Once PL molecules are oxidized, they generate a multitude of different oxidation products that remain esterified to the parent PL molecule. OxPLs gain bioactive properties that were not attributed to their precursors as a result of oxidation. OxPLs are able to induce cell-signaling pathways and cause an active cell response. Studies of human aortic endothelial cells (HAECs) indicate that just a brief exposure to a small number of OxPCs that are generated in vivo will affect the transcription of >1,000 genes involved in inflammation, pro-coagulant activity, redox reaction, sterol metabolism, cell cycle, unfolded protein response, and angiogenesis [36]. Likewise phenotypic changes of cells are also observed and were demonstrated within macrophage populations within atherosclerotic plaques [37]. One of the first defined OxPCs were the fragmented PAF-like lipids that through a

G-protein mediated pathway resulted in cellular activation [38]. Since the initial discovery of OxPC molecules, there have been other classes of phospholipids that have been shown to undergo oxidative modification in parallel with choline phospholipids, forming homologous products. Phosphatidylserine (PS) oxidation, in particular, has a distinct and key role in mitochondrial dysfunction, apoptosis, and recognition of apoptotic cells [39]. Ethanolamine phospholipids are oxidized during platelet activation and are the sites of prostanoid formation [40].

3.5 Oxidized Phospholipid Receptors

Due to increased polarity, OxPLs interact with membrane proteins resulting in binding to a wide variety of inflammatory receptors [41–43]. OxPLs were shown or hypothesized to stimulate several types of signal-transducing receptors located on the cell surface or in the nucleus, including G protein-coupled receptors, receptor tyrosine kinases, Toll-like receptors, receptors coupled to endocytosis, and nuclear ligand-activated transcription factors such as PPARs. The specificity of OxPL receptor binding is likely a result of the chemical similarity of the OxPL to the receptor ligand. OxPCs containing esterified isoprostaglandins (PEIPC) activate receptors recognizing prostaglandins E2 and D2 by EP2 and DP receptors, respectively [20]. The EP2 receptor is expressed in all cell types relevant to atherosclerosis including endothelial cells (ECs), monocytes, macrophages, and vascular smooth muscle cells (VSMCs). Activation of EP2 receptor on ECs results in activation of β1 integrin and increased binding of monocytes to ECs similar to that induced by OxPC, while EP2-receptor antagonists inhibit the action of OxPC.

Innate immune responses to OxPL are mediated by natural antibodies (N-Ab), C-reactive protein (CRP), and CD36 on macrophages [44]. PAF receptor and TLRs are well studied initiators of OxPL signaling and impact cascades like PI3K, Akt, JAK, ERK1/2, and MAPK signaling [44, 45]. Multiple other receptors exist to mediate cellular activity of OxPL including EP2, VEGFR2, and SR-B1 [46–48]. The N-Ab against OxPL are encoded in germ line tissue and are produced by B-cells as IgM immunoglobulins [49, 50]. They are able to bind antigens that represent pathogens and stress-induced self-antigens as part of the humoral arc of innate immunity [51, 52]. N-Ab have shown affinity for OxPL in studies that used T15/E06 N-Ab to block the effects of OxPL on macrophage uptake of OxLDL [53, 54]. Complement response to OxPL is mediated by interaction with the defense molecule CRP. High levels of CRP are used to identify an active inflammatory response [45]. CRP has been shown to bind specifically OxPL within OxLDL [55]. The complex of CRP bound to OxLDL, by the cleaved product of OxPC, lysoPC, was shown to mediate the suppression of inflammation in macrophages via reduced activation of the inflammatory transcription factor NF-κB [56]. Macrophage activation is central to inflammation. OxPLs bind the macrophage by scavenger receptors specifically by CD36 which is the primary scavenger receptor capable of binding OxLDL and has been shown to bind OxPL [57]. The binding of OxLDL

with CD36 is integral to the development of "foam cells" which are macrophages with large depositions of OxLDL including its lipid-rich core. These foam cells are believed to be the initial step in the generation of fatty streak resulting in atherosclerotic plaque formation [58].

OxPLs have also been shown to play a role in the thrombosis and the clotting cascade through two particular receptors, tissue factor pathway inhibitor (TFPI) and PAF receptor. The accumulation of the PAF-like (alkyl-acyl) OxPLs and lysophospholipids (alkyl-hydroxyl) in plaques leads to platelet aggregation [4, 59]. OxPL induces increased expression of P-selectin causing a change in the platelet shape which favors aggregation of platelets. In concert with ADP and other agonists of platelet accumulation, the diacyl-OxPLs appear to be active in inducing significant platelet aggregation, but by themselves are only weak inducers of clotting factors [60]. Other OxPLs are able to increase transcription of the "master-switch" of coagulation, the tissue factor protein, and block the inhibitor TFPI, causing clotting signaling to be activated [44].

3.6 Cell Signaling Cascades Influenced by Oxidized Phospholipids

Transmission of signaling cascades initiated by OxPL has widespread effects. Inflammation, cell cycle, and cell death pathways can be up-regulated or down-regulated when OxPLs bind to the cell [61]. There are multiple secondary messengers, like cAMP and Ca^{2+}, that are increased by OxPL. Transcription factors, like NF-κB and STAT3, and modifying enzymes, like kinases and phosphatases, are also activated by OxPL. Together these influence diverse tissue and cell-specific responses [44]. OxPLs have been shown to influence PI3K/Akt signaling to mediate inflammation by nitric oxide production by NADPH oxidases and endothelial nitric oxide synthase [62]. The study also demonstrated up-regulation of IL-8, a pro-inflammatory cytokine, was generated in endothelial cells by this process. The *Jun* N-terminal kinase pathway can be up-regulated by OxPC while there is a simultaneous down-regulation of phosphorylated-Akt signaling during oxidative stress within rat oligodendrocytes [63]. These pathways are influenced specifically by POVPC causing induction of neutral sphingomyelinases. The down-stream apoptotic signaling up-regulates caspase 3 and caspase 8 which are important for the completion of apoptosis. Inflammatory genes and the unfolded protein response are pathways in which transcriptional activation occurs in response to OxPL. Activating transcription factor-6 (ATF-6) and X-box binding protein-1 (XBP-1) are transcription factors activated by OxPL that target inflammation genes. ATF-6 induces XBP-1 mRNA and splicing is mediated by the ER membrane protein inositol requiring 1 (IRE1) allowing modulation in the nucleus [64]. Another mechanism described is the phosphorylation of eIF2α catalyzed by double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK) leading to the presence of ATF-4 acting as a transcription factor [36]. XBP-1 and ATF-4 bind to promoter regions upstream of the target

IL-6 and IL-8 inflammatory signals causing them to be up-regulated. TLR signaling modulates the inflammatory pathways relating to the innate immune system. OxPLs were able to initiate TLR-4 signaling through MAPK cascade to NF-κB and influence lipid metabolism and inflammation [65]. When TLR-4 is activated, Bcl-2 family proteins in the mitochondria, Bid, Bad, Bax, and the nuclear transcription factor NF-κB shut down oxidative phosphorylation within the mitochondria and act together to increase the expression of pro-inflammatory cytokines [66, 67]. This process induces the pathways of inflammation through a caspase 1-mediated mechanism to increase active IL-1β and IL-18 in the extracellular spaces [66]. This pro-inflammatory and pro-apoptotic environment catalyzed by OxPL catapults the cells into cell stress culminating in inflammation or apoptosis if not reversed. Cells exposed to modified and OxLDL demonstrate up-regulation of two adhesion molecules, β1-integrin [68] and P-selectin [69], that specifically promotes monocyte adhesion to these cells. Infiltration of macrophages past adjacent endothelial cells is also promoted during lung injury by disruption of adherens junctions. A short chain fragmented PC produced during oxidative stress, PGPC (1-palmitoyl-2-glutaroyl-s*n*-glycero-3-phosphocholine) was demonstrated to modulate the phosphorylation of VE-cadherin via activation of Src kinase that phosphorylated tyrosine residues important for adherens junctions stability [70].

Chemokines are important to modulate the inflammatory response and OxPLs are able to target several chemokines that modulate the immune system. The chemokines MCP-1, MCP-3, MCP-5, MIP-1α, MIP-1β, MIP-2β, IL-6, IL-8, and GROα [36, 44, 71, 72] are influenced upon exposure to OxPL. MCP and MIP proteins are able to attract and activate macrophages causing sustained IL-8 production causing positive feedback to the inflammatory response induced by OxPL. IL-6 is particularly important in the acute phase inflammation as *IL-6*$^{-/-}$ knock-out mice demonstrate an impaired immune response [73]. These pro-inflammatory signals cooperate to modulate other cell types in response to these stresses.

3.7 Apoptosis

Irreversible cell loss occurs through apoptosis signaling, a programmed sequence of cellular events that result in controlled cellular death [74]. What is central to the intrinsic cellular death pathway is the increase in the permeability of mitochondria, a result of apoptotic signals and caspase 3 activation [75]. Caspase 3 is a central apoptotic activator that allows for triggering the enzymatic cascade that leads to cell death [76]. Recently, it has been shown that PAzPC (1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine) has a receptor-independent cytotoxic effect on promyelocytic HL60 cells and HUVECs [77]. PAzPC-induced changes in cell morphology typical for apoptosis triggered phosphatidylserine exposure on the outer leaflet of the plasma membrane which then stimulated the release of mitochondrial cytochrome C, apoptosis-inducing factor and activated caspase 3 [67]. In a caspase 3-mediated pathway, truncated OxPC molecules such as POVPC can

produce VSMC apoptosis. In rat oligodendrocytes, POVPC was pro-apoptotic through activation of the caspase 3 pathway [63]. Macrophages, VMSCs, and dendritic cells are confirmed to demonstrate increased apoptotic signaling in the presence of OxPL [36, 63, 78, 79]. There are certain Bcl-2 family proteins like, Bax a mitochondrial pro-apoptotic protein, which are able to interact with OxPL potentially activating it at mitochondria exposed to oxidative stress [80]. This is clear activation of the intrinsic apoptotic pathway leading to mitochondrial dysfunction and caspase activation [67, 81].

3.8 Oxidized Phospholipids and Phospholipases

Phospholipases functionally impact the structures of OxPLs by hydrolyzing either the oxidized fatty acid or the functional head group of the phospholipids resulting in the generation of lysophospholipids, phosphatidic acids, and oxidized fatty acids (Fig. 3.3). There is growing evidence that phospholipases play a role in the mediation of OxPL activity.

Oxidatively truncated phospholipids, but not their biosynthetic phospholipid precursors, are substrates for a class of phospholipases A2, the group VII class of PAF acetylhydrolases. These enzymes not only selectively recognize the *sn*-2 acetyl residue of PAF, but also specifically hydrolyze the fatty acyl fragment that remains esterified in the *sn*-2 position of the phospholipid glycerol backbone after fragmentation of the oxidized fatty acyl residue [82]. These phospholipases are highly specific for OxPL recognition and cleavage. These enzymes are believed to be conserved over 100 Ma of evolution while maintaining their specialized function, which demonstrates the continuing importance of specifically removing phospholipid oxidation products within aerobic organisms.

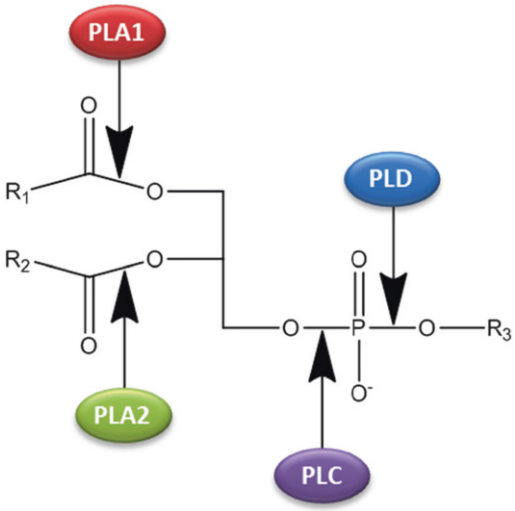

Fig. 3.3 Each phospholipase class cleaves at a particular site. Locations on phospholipid backbone associated with phospholipase cleavage by each class of phospholipase resulting in different products. *PLA1* phospholipase A1, *PLA2* phospholipase A2, *PLC* phospholipase C, *PLD* phospholipase D

As an example, lipoprotein-associated phospholipase A2 (Lp-PLA2), associated with low-density lipoprotein (LDL) was found to bind OxPCs that are recognized by E06 antibodies [83]. The phospholipid pools within LDL are also influenced by the presence of Lp-PLA2 as noted when the oxidation of LDL in the presence of an irreversible Lp-PLA2 inhibitor, SB222657, resulted in the accumulation of short chain OxPCs but reduction of lysoPC species. This identified short chain OxPC as the substrate for Lp-PLA2 and various saturated and mono-unsaturated lysoPC as the products [84]. Given that the oxidative modification of phospholipids occurs at the *sn*-2 position we will limit out discussion to PLA2 enzymes and their activity towards OxPL molecules.

3.9 Phospholipase A2 Affinity for OxPL

There are more than 20 different PLA2 enzymes. The three main groups are the calcium-dependent cytosolic, secretory PLA2, and calcium-independent PLA2. PLA2 enzymes bind phospholipids and their oxidized products to cleave at the *sn*-2 position releasing free oxidized fatty acids and lysoPL. LysoPLs are further broken down into lysophosphatidic acids which are themselves bioactive and exert their activity via the G-protein coupled receptors targeting adenylyl cyclase, ERK kinase, phospholipase C, phosphoinositol 3-kinase, and the Rho GTPase [85]. PLA2 has been proposed to serve as a secondary defense mechanism against the oxidative damage of phospholipids within membranes. However, extensive activation of this enzyme can also lead to membrane hydrolysis and loss of membrane integrity in the setting of membrane peroxidation. Increase in PLA2 activity following PL oxidation and disturbance in the lipid bilayer appears to support this hypothesis. This increase in activity has been seen for many PLA2 enzymes which appear to have substrate specificity towards OxPL molecules with an oxidized fatty acid at the *sn*-2 position [86]. Contributing to the increased levels of PLA2 activity at site of PL oxidation are the increases in intracellular Ca^{2+} levels that occur concurrently. Also the change in the physiochemical structure of the phospholipid bilayer results in exposure of the oxidized fatty acid to PLA2. This specificity was originally shown in vesicles containing oxidized soy bean PC which results in increased PLA2 activity when compared to the vesicles containing non-oxidized PC molecules. This increased hydrolysis occurred at calcium concentrations of 10 μM and below, indicating that at physiological Ca^{2+} concentrations there is an increased specificity towards OxPC molecules by PLA2.

This increased PLA2 activity has also been shown within atherosclerotic tissue. Lipidomic analysis of atherosclerotic tissue has shown an increase in lysoPL as the plaque progresses from fatty streaks to necrotic cores in proportion to the OxPL levels [19]. PLA2 have also been shown to modulate the generation of OxPLs during LDL oxidation. In presence of PLA2 inhibitor, LDL oxidation progresses more rapidly with generation of larger amounts of OxPCs resulting in a more atherogenic particle [84]. The Lp-PLA2 is the main phospholipase present within LDL

that has specific affinity for fragmented OxPLs generated during LDL oxidation [84]. This specificity has recently been shown to include the oxidized phosphatidylserines (OxPS) [87]. The interaction of Lp-PLA2 with different oxidized and non-oxidized PS species is mechanistically selective for hydrolysis based on the structure of the fragmented OxPS. His and Asp residues represent a catalytic dyad, and an essential Ser273 residue is present in Lp-PLA2 allowing for catalytic hydrolysis, the His/Asp dyad is also found in two important cytosolic PLA2s, GIVA, and GVIA [88]. In Lp-PLA2, Ser273 acts as a nucleophile that attacks the *sn*-2 ester bond of phospholipids within the active site which is composed of the catalytic triad involving Ser, His, and Asp [89]. This is the likely mechanism by which various PLA2 enzymes have different affinities for different OxPLs depending on the presence of the dyad or the triad catalytic site. In particular the *sn*-2 ester bond's proximity to the Ser273 residue in the active site determines the specificity and efficiency of Lp-PLA2 hydrolysis. It appears that in OxPS species which are hydrolyzed preferentially by Lp-PLA2, particularly 9-hydroxy or 9-hydroperoxy fatty acid chains, the *sn*-2 ester bond is closer than 3 Å to Ser273 compared to other species [87]. This suggests that Lp-PLA2 is more likely to hydrolyze a species that has an oxygen group closer to the *sn*-2 ester bond than one further away. PLA2 is activated by 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (PONPC), a species with a terminal aldehyde at the C9 position [90]. Due to the activity of PONPC and the oxidized fatty acid, they may interact with lysine residues that are important for the interaction of PLA2 with membranes; this allosteric modification as a Schiff base could cause cross-linking and permanent activation of PLA2 enzymes [91]. The anti-inflammatory effects of secretory PLA2 group IIA (sPLA2(IIA)) are seen as increased levels of OxLDL within atherosclerotic tissue results in a marked increase in enzymatic activity. As oxidized lipoproteins contain significant amounts of PC, OxPC, sphingomyelins (SM), and cholesterol, studies correlating these lipids to sPLA2(IIA) activity demonstrated no enzymatic effect from native PC and cholesterol, but opposing effects by OxPC and SM, stimulatory and inhibitory effects respectively [90, 92, 93]. OxPC and SM have similar PC head groups and it reasonably follows that there is a competitive binding that could occur for the sPLA2(IIA) binding site. Experiments that incorporated various ratios of OxPC and SM into LDL proved that OxPC could out-compete SM, blocking the inhibitory effect on the activity of sPLA2(IIA), and SM could eliminate the stimulatory effect of OxPC in dose-dependent fashion [94]. However, OxPC exhibited a much more potent effect to stimulate sPLA2(IIA) activity as 1 nmol could overcome the inhibitory effect of 2 nmol of SM, while SM required 8 nmol to suppress 1 nmol of OxPC activation. This potent activation of sPLA2(IIA) shows how strong OxPCs are able to influence inflammation. It is not simply due to the modification that PLA2 are activated by the OxPL. Halogenated PL produced by myeloperoxidase, and hypohalous acids during inflammation are actually inhibitory to the enzyme. High concentrations of chlorinated and brominated PC molecules decreased sPLA2(IIA) activity twofold [95]. There appears to be a differential ability of stimulating versus inhibiting molecules on the regulation of sPLA2(IIA). The researchers concluded that the swift activation of the PLA2 enzyme is essential to eliminate OxPL during the initial stages

of the inflammatory response which means that low concentrations of the activators should be strong signals for activation. When the OxPLs have been sufficiently removed from the tissue, the inhibitors are increasing in concentration during the final stages of the inflammatory process. These inhibitors, like the halogenated PL, are in high concentrations in the atherosclerotic tissue and they could be the reason for the activity of sPLA2(IIA) decreasing considerably in the final stages, when OxPL activators decrease enough to be out-competed by either SM or halogenated PL [95]. This interplay could be important for other diseases as other sPLA2 enzymes are also important to disease progression including neoplasms and neurodegenerative disorders. In breast cancer patients a sPLA2, human group X secreted PLA2 (hGX-sPLA2), induced lipid droplet (LD) formation within breast cancer cells, causing them to have an extended survival time during serum deprivation [96]. Significant metabolic transformations were identified to be induced by hGX-sPLA2 in highly invasive breast cancer cells. The enzyme was stimulatory of β-oxidation by supplying free fatty acids which could produce energy for the production of triacylglycerides that would cause the aggregation of cytosolic LDs that could serve as an energy source in cell survival. Interestingly, recent studies revealing that mitochondria form contact sites with nascent LDs and participate in phospholipid and TAG synthesis during their biogenesis [30] are in line with a possible association between β-oxidation and LD formation. LysoPC, in particular, are influenced in cancer as circulating plasma concentrations appear to be higher in women with malignant breast tumors than healthy women [97]. In Alzheimer's disease a pro-inflammatory sPLA2 is up-regulated in response to IL-1β and is present in the hippocampus and inferior temporal gyrus in humans [98]. With this in mind, there continues to be a paradox when considering PLA2. The enzymes could be a physiological mechanism to prevent high OxPL levels from causing damage; however the lysoPLs are also part of progressing diseases. In addition to this, the allosteric regulation of PLA2 by OxPL continues to be investigated as well as the cell-signaling capabilities of PLA2 during inflammation when the enzyme is bound to OxPL. The mechanism by which the PLA2 class of enzymes could impact OxPL in disease progression or prevention is still under investigation.

3.10 Conclusions

There is a growing body of evidence supporting the role of OxPL in inflammation. It is apparent that OxPLs are not bystanders, but are biologically active molecules. These molecules mediate a host of diverse signaling pathways, the net effect of which is contributing to the inflammatory process. Only recently, and with the advent of improved experimental techniques, have we gained a better understanding of the individual characteristics and roles of these molecules. The key enzyme in attenuating the pathological effects of OxPL is phospholipases. By better understanding the specific affinities and interactions of phospholipases towards OxPL molecules, we can tailor therapies that will allow for neutralization of OxPLs.

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Chapter 4
Phospholipases in Cardiovascular Disease

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Abstract Cardiovascular diseases comprise a frequent cause of morbidity and mortality in the modern world. Atherosclerosis, the most common pathophysiological process leading to cardiovascular disease, is a complex process involving many different pathways some of which are still under investigation. It has been shown that traditional risk factors are not sufficient in predicting cardiovascular events in the general population. Present research for the detection of substances that play a role in the atherogenic process has linked phospholipases with cardiovascular disease. Phospholipases, such as secretory phospholipase A_2 and lipoprotein-associated phospholipase A_2 (Lp-PLA_2), have been considered as markers of vascular inflammation and could therefore play an important role in cardiovascular disease. Furthermore, it has been shown that pharmacological inhibition of Lp-PLA_2 activity could exert beneficiary effects on the atherosclerotic process, offering a putative novel target for the management of these patients. This chapter summarizes current knowledge regarding various phospholipases and their role in atherogenesis. Studies involving these molecules will be investigated in order to enlighten the putative pathophysiologic mechanisms by which these proteins exert their effect on cardiovascular function. Additionally, the pharmacological interventions that influence phospholipase activity will be analyzed, proposing a putative new pharmacological approach for the treatment of atherosclerosis.

Keywords Secretory phospholipase A_2 • Lipoprotein-associated phospholipase A_2 • Platelet-activating factor acetylhydrolase • Cardiovascular disease

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_4,

4.1 Introduction

Cardiovascular diseases [CVDs: coronary artery disease (CAD), stroke, peripheral artery disease] comprise a frequent cause of morbidity and mortality in the modern world. Atherosclerosis is the common pathophysiological process of CVD, leading to various clinical manifestations according to the vessel affected. Atherosclerosis is a complex process involving many different pathways some of which are still under investigation. It has already been shown from the Framingham Heart Study that traditional risk factors were not sufficient in predicting cardiovascular events in the general population [1]. The ongoing research for the detection of substances that play a role in the atherogenic process has linked phospholipases with CVD. Phospholipases, such as secretory phospholipase A_2 ($sPLA_2$) and lipoprotein-associated phospholipase A_2 (Lp-PLA_2), have been considered as markers of vascular inflammation and could therefore play an important role in CVD. Furthermore, recent research has shown that direct pharmacological inhibition of Lp-PLA_2 activity exerts beneficiary effects on the atherosclerotic process. These findings are important since they could offer a novel target for therapeutic intervention and facilitate CVD prevention.

4.2 Secretory Phospholipase A_2

Phospholipase A_2 (PLA_2) is an enzyme that catalyzes the hydrolysis of the fatty acyl ester bond at the sn-2 position of phospholipids to produce free fatty acids and lysophospholipids. Secretory PLA_2 ($sPLA_2$) represents a class of enzymes that hydrolyze phospholipids from cellular membranes and lipoproteins, resulting in proatherogenic actions in the vessel wall [2]. It belongs to the same family of phospholipases as Lp-PLA_2. This enzyme is a 14 kDa calcium-dependent lipase that is produced from macrophages and arterial wall smooth muscle cells. $sPLA_2$ has been considered a marker of vascular inflammation. However, in contrast to Lp-PLA_2, the levels of this enzyme are determined by the levels of other markers of inflammation, such as IL-1, IL-6, and TNF-α.

Secretory nonpancreatic type II phospholipase A_2 ($sPLA_2$-IIa) has been shown to contribute to the pathogenesis of various inflammatory diseases [3], as well as various forms of cancer [4, 5]. Previous studies have shown an important role of $sPLA_2$ in CAD. Levels of $sPLA_2$ were found to be increased in 142 patients with CAD in comparison to healthy individuals, and they were positively correlated with increased levels of CRP. Furthermore, individuals with high levels of $sPLA_2$ had an increased probability of developing an acute coronary event, implying that this biomarker could be used as a risk factor conferring prognostic information [6]. The same group of researchers have also shown that $sPLA_2$ plays an important role in coronary artery spasm, a fact that, according to the authors, could reflect vascular inflammation in the coronary arteries, as expressed by high circulating levels of this enzyme [7].

4.3 Lipoprotein-Associated Phospholipase A_2

Lp-PLA_2, also known as platelet-activating factor acetylhydrolase (PAF-AH), is an enzyme that belongs to the A_2 phospholipase superfamily and is produced by inflammatory cells, primarily by macrophages [8], but also by monocytes, T-lymphocytes, and mast cells [9] that are all involved in the process of atherogenesis [10]. Lipoprotein-associated PLA_2 is a calcium-insensitive lipase. This 50 kDa protein resides mainly on LDL in human plasma, in a percentage of approximately 80 %. Lp-PLA_2 has been shown to play an active role in the oxidation of LDL [11]. The oxidative process transforms phosphatidylcholine (PC) to oxidative-modified PC that acts as a substrate for Lp-PLA_2. The interaction between oxidative-modified PC and Lp-PLA_2 generates the oxidized fatty acids (OxFA) and lysophosphatidylcholine (Lyso-PC) [12]. Lyso-PC and OxFA exert many proinflammatory actions (upregulation of adhesion molecules, cytokine and CD40 ligand expression, promotion of endothelial cell dysfunction, stimulation of macrophage proliferation, chemoattraction of inflammatory cells) leading to atherosclerotic plaque formation. Lp-PLA_2 molecules are expressed in and around the necrotic core of advanced human atheroma [13], and as the atheromatic plaque grows, its concentration in Lp-PLA_2 is increased [14].

Experimental studies as well as studies in Lp-PLA_2-deficient individuals had proposed antiatherogenic properties of Lp-PLA_2 [15]. However, current data suggests a proatherogenic role for this protein. Lp-PLA_2 activity has been shown to be upregulated in atherosclerotic lesions, particularly in complex plaques [16]. Furthermore Lp-PLA_2 mass or activity has been linked to increased cardiovascular risk [17]. Lp-PLA_2 serves as a marker of vascular inflammation and it appears to be involved in the initiation of the early stages of the vascular inflammatory process. The detection of Lp-PLA_2 as an emerging inflammatory biomarker implicated in atherosclerosis [18] comes to serve the up to now unmet need for cardiovascular risk prediction and possibly to offer a future target for therapy [19].

4.4 Clinical Implications of Phospholipases in CVD

The enzymes of the phospholipase superfamily have been studied extensively because of their implication in the atherosclerotic process. The EPIC-Norfolk Prospective Population Study investigated the prospective relationship between serum levels of type II $sPLA_2$ and the risk of future CAD in apparently healthy men and women. The study was a prospective nested case-control study among 3,314 apparently healthy men and women aged 45–79 years old. $sPLA_2$ levels were significantly higher in cases of people in whom fatal or nonfatal CAD developed during follow-up than controls (9.5 ng/mL; interquartile range [IQR], 6.4–14.8 vs. 8.3 ng/mL; IQR, 5.8–12.6; $p < 0.0001$). After adjusting for body mass index, smoking, diabetes, systolic blood pressure, low-density lipoprotein cholesterol,

HDL cholesterol, and CRP levels, the researchers found that the risk of future CAD was 1.34 (1.02–1.71; $p=0.02$) for people in the highest $sPLA_2$ quartile, compared with those in the lowest [20].

An increase of $sPLA_2$-IIa in peripheral plasma levels has shown a significant prognostic value in patients with CAD. In a recent study, Xin et al. examined the prognostic value of $sPLA_2$ levels after an acute myocardial infarction (AMI). Serum levels of $sPLA_2$-IIa were measured by ELISA in 964 post-AMI patients with serum samples collected in the convalescent stage. Patients with elevated $sPLA_2$-IIa (>360 ng/dL, $n=164$) had a significantly higher prevalence of death (18.3 % [30/164] vs. 2.75 % [22/800], $p<0.001$) and readmission for heart failure (14 % [23/164] vs. 2.1 % [17/800], $p<0.0001$). The authors conclude that a cut-off level of 360 ng/dL for $sPLA_2$-IIa during the convalescent stage after discharge of patients with AMI independently predicts long-term mortality and readmission for heart failure [21].

The West of Scotland Coronary Prevention Study (WOSCOPS) enrolled 6,595 men with hyperlipidemia, aged 45–65 years old, for a follow-up period of 5 years. Markers of inflammation, including fibrinogen, CRP, and $Lp\text{-}PLA_2$, were measured. Participants with elevated $Lp\text{-}PLA_2$ mass had approximately a twofold risk for future cardiovascular events (relative risk of 1 SD increase = 1.20, 95 % confidence interval [CI]: 1.08–1.34, $p=0.0008$) [22]. $Lp\text{-}PLA_2$ was the strongest predictor of an adverse outcome and was independent of traditional and emerging risk factors, including CRP (relative risk of 1 SD increase = 1.18, 95 % CI: 1.05–1.33, $p=0.005$) [23].

In the Atherosclerosis Risk in Communities (ARIC) Study approximately 16,000 middle-aged individuals of both sexes were enrolled. By multivariate analysis, $Lp\text{-}PLA_2$ was a significant predictor of risk after adjustment for interaction with LDL. The researchers found that patients with an $Lp\text{-}PLA_2$ level in the second and third tertiles had statistically significant increases in their risk ratios for an incident coronary heart disease (CHD) compared to patients with $Lp\text{-}PLA_2$ level in the first tertile. Among ARIC individuals with a low LDL level (<130 mg/dL), those with an $Lp\text{-}PLA_2$ level in the second and third tertiles had a statistically significant increased risk ratio of about twofold, after adjusting for other relevant variables. CRP in the third risk category also resulted in a statistically significant increase in the risk ratio of an incident CHD event. Furthermore, individuals with a combination of high levels of $Lp\text{-}PLA_2$ and CRP were at a greater risk than those with only one elevated inflammatory marker. High CRP and $Lp\text{-}PLA_2$ were additive predictors of increased risk for first CHD event in the ARIC cohort participants with LDL-C <130 mg/dL [24].

In the Rotterdam study, $Lp\text{-}PLA_2$ activity was shown to be an independent predictor for the risk of CAD and ischemic stroke in a population of 7,983 middle-aged persons. Compared with the first quartile of $Lp\text{-}PLA_2$ activity, multivariate-adjusted hazard ratios (HRs) of the second, third, and fourth quartiles were 1.39 (95 % CI, 0.92–2.10), 1.99 (95 % CI, 1.32–3.00), and 1.97 (95 % CI, 1.28–3.02), respectively (p for trend = 0.01), for the risk of coronary heart disease and 1.08 (95 % CI, 0.55–2.11), 1.58 (95 % CI, 0.82–3.04), and 1.97 (95 % CI, 1.03–3.79) (p for trend = 0.03) for the risk of ischemic stroke [25]. The monitoring of trends and determinants in cardiovascular disease (MONICA) study enrolled 934 apparently healthy middle-aged men. Increased levels of $Lp\text{-}PLA_2$ were associated with increased risk of future

coronary events (hazard ratio 1.23, 95 % CI: 1.02–1.47), after controlling for potential confounders, however, inclusion of C-reactive protein in the multivariable model eliminated Lp-PLA_2's additive predictive value [26]. In the Bruneck study, Lp-LPA_2 activity was shown to be related with lipid and inflammatory markers, as well as with incident fatal and nonfatal CVD [age- and sex-adjusted hazard ratio (95 % CI) 2.9 (1.6–5.5); third vs. first tertile group; $p<0.001$]. However, non-cardiovascular mortality was not associated with increased Lp-PLA_2 activity in this study [27].

The Rancho Bernardo study demonstrated that Lp-PLA_2 levels are positively correlated with age, body mass index, LDL, triglycerides, and CRP and negatively correlated with HDL in 1,077 apparently healthy men and women. Lp-PLA_2 levels in the second, third, and fourth quartiles predicted an increased risk of CHD compared with the lowest quartile (hazard ratios 1.66, 1.80, and 1.89, respectively; $p=0.05$ for each) after adjusting for C-reactive protein and other CHD risk factors [28].

Since several studies demonstrated an additive prognostic value of Lp-PLA_2 levels to traditional atherosclerotic risk factors, there have been efforts to incorporate Lp-PLA_2 measurements in multimarker panels in order to improve the prognostic value for cardiovascular events. In one study, NT-Pro-BNP, whole blood choline (WBCHO) and LpPL-A_2 were found to be the optimal combination for risk stratification in 432 patients presenting with an acute ischemic episode in the emergency department [29]. The incremental value of Lp-PLA_2 for prediction of cardiovascular events was also examined after addition of Lp-PLA_2 measurements to a model including traditional risk factors, renal function as assessed by cystatin C, and hemodynamic stress as assessed by NT-Pro-BNP. The study monitored the number of cardiovascular events (death, nonfatal MI, stroke) in 1,051 patients with CAD during 4 years of follow-up. The addition of cystatin C and NT-Pro-BNP measurement to the basic model improved its predictive accuracy (Area Under the Curve (AUC): 0.71 from 0.69) and when Lp-PLA_2 levels were added on the top of cystatin C and NT-Pro-BNP, the AUC showed a small increase (0.73 from 0.71). In the multivariable analyses there was a near twofold increased risk for future cardiovascular events in patients in the top two tertiles of Lp-PLA_2 mass compared to the lower tertile, after adjustment for markers of inflammation, renal dysfunction, and hemodynamic stress [30]. Furthermore, in the PEACE trial, elevated Lp-PLA_2 and hs-CRP levels were shown to predict acute coronary syndromes in patients with stable CAD ($p<0.005$ and 0.001, respectively), whereas only Lp-PLA_2 was a significant predictor for coronary revascularization during 4.8 years of follow-up [31].

The research in this field is ongoing and current knowledge is growing rapidly in a way that aids in the understanding of the complex pathophysiological process of atherosclerosis. It was recently shown that the binding of Lp-PLA_2 to certain lipoproteins can alter its characteristics. Rallidis et al. studied the cardiovascular events in 477 patients with stable CAD during a follow-up period of 34 months. Total plasma Lp-PLA_2 and high-density lipoprotein-linked Lp-PLA_2 mass and activity measurements were obtained at baseline. After the follow-up period 123 cardiovascular events were recorded. As expected, total plasma Lp-PLA_2 mass and activity were predictors of cardiac death (hazard ratio [HR]: 1.013; 95 % confidence interval [CI]: 1.005–1.021; $p=0.002$; and HR: 1.040; 95 % CI: 1.005–1.076; $p=0.025$,

respectively). However, HDL-Lp-PLA_2 mass and activity were shown to confer a lower risk for cardiac death (HR: 0.972; 95 % CI: 0.952–0.993; $p=0.010$; and HR: 0.689; 95 % CI: 0.496–0.957; $p=0.026$, respectively) after adjustment for traditional risk factors for CVD [32], suggesting a protective role of HDL-linked vs. LDL-linked Lp-PLA_2.

A large number of epidemiological studies suggest an association between measurements of phospholipase levels and CVD. These findings have been incorporated in the Adult Treatment Panel III (ATP III) scientific board proposals. Therefore, the addition of measurement of Lp-PLA_2 levels could be considered in patients with family history of coronary heart disease and relatively normal lipid values or patients that show a combination of risk factors that places them just below current guideline cut-off levels for treatment, Thus, elevated Lp-PLA_2 in these categories of patients would indicate the need of a more aggressive treatment. However, Lp-PLA_2 is not currently advocated as a routine screening test.

4.5 Possible Therapeutic Implications

Various effective cardiovascular medications have been shown to exert anti-inflammatory actions, i.e., statins. However, no medication is specifically designed to target vascular inflammation. In the Jupiter study [33] the administration of rosuvastatin was shown to offer additional benefit for persons with high hsCRP levels and low LDL levels regarding the combined primary end point of myocardial infarction, stroke, arterial revascularization, hospitalization for unstable angina, or death from cardiovascular causes. Also, anti-inflammatory medications, used for other purposes (i.e., autoimmune diseases), have been found to exert beneficiary actions in CVDs. Anakinra, a human recombinant IL-1 receptor antagonist, has documented beneficial effects on the coronary flow, endothelial, and myocardial function [34]. Medications especially designed to target vascular inflammation, such as PLA_2 inhibitors, have shown positive results in indices of atherosclerosis. A theoretical goal of treatment with these medications would be to modulate the inflammatory processes within the vessel wall without affecting host defenses, thus exerting the maximum potential vascular benefit.

sPLA_2 serves as a marker of vascular inflammation. Varespladib, an inhibitor of sPLA_2, has been developed and originally tested for its anti-inflammatory properties on pancreatitis [35], rheumatoid arthritis [36], and sepsis [37]. Since sPLA_2 is implicated in vascular inflammation and the progression of atherosclerosis, this inhibitor was also tested for its potential antiatherogenic properties. In animal studies varespladib was shown to reduce markers of inflammation (IL-10, IL-12 GM-CSF), as well as cholesterol accumulation and atherosclerotic lesions of the aorta [38]. Phase II clinical trials of varespladib did not demonstrate a good efficacy profile in patients with rheumatoid arthritis, asthma and ulcerative colitis, whereas in patients with CAD, varespladib methyl consistently reduced LDL-cholesterol levels [39]. FRANCIS-ACS [40], a phase III trial for the use of varespladib in

patients with acute coronary syndrome, is in progress and its results will enlighten our knowledge regarding this potentially useful medication.

Recently, clinical studies have demonstrated that Lp-PLA_2 levels can be reduced with pharmacological intervention. Statins, when used to treat hyperlipidemia, have been shown to lower Lp-PLA_2 levels. A 17 % reduction in Lp-PLA_2 levels was demonstrated in subjects treated with pravastatin in the WOSCOPS study [41]. In accordance to these results, Tsimihodimos et al. have shown that atorvastatin reduced Lp-PLA_2 activity by 28–42 % [42]. Additionally, fibrates were also shown to have an impact on Lp-PLA_2 levels (fenofibrate treatment reduced Lp-PLA_2 levels by 22–28 % in patients with small dense LDL particles) [43]. However, it has not yet been demonstrated whether lowering Lp-PLA_2 levels has a significant effect on patients' outcomes, though Lp-PLA_2 has been shown to be predictive of CHD risk.

Darapladib is a novel medication that acts as an Lp-PLA_2 inhibitor. It inhibits Lp-PLA_2 activity over a 24-h dosing interval and its concentrations in patient's plasma are stable over 24 h, with C_{max} at ~6 h post-dose. It is metabolized in the liver (CYP3A4), produces minimal inhibition of other PLA_2 isozymes, and no clinically significant drug–drug or drug–food interactions have been noted [44]. Furthermore, there is no need for dose adjustment according to age, gender, ethnicity, and mild-to-moderate renal impairment. Animal studies have shown a marked inhibition of plasma and lesion Lp-PLA_2 activity and reduced lesion Lyso-PC content with darapladib, as well as a reduced development of advanced coronary atherosclerosis in diabetic and hypercholesterolemic swine [45].

In humans, the addition of darapladib to intensive statin therapy in CHD patients was shown to decrease Lp-PLA_2 activity and also to reduce systemic inflammation (as expressed by reduced CRP, IL-6 levels). Darapladib 40, 80, and 160 mg inhibited Lp-PLA_2 activity by approximately 43 %, 55 %, and 66 %, respectively, compared with placebo. No unexpected clinical or laboratory adverse effects were reported. This study, however, did not investigate the clinical impact of Lp-PLA_2 activity reduction in the atheromatic plaque [46]. The Integrated Biomarkers and Imaging Study-2 (IBIS-2) was designed to examine the impact of darapladib therapy on the vessel wall. Patients with angiographically proven CAD were randomized to receive darapladib 160 mg or placebo once daily. The investigators incorporated the use of novel intravascular ultrasound techniques (palpography and virtual histology) to measure the mechanical properties and the components of the atherosclerotic plaque. It was shown that Lp-PLA_2 inhibition prevented necrotic core expansion, a key determinant of plaque vulnerability, after 12 months of treatment (necrotic core volume increased significantly in the placebo arm (4.5 ± 17.9 mm^3; $p = 0.009$), whereas darapladib halted this increase (-0.5 ± 13.9 mm^3; $p = 0.71$ in the darapladib arm). Even though changes in plaque composition, like the ones reported, do not always translate to actual benefit in cardiovascular end points, the findings of this study suggest that inhibition of Lp-PLA_2 could represent a novel therapeutic intervention [47].

Pharmaceutical interventions to reduce Lp-PLA_2 activity may result in additional anti-inflammatory effect in patients with atherosclerosis, as was shown for darapladib administration and its effect on reducing high-sensitivity CRP and IL-6 levels.

Two studies have been designed to test the efficacy of darapladib in coronary heart disease. The STabilization of Atherosclerotic plaque By Initiation of darapLadIb TherapY (STABILITY) [48] aims to enrol 15,500 patients with chronic CHD. Its objective is to show whether darapladib treatment, when added to standard of care, will result in a reduction in the incidence of first occurrence of the composite of major adverse cardiovascular events (i.e., cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke). The Stabilization Of pLaques usIng Darapladib-Thrombolysis In Myocardial Infarction 52 Trial (SOLID-TIMI 52) [49] aims to investigate the role of darapladib in the setting of an acute coronary event (myocardial infarction, unstable angina). Results from these studies will answer the question whether the extensive research in the preclinical field will translate in better outcomes in patients with CHD.

4.6 Conclusions

Even though current medicine provides tools for cardiovascular risk stratification, there continues to be a large unmet need for the detection, assessment, and treatment of CVDs. The role of inflammation in atherosclerosis is well established and inflammatory markers are already in use in everyday clinical practice. The enzymes of the phospholipase superfamily have been studied extensively because of their implication in the atherosclerotic process and could serve as emerging biomarkers of vascular inflammation. sPLA2 and Lp-PLA_2 could serve the up to now unmet need for cardiovascular risk prediction.

Furthermore, even though various effective cardiovascular medications have been shown to exert anti-inflammatory actions, no medication is specifically designed to target vascular inflammation. Varespladib, an inhibitor of sPLA2, and darapladib, a novel medication that acts as an Lp-PLA_2 inhibitor, have shown promising results in indices of atherosclerosis. Phospholipase inhibition could putatively serve as a promising target for CVD management. These specific inhibitors of vascular inflammation are already being tested and the results of these studies are expected to shed light to our understanding of the pathogenesis and management of CVD. Results from current trials are expected in order to investigate whether the inhibition of phospholipases action is associated with a morbidity and mortality benefit.

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Part II
Role of Phospholipase A

Chapter 5
The Structures and Functions of Intracellular Phospholipase A_1 Family Proteins

Katsuko Tani, Takashi Baba, and Hiroki Inoue

Abstract Phospholipase A_1 is an enzyme that hydrolyzes phospholipids, producing 2-acyl-lysophospholipids and fatty acids. Intracellular phospholipase A_1 ($iPLA_1$) functions inside cells. Except for a short lipase consensus sequence (G–X–S–X–G), the overall primary structures of $iPLA_1$ proteins differ from those of other phospholipases. While yeast, nematode, fruit fly, and *Arabidopsis* each have only one $iPLA_1$ gene, mammals including humans possess three $iPLA_1$ genes (phosphatidic acid-preferring phospholipase A_1 (PA-PLA_1)/DDHD1/$iPLA_1\alpha$, p125/Sec23IP/$iPLA_1\beta$, and KIAA0725p/DDHD2/$iPLA_1\gamma$). The three mammalian $iPLA_1$ proteins are localized in different subcellular compartments, suggesting their different roles. All the $iPLA_1$ family proteins have a domain named DDHD, in addition to a lipase consensus sequence, and some of them have a sterile alpha motif (SAM). Studies of the three mammalian $iPLA_1$ proteins have demonstrated that the lipase consensus sequence and the DDHD domain are involved in their enzymatic activity, and that the tandem SAM-DDHD domain is important for binding to intracellular membranes. Recent studies have revealed the physiological functions of the $iPLA_1$ proteins. p125 plays a role in vesicular transport and seems to be involved in spermiogenesis. As to human diseases, mutations of the PA-PLA_1 and KIAA0725p genes are responsible for hereditary spastic paraplegia, a neurodegenerative disorder. In this chapter, we focus on the mammalian $iPLA_1$ proteins and provide an overview of their structures and functions. We also briefly mention the physiological functions of nonmammalian $iPLA_1$ family proteins.

Keywords Intracellular phospholipase A_1 • Phosphatidic acid • Phosphatidylinositol • Hereditary spastic paraplegia • Membrane trafficking

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_5,

5.1 Introduction

Phospholipases are involved in a variety of cellular functions such as membrane synthesis and turnover, production of signaling molecules, organelle biogenesis, and vesicular transport. Phospholipase A_1 (PLA_1) is an enzyme that hydrolyzes phospholipids, producing 2-acyl-lysophospholipids and fatty acids (Fig. 5.1a). PLA_1 enzymes can be divided into two groups based on their cellular localization: group 1 consists of extracellular enzymes, and group 2 of intracellular enzymes [1, 2]. The group 1 enzymes, which belong to the pancreatic lipase gene family, are synthesized with a signal peptide and function outside cells [3]. Currently, six extracellular PLA_1 molecules are known in mammals. The following review provides details on these molecules [4].

The group 2 enzymes, intracellular PLA_1s ($iPLA_1$s), were relatively recently discovered. In the mid-1990s, Glomset and his colleagues first identified an $iPLA_1$ and called it phosphatidic acid-preferring phospholipase A_1 (PA-PLA_1) [5]. To date, it is known that mammals including humans possess three $iPLA_1$ proteins (PA-PLA_1/DDHD1/$iPLA_1\alpha$ [6], p125/Sec23IP/$iPLA_1\beta$ [7], and KIAA0725p/DDHD2/$iPLA_1\gamma$ [8]), while yeast, nematode, *Arabidopsis*, and *Drosophila* each have only one $iPLA_1$. Figure 5.1b shows a phylogenetic tree. Figure 5.2 illustrates the domain structures of $iPLA_1$ family proteins. All the $iPLA_1$ family proteins have a lipase consensus sequence G–X–S–X–G (or S–X–S–X–G). Except for the lipase consensus sequence, the overall primary structures of $iPLA_1$ proteins differ from those of other phospholipases and lipases. This family has a conserved characteristic sequence called the DDHD domain, which is about 180-amino acids long and characterized by four conserved amino acid residues (three Asp and one His; hence it is named "DDHD"). This domain was first found in *Drosophila* retinal degeneration B proteins, which are lipid transfer proteins. Although the function of the DDHD domain remains elusive, it is supposed to mediate interactions between proteins. p125 and KIAA0725p also have a sterile alpha motif (SAM). The SAM, which comprises around 70 residues, is a putative protein interaction module present in a wide variety

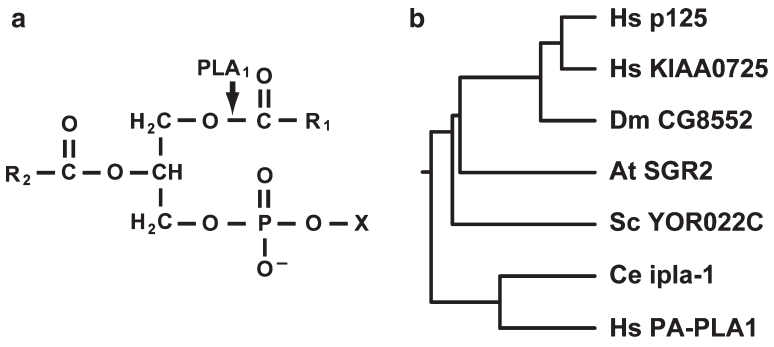

Fig. 5.1 $iPLA_1$ family proteins. (**a**) Hydrolytic site of PLA_1s. (**b**) Phylogenetic tree for $iPLA_1$ family proteins

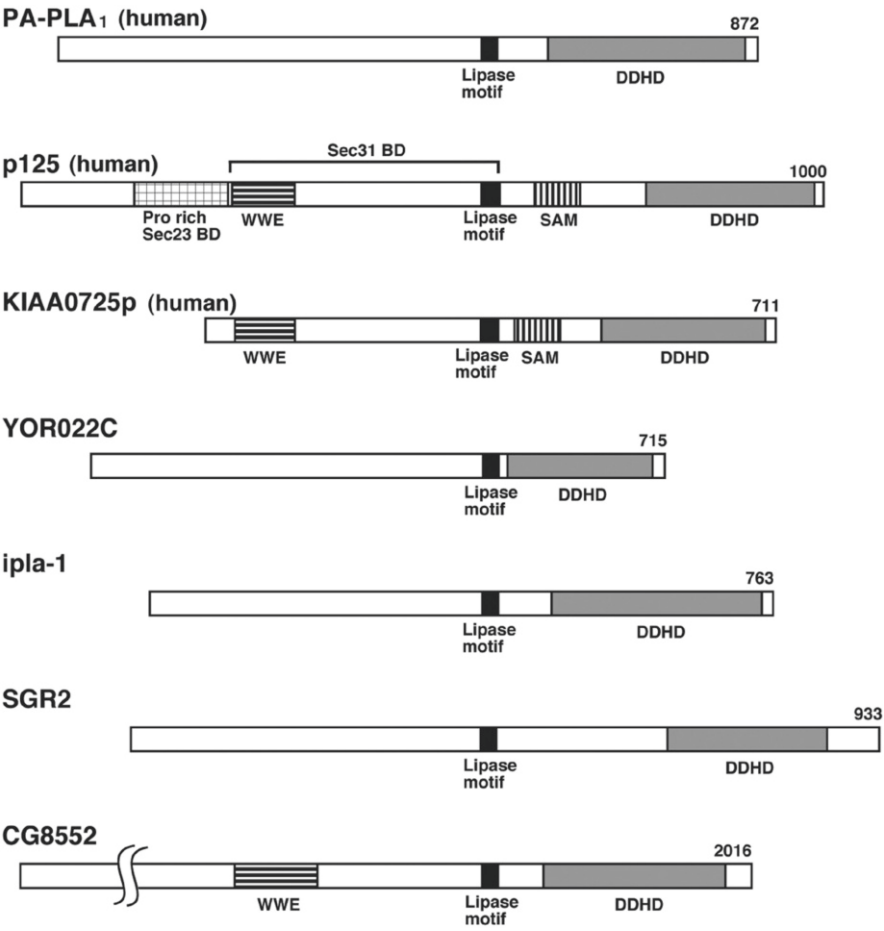

Fig. 5.2 Schematic representation of the domain structures of $iPLA_1$ family proteins. The numbers at the *upper right* indicate the numbers of amino acid residues in the respective proteins

of proteins. Recent research has revealed the characteristics and physiological roles of the $iPLA_1$ family proteins. This chapter focuses on the mammalian $iPLA_1$ family proteins and has the following sections: (1) Enzymatic activity of $iPLA_1$; (2) Intracellular localization; and (3) physiological functions. We also briefly mention nonmammalian $iPLA_1$ family proteins in Sect. 5.5.

5.2 Enzymatic Activity of $iPLA_1$

$PA\text{-}PLA_1$ was identified using a Triton X-100 mixed micelle system, which is an in vitro assay system containing Triton X-100, phosphatidic acid (PA) being used as a substrate to screen for PLA_1 activity [5]. Subsequent analysis revealed that $PA\text{-}PLA_1$

cleaves various phospholipids in vitro depending on the assay conditions [9–11]. PS-PLA_1, and mPA-$PLA_1\alpha$ and β, which belong to the extracellular PLA_1 family, exhibit remarkably high substrate specificity toward phosphatidylserine (PS) and PA, respectively [4]. (Note: Although the nomenclature is somewhat confusing, mPA-PLA_1 is short for membrane-associated phosphatidic acid-selective phospholipase A_1 and differs from PA-PLA_1.) Differing from the above extracellular PLA_1 proteins, the iPLA_1 proteins apparently exhibit low substrate specificity. Some studies have shown that PA and phosphatidylinositol (PI) are promising target substrates for the iPLA_1s.

Several research groups have reported that PA-PLA_1 hydrolyzes PA [5, 6, 8, 9, 11, 12]. According to Glomset and his colleagues, the activity of purified PA-PLA_1 toward PA is four to tenfold greater than those toward phosphatidylethanolamine (PE), phosphatidylcholine (PC), PS, and PI in the Triton X-100 mixed micelle system, whereas in the absence of Triton X-100, PE as well as PA is a good substrate. Recently, Yamashita et al. reported that PA-PLA_1 cleaves PI as well as PA in an in vitro system involving ectopically expressed PA-PLA_1 [11]. They proposed the model that PA produced by phospholipase D binds to PA-PLA_1 and thereby augments its PLA_1 activity toward PI. It is intriguing that ipla-1, which is a unique iPLA_1 family protein in *Caenorhabditis elegans* (*C. elegans*), exhibits the highest homology to PA-PLA_1 among the three mammalian iPLA_1 proteins (see Fig. 5.1b) and hydrolyzes PI but not PA in vitro [13]. Imae et al. analyzed lipids in an *ipla-1* mutant by mass spectrometry and demonstrated that PI is an in vivo substrate for ipla-1.

Meanwhile, KIAA0725p exhibits high lipase activity toward PA in the presence of Triton X-100, whereas it exhibits high activities toward PA and PE and low activities toward PS and PC in the absence of Triton X-100 [8]. Its specific activity is much lower than that of PA-PLA_1 [12]. In contrast, no enzymatic activity has been detected for p125 [8]. Thus, p125 is a unique protein in the iPLA_1 family proteins, and its details are given below. All the enzymatic activities of the mammalian iPLA_1s have been analyzed in an in vitro system. However, in vivo substrate analysis has yet to be carried out. In addition, the regulation of iPLA_1 enzymatic activity remains unclear. Han et al. suggested that phosphorylation may regulate the enzymatic activity of PA-PLA_1 [14].

The following illustrates the relationship between the enzymatic activity and the domain structure. Initial studies [6, 8] showed the importance of the lipase consensus sequence for the enzymatic activity. Higgs mutated serine 540 in bovine PA-PLA_1 to alanine and Nakajima mutated serine 351 in human KIAA0725p to alanine. Both are central serine residues in the lipase consensus sequence. The two mutated proteins had completely lost the PLA_1 activity toward PA. Hence, the central serine residue within the lipase consensus sequence is indispensable for the enzymatic activity. Recently, Inoue et al. showed the DDHD domain is also important for the enzymatic activity [12]. They showed that the phospholipase activity of KIAA0725p and PA-PLA_1 is markedly reduced by deletion of the DDHD domain or point mutations of conserved aspartate or histidine residues in the domain. In view of the above, the enzymatic activity must require both the lipase consensus sequence and the DDHD domain.

5.3 Intracellular Localization

Cell biological analysis involving cultured cells revealed that the three mammalian $iPLA_1$ proteins are localized in different cellular compartments (Fig. 5.3). PA-PLA_1 is localized to the cytosol. In addition to the cytosol, p125 is localized to endoplasmic reticulum (ER) exit sites, which represent an ER subdomain [15]. KIAA0725p is localized to the *cis*-Golgi and possibly the ER-Golgi intermediate compartment (ERGIC), as well as the cytosol. Accordingly, part of p125 or KIAA0725p associates with an intracellular membrane structure. The results of FRAP analysis suggested that the two proteins are rapidly cycled between the membrane-associated and cytosolic pools. Membrane binding of p125 and KIAA0725p seems to correlate with their phosphatidylinositol phosphate (PIP)-binding ability [12]. p125 and KIAA0725p, but not PA-PLA_1, bind to PIPs in vitro. This binding is independent of phospholipase activity. That is, an activity-deficient mutant exhibits the same binding activity as the wild-type protein. Among the PIPs, phosphatidylinositol 4-phosphate (PI(4)P) is known to be abundant in Golgi membranes [16]. The forced expression of Sac1, a phosphoinositide phosphatase that prefers PI(3)P and PI(4)P, in the Golgi, causes drastic redistribution of KIAA0725p from the Golgi to cytoplasmic punctate structures, suggesting the importance of PIPs for the localization of KIAA0725p. The PIP-binding site has been mapped to the tandem SAM-DDHD domain of KIAA0725p. The lack of the SAM domain in PA-PLA_1 may explain its inability to bind to cellular membranes (Table 5.1).

In the case of KIAA0725p, not only its PIP binding ability but also its catalytic activity contributes to its membrane binding [17]. Wild-type KIAA0725p and the activity-deficient S351A mutant are equally targeted to the Golgi-like structure in control cells. In cells depleted of endogenous KIAA0725p with siRNA, the mutant was much less efficiently targeted to the Golgi-like structure compared with the wild-type protein. These results suggest that lysophospholipids (LPs) produced by KIAA0725p support the association of KIAA0725p with membranes. The above idea is also supported by a study involving an acyltransferase inhibitor, CI-976 [18].

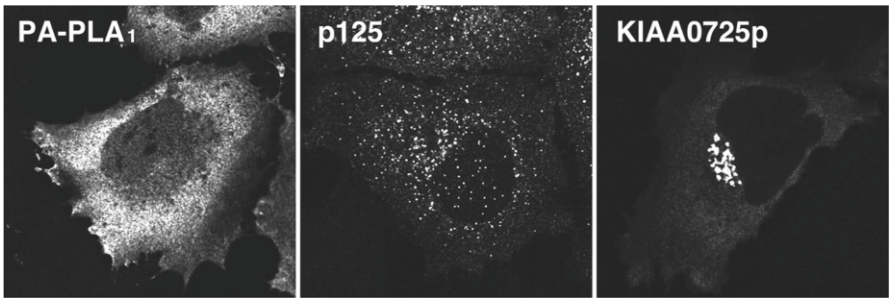

Fig. 5.3 Subcellular localization of the three mammalian $iPLA_1$ proteins. The three FLAG-tagged mammalian $iPLA_1$ proteins (PA-PLA_1, p125, and KIAA0725p) were ectopically expressed in HeLa cells and then analyzed by immunofluorescence microscopy

Table 5.1 Functions of iPLA$_1$ family proteins

iPLA$_1$ proteins	Intracellular localization	Functions
PA-PLA$_1$ (mammals)	Cytosol	Mitochondria formation?
		Mutations are responsible for HSP
KIAA0725p (mammals)	*cis*-Golgi and ERGIC	Protein transport from the Golgi
	Cytosol	Mutations are responsible for HSP
p125 (mammals)	ER exit sites	Organization of ER exit sites
	Cytosol	Protein transport from the ER
		Spermiogenesis?
ipla-1 (*C. elegans*)	Cytosol?	Fatty acid remodeling of PI
		Vulval formation
SGR2 (*A. thaliana*)	Vacuoles	Vacuole formation
	Cytosol	Shoot gravitropism
YOR022C (*S. cerevisiae*)	Mitochondria?	Vacuole formation?
	Cytosol?	Mitochondria formation?
CG8552 (*D. melanogaster*)	?	Synapse formation?

The iPLA$_1$ protein functions so far reported are listed

The remodeling of fatty acid side chains is caused by cleavage of the fatty acid side chains by phospholipases, followed by the action of acyltransferases [19, 20]. Once the reaction with acyltransferase is inhibited by CI-976, lyso forms are likely to accumulate inside cells. In CI-976-treated cells, KIAA0725p was found to accumulate in specific membrane structures, which were not colocalized with canonical organelle markers including Golgi proteins [18]. This is because KIAA0725p seems to recognize structures containing LPs. Although the p125 and KIAA0725p molecules exhibit high homology (see Fig. 5.1b), p125 is localized to ER exit sites and KIAA0725p to the Golgi. Analyses of truncation and chimeric proteins have revealed that the N-terminal region specific to p125 regulates its membrane specificity, namely its localization to the ER exit sites [15].

5.4 Physiological Functions

5.4.1 PA-PLA$_1$ and KIAA0725p

It has been speculated that PA-PLA$_1$ is involved in spermatogenesis or sperm function since it was first identified. This is based on its expression pattern. PA-PLA$_1$ is highly expressed in brain and testis. In addition, mature testis was found to exhibit tenfold or more activity than newborn calf testis [5]. To date, however, there is no direct proof that PA-PLA$_1$ is involved in spermatogenesis. Yamashita et al. have proposed the production of lyso-PI, a signaling lipid, as a physiological function of PA-PLA$_1$ [11]. They presented the hypothesis that PA-PLA$_1$ plays a role in the activation of GPR55, a G-protein-coupled putative cannabinoid receptor, by producing its agonist, 2-arachidonoyl-lysoPI.

KIAA0725p is expressed in various organs at substantially the same level. Since KIAA0725p is localized to the Golgi and exhibits high structural homology to p125, the functions of KIAA0725p in membrane trafficking have been extensively investigated. In cultured cells, overexpression of KIAA0725p causes dispersion of the Golgi apparatus and ERGIC, suggesting its involvement in the early secretory pathway. Morikawa et al. [21] suggested that KIAA0725p is involved in retrograde trafficking from the Golgi to the ER, based on the results of a knockdown study. Later, Sato et al. suggested that the results of Morikawa et al. may be artifacts due to an off-target effect. Using several siRNAs, Sato et al. demonstrated that knockdown of KIAA0725p does not inhibit the retrograde trafficking from the Golgi, but causes a partial defect in the trafficking from the Golgi to the plasma membrane [17].

Recently, mutations of PA-PLA_1 [22] and KIAA0725p [23, 24] have been reported to be involved in hereditary spastic paraplegia (HSP). HSPs comprise a genetically heterogeneous group of inherited neurodegenerative disorders, in which lower extremity weakness and spasticity are predominant symptoms [25, 26]. The symptoms are the consequence of corticospinal-tract degeneration. HSPs are classified clinically as "uncomplicated or pure HSP" (characterized by lower extremity spasticity and weakness, and subtle lower extremity dorsal column impairment), and "complicated or complex HSP" (in which spastic paraplegia is associated with additional neurologic or systemic abnormalities such as ataxia, mental retardation, and neuropathy). More than 50 distinct loci and more than 20 gene products have been identified to date. These gene products have a wide variety of functions and include proteins involved in axonal transport, membrane trafficking, ER morphology, mitochondrial regulation, myelination, lipid/sterol modification, and axon pathfinding. Thus, deterioration and deficiencies of various functions in neurons lead to the onset of HSP. Inheritance can be X-linked recessive, autosomal dominant, or autosomal recessive. PA-PLA_1 has been designated as spastic paraplegia gene (SPG) 28, and KIAA0725p as SPG54. The two loci exhibit autosomal recessive inheritance. The details of the HSPs are given below.

Bouslam et al. [27] found a consanguineous Moroccan family with autosomal-recessive forms of pure HSP and determined the genetic locus in 14q.11, calling it SPG28. Tesson et al. [22] sequenced all exons of SPG28 and verified that mutations of PA-PLA_1 are responsible for SPG28. It has been observed that lymphoblasts derived from HSP patients with a PA-PLA_1 mutation exhibit decreased cell respiration and ATP contents, and increased H_2O_2 accumulation. From these phenomena, Tesson et al. suggested that the mutation in PA-PLA_1 causes decreased mitochondrial functions, which may lead to HSP.

Schuurs-Hoeijmakers et al. reported on four families exhibiting a clinical presentation of complex HSP due to mutations of KIAA0725p [23]. The core phenotype of this HSP syndrome consists of early-onset spastic paraplegia, intellectual disability, and a specific pattern of brain abnormalities on cerebral imaging. All identified mutations affect the protein's DDHD domain. As described above, the mutations in the DDHD domain affect the lipase activity. Indeed, they detected an abnormal lipid peak indicating accumulation of lipids on cerebral magnetic resonance spectroscopy, suggesting the functioning of KIAA0725p in lipid metabolism in the central

nervous system. Soon after Schuurs-Hoeijmakers's report, Gonzalez et al. reported two deleterious mutations of KIAA0725p in two other families with complex HSP [24]. Their phenotype is quite similar to that reported by Schuurs-Hoeijmakers. Both groups suggested that both membrane trafficking and lipid metabolism are involved in the onset of HSP caused by the mutations of KIAA0725p.

These studies first delineated a relationship between mammalian iPLA$_1$s and human diseases. It is possible that deficiencies of the two proteins may induce deregulated lipid metabolism in a similar manner. As mentioned above, PA-PLA$_1$ is highly expressed in the brain and testis. KIAA0725p, however, is ubiquitously expressed at substantially the same level. Of note is that the symptoms of the two HSPs are not completely the same (pure for SPG28 but complex HSP for SPG54). There remains a possibility that the two proteins play somewhat different roles in the human nervous system. How the mutations of the two proteins lead to the onset of HSP and what kind of mechanisms are involved in this process have yet to be determined.

5.4.2 p125

p125 was discovered not as an enzyme but as a component implicated in vesicular transport from the ER. The export of newly synthesized proteins from the ER is mediated by transport vesicles called coat protein complex II (COPII)-coated vesicles, which are produced in a specialized ER subdomain known as ER exit sites [28–30]. COPII consists of two heterodimeric complexes, Sec23–Sec24 and Sec13–Sec31, and a low-molecular-weight GTP-binding protein, Sar1. Sec23–Sec24 and Sec13–Sec31 form the inner and outer layers of COPII coat, respectively, and the Sar1-GTP cycle regulates coat assembly.

p125 was isolated as a mammalian Sec23-interacting protein by affinity chromatography using GST-tagged mouse Sec23-coupled resin [7]. p125 comprises an N-terminal proline-rich region responsible for the interaction with Sec23, and central and C-terminal regions, which exhibit high homology to those of iPLA$_1$ proteins (Fig. 5.2). p125 is localized to ER exit sites. Overexpression and knockdown studies suggested that p125 is involved in the architecture of ER exit sites [15]. Later, Ong et al. [31] showed that p125 binds to Sec31 as well as Sec23. The Sec31-interacting domain of p125 lies within residues 260–600 and differs from the region responsible for the binding to Sec23, indicating that p125 interacts with the two proteins using different regions. They proposed a model in which p125 bridges the inner and outer layers of the COPII coat. Note that although p125 possesses a lipase consensus sequence, GHSLG, and a DDHD domain, no phospholipase activity has been detected [8]. Even when the p125-specific N-terminal domain is deleted, no enzymatic activity is detected, indicating that this domain has nothing to do with regulation of the enzymatic activity. There is no clear-cut explanation for this lack of enzymatic activity. It is possible that the phospholipase activity of p125 was lost during the course of evolution, and instead it may have acquired some specific functions, including a role in the organization of ER exit sites. Consistent with this idea,

p125 only exists in metazoans, whereas PA-PLA_1 is conserved throughout eukaryotes from yeast to mammals. Perhaps p125 serves as an accessory protein that regulates the functions of COPII components rather than as an enzyme. Increasing evidence suggests the importance of COPII accessory proteins in the regulation of transport [28].

A gene-targeting study showed p125 is involved in spermatogenesis [32]. Male p125-KO mice are subfertile. Many sperm of p125-deficient mice were found to have a round-shaped head and an abnormal mitochondrial sheath, and to lack an acrosome. This phenotype is similar to that observed in males with globozoospermia, a rare disorder of male infertility. The acrosome is a specialized secretory organelle responsible for fertilization and is localized in the head of mammalian sperm. The acrosome contains digestive enzymes, which dissolve the jelly coat of eggs. In the late spermatocyte stage, acrosomal components are first expressed, and in the spermatid stage the acrosome is formed through the fusion of pro-acrosomal vesicles derived from the *trans*-Golgi network [33]. While p125 is present in spermatocytes and spermatids, mature sperm exhibit no p125 expression. In spermatocytes, p125 is localized in a certain population of ER exit sites. p125 may facilitate the transport of certain proteins that are critically required for acrosome formation.

5.5 $iPLA_1$ Family Proteins in Other Species

Saccharomyces cerevisiae (*S. cerevisiae*), *C. elegans*, *Arabidopsis thaliana* (*A. thaliana*), and *Drosophila melanogaster* each have one $iPLA_1$ protein. Thus, it is unclear as to which mammalian $iPLA_1$ corresponds to the above $iPLA_1$ or whether or not the functions of the above $iPLA_1$ overlap those of the three mammalian $iPLA_1$ proteins. The following provides an overview of the respective characteristics so far reported.

In *S. cerevisiae*, the *YOR022C* gene encodes an $iPLA_1$ protein. A null mutation of *YOR022C* keeps the cells viable but reduces the growth rate with respiration-dependent carbon sources. In addition, the *YOR022C*-deficient yeast shows an abnormal vacuolar morphology. This is intriguing because SGR2, the $iPLA_1$ protein in *A. thaliana*, is involved in vacuolar formation. In fact, YOR022C and SGR2 are closely mapped on the phylogenic tree. A recent genome-wide interaction study showed that *YOR022C* genetically interacts with several genes related to mitochondria [34], including *MMM1* for a component of the ER–mitochondria encounter structure, *UGO1* for an outer membrane component implicated in mitochondrial membrane fusion, and *UPS1* for a lipid transfer protein that can shuttle PA between mitochondrial membranes. Localization of the YOR022C protein to mitochondria has also been reported [35].

C. elegans possesses one $iPLA_1$ family protein named ipla-1, which exhibits the highest homology to PA-PLA_1 among the mammalian $iPLA_1$ proteins. Arai's group proposed that ipla-1 is involved in the remodeling of fatty acids at the *sn*-1 position of PI [13]. They showed that the *sn*-1 fatty acid of PI is determined by *ipla-1* and

three acyltransferases (*acl-8*, *-9*, and *-10*) in vivo. *ipla-1* mutant alleles yield vulval defects caused by disturbance of the terminal asymmetric division of seam cells, stem cell-like epithelial cells [36]. β-Catenin [37] is a component of the Wnt/β-catenin asymmetry pathway, which determines the polarity of the asymmetric division of stem cells. They suggested that the mutation of *ipla-1* causes alteration of the molecular species of PI. This, in turn, causes abnormal membrane trafficking, leading to mislocalization of β-catenin. Since a relationship between an $iPLA_1$ family protein and PI metabolism has been revealed at the organism level, their study is significant.

A higher plant, *A. thaliana*, possesses one $iPLA_1$ protein named Shoot Gravitropism 2 (SGR2). While *sgr2* mutants exhibit abnormal gravitropism with respect to the shoots, the positive root gravitropism is normal [38]. Endodermal cells of the shoots are considered to be gravity-sensing cells. In the endodermal cells, amyloplasts, which sediment in the direction of gravity, act as statoliths [39]. In the *sgr2* mutants, amyloplast sedimentation is abnormal. Shoot endodermal cells are mostly occupied by a large central vacuole. SGR2 is localized to the vacuole and a defect of it causes disorganization of the vacuole, suggesting that its function is closely associated with vacuolar biogenesis [40]. The expression of the wild-type SGR2 protein but not the mutant protein, which lacks phospholipase activity, can rescue the phenotype, suggesting the importance of the enzymatic activity. *Drosophila* possesses one $iPLA_1$ gene, *CG8552*. Knockdown of *CG8552* reduced the number of active zones at synaptic terminals in *Drosophila*, suggesting its involvement in synaptic functioning [23]. No obvious motor abnormality, however, was observed in these flies.

5.6 Conclusions

The three mammalian $iPLA_1$ proteins exhibit different intracellular localizations. Accordingly, it has been considered that the $iPLA_1$ proteins may be involved in the formation of different organelles and membrane trafficking. In fact, cellular analysis has provided experimental data showing that p125 is involved in the formation of ER exit sites and that KIAA0725p is involved in membrane trafficking from the Golgi. Further, analysis at the organism level also indicated that PA-PLA_1 is involved in mitochondrial functions and SGR2 is involved in formation of vacuoles. Perhaps, the $iPLA_1$ family proteins play critical roles in membrane trafficking and organelle formation. In general, the $iPLA_1$ family proteins exhibit decreased substrate specificity and are localized to the cytosol. Hence, the proteins may affect all the intracellular membranes facing the cytosol. In the future, we should investigate: (1) How the specificity of $iPLA_1$ is determined on membranes; (2) what is an in vivo substrate; (3) how the activity is regulated; and (4) what mechanisms are used to regulate membrane trafficking and organelle formation. To do this, we should elucidate the relationships between $iPLA_1$s and other lipid-metabolizing enzymes, such as phospholipase D, which produces PA, or acyltransferase, which consumes the

$iPLA_1$ products, LPs. As described above, Arai et al. have shown a relationship between ipla-1 and three acyltransferases and have proposed that ipla-1 plays a role in the remodeling of the fatty acid side chains of PI. Many studies suggest the involvement of phospholipase D in membrane trafficking and formation of the Golgi [41, 42]. In addition, mitochondria contain MitoPLD, which cleaves cardiolipin to produce PA [43]. Elucidation of the overall lipid metabolic pathways including those involving the $iPLA_1$ family proteins will provide a new level of understanding of the intracellular membrane system.

Meanwhile, recent studies have shown various physiological functions of the $iPLA_1$ family proteins. Mutations of PA-PLA_1 and KIAA0725p are responsible for HSP. HSP, however, has various causes, so that the mechanism leading to the onset of HSP is unknown in both cases. Understanding the mechanisms underlying the onset of HSP in both cases will not only provide information on the $iPLA_1$ protein functions, but also lead to the discovery of new characteristics of the human nervous system.

Acknowledgment We thank Tomoyuki Shishido for critical reading of the manuscript.

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Chapter 6
Phospholipase A and Breast Cancer

Warren Thomas

Abstract Dysregulated eicosanoid signalling is emerging as a crucial factor in diverse disease processes that include oncogenesis. Breast carcinoma progression is the result of the subversion of multiple intracellular signalling pathways found in normal mammary tissue that impact upon the differentiation, proliferation and survival of tumour cells, as well as the stimulation of angiogenesis. Phospholipase A (PLA), as the enzyme initiating arachidonic acid release from membrane phospholipids, is located at a critical junction between hormone- and growth factor-regulated signalling cascades. PLA itself or the other enzymes that catalyse downstream eicosanoid metabolism may provide novel therapeutic targets for the treatment of breast carcinoma. This review describes the contribution of PLA and its products to the progression of breast carcinoma and the interaction of eicosanoid signalling with other cascades modulated by oestrogen, epidermal growth factor, signal transducers and activators of transcription, mammalian target of rapamycin and also cell metabolism in tumour cells.

Keywords Phospholipase A • Breast cancer • Oestrogen • Eicosanoid • Cyclooxygenase • Lipoxygenase

6.1 Introduction

Breast carcinoma is the most common malignancy experienced by women and has a 1 in 8 lifetime risk for the global female population. Breast carcinoma accounts for 23 % of all malignancies diagnosed in women and accounts for 15 % of all female

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_6,

cancer-related mortality [1]. In the year 2008, 1.38 million women were newly diagnosed with this disease worldwide. In particular breast carcinoma is a significant cause of death among post-menopausal women in developed countries, where the rate can be five times higher than that for women in developing countries. The higher rate in developed countries is believed to relate to lifestyle choices in reproductive behaviour including delayed and infrequent pregnancy; the use of hormonal contraceptives and also dietary changes that impact on body weight. In more recent years the mortality rates have declined especially in younger patients as a result of earlier diagnosis and effective therapies [2]. In the United Kingdom the age-standardised 5-year survival increased from 52 to 85.1 % over the period 1971–2009. Five-year survival for patients diagnosed with stage I breast carcinoma is sixfold higher compared to patients diagnosed with stage IV malignancy (88 % vs. 15 %). The increasing prevalence of breast carcinoma in the aging population of developed countries has led to considerable investment in investigating the aetiology and progression of this malignancy. The focus on personalised medicine to target the disease as it manifests itself in individual patients will facilitate the development of more effective interventions, particularly for later stage malignancy with worse prognosis, and also where resistance to existing therapies develops over time.

Genetic and epigenetic changes result in the transition from a normal to a malignant cell phenotype. Characterisation of the molecular basis of these changes and the profiling of individual tumours has made us aware of the heterogeneity of breast carcinoma and has become an important clinical tool in predicting the progression of the malignancy and identifying the most effective treatment approach for individual patients [3–5]. For example, patients with oestrogen receptor (ER)-positive tumours can be treated with adjuvant endocrine therapy to suppress the growth-promoting actions of ERα. Current ER-targeted pharmacological interventions include Tamoxifen and Fulvestrant [6]. Patients whose tumours express HER2 can benefit from treatment with specific antagonists of this receptor such as Lapatinib and Trastuzumab (Herceptin) [7]. The majority of patients treated with adjuvant systemic therapy respond poorly to treatment or go on to develop acquired resistance, rendering the therapy ineffective. The subset of patients whose tumours are ER-negative, progesterone receptor (PR)-negative, and HER2-negative (triple-negative, or basal-like cancers) do not have a standard adjuvant intervention and can only be treated with conventional chemotherapy [8]. It is highly desirable to identify therapeutic targets that can be used to treat this subgroup of patients and also where resistant to standard intervention develops.

The phospholipase A_2 (PLA_2) family of enzymes catalyse the hydrolysis of the sn-2 bond of membrane phospholipids to release arachidonic acid (AA) and lysophospholipid secondary messengers under the influence of various stimuli including circulating hormones and growth factors. There are three principal subgroups of PLA_2 isoforms: the Ca^{2+}-dependent secretory PLA_2 ($sPLA_2$), the intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$), and the intracellular Ca^{2+}-dependent PLA_2 ($cPLA_2$) [9]. The ubiquitously expressed $cPLA_2\alpha$ isoform has high selectivity for membrane phospholipids that contain AA that can be metabolised to growth-promoting eicosanoids. This has resulted in a number of studies that link $cPLA_2\alpha$ activity to

tumorigenesis [10]. cPLA$_2\alpha$ has a cytoplasmic distribution when inactive, but translocates to intracellular membranes once activated by concurrent Ca^{2+} binding and phosphorylation at serine residue 505 [11]. cPLA$_2\alpha$ released AA is a potent cytotoxic compound, inducing cell death through stimulation of the mitochondrion-mediated apoptosis and the sphingomyelin phosphodiesterase (SMase)-ceramide pathways unless the AA is subjected to further metabolism [10].

6.2 Eicosanoid Signalling Defects and Breast Carcinoma

Free fatty acids are used as an energy source by mammary gland cells and to synthesise milk lipids; however, excess dietary fatty acid intake is associated with breast carcinogenesis [12, 13]. AA is an essential fatty acid synthesised from linoleic acid that is metabolised into various eicosanoid signalling intermediates. The role of eicosanoid signalling in breast cancer development has been the subject of investigation for more than 30 years, since the up-regulation of cyclooxgenase-2 (COX-2) was observed in breast cancer cells [14, 15]. The merit of perturbing COX activity in the treatment of other cancers has been investigated with mixed outcomes, where the efficacy demonstrated in experimental models has not always been replicated in clinical trials [16, 17]. The published data is often conflicting in its attribution of promotional or inhibitory effects to eicosanoid signalling intermediates on breast cancer progression, and in distinguishing the contribution of the different branches of the eicosanoid biogenesis pathway to carcinogenesis. Recent data has suggested a link between eicosanoid signalling and oestrogen-stimulated signalling events in breast cancer cells both at the level PLA and COX activity.

In general terms tumour progression results from the development of enhanced survival potential and growth autonomy by malignant cells that renders them refractive to normal control. The dysregulation of homeostatic cell signalling and metabolic pathways facilitates this transition into malignancy. Perturbation of eicosanoid metabolism is emerging as a significant driver in carcinogenesis. AA can be converted into various biologically active eicosanoid mediators (Fig. 6.1) including prostaglandins (PGs), hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs) by cytochrome P450 monooxygenase, COX isoforms and lipoxygenases (LOXs) [18, 19]. PGE_2 contributes to the regulation of diverse cellular actions including proliferation, metabolism and differentiation; consequently, the AA-based eicosanoid signalling pathway has been implicated in the development and progression of cancer in different human tissues, including the breast [10, 20].

PGs regulate key physiological processes through the activation of specific G-protein-coupled receptors (GPCRs) [21], which results in the production of secondary signalling intermediates to induce proliferation, migration, apoptosis and angiogenesis [22]. PGE_2 abundance is elevated in many malignancies and correlates with tumour development. PGE_2 promotes the expression of growth-promoting genes such as c-fos and vascular endothelial growth factor (VEGF) [23] and can stimulate COX-2 gene expression in colorectal cancer, breast cancer and in normal

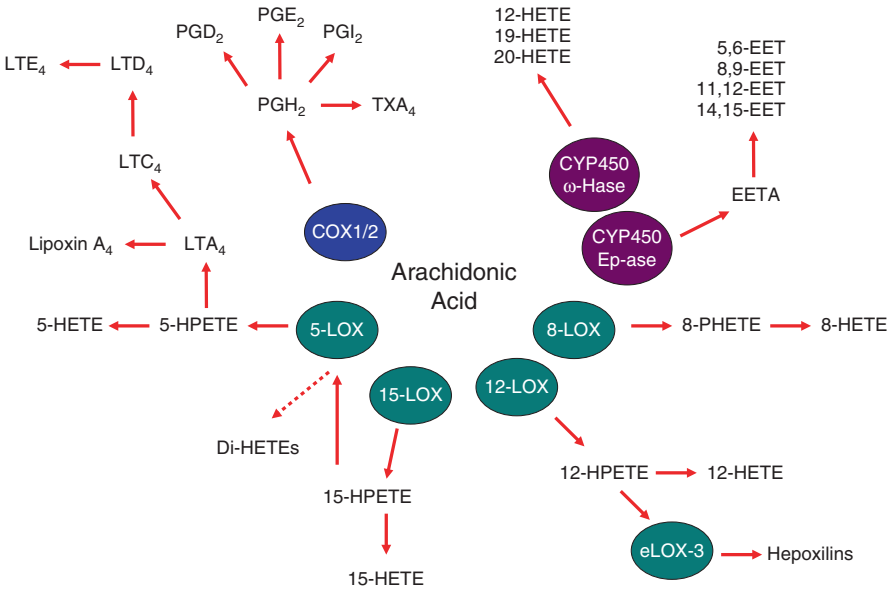

Fig. 6.1 Arachidonic acid (AA) can be metabolised into a diverse family of signalling intermediates. Cyclooxygense (COX) enzymes catalyse the first step in prostaglandin (PG) biosynthesis with the conversion of AA to PGH_2 which can then be metabolised to form other PGs or Thromboxane A_4. The lipoxygenase (LOX) family of enzymes convert AA into hydroperoxyeicosatetraenoic acids (HPETEs) which are further metabolised to hydroxyeicosatetraenoic acids (HETEs), hepoxilins, leukotrienes (LTs) or lipoxins. Cytochrome P450 (CYP450) family enzymes can catalyse the formation of other HETEs from AA through ω-hydrolase (ω-Hase) activity or the conversion of AA to epoxyeicosatrienoic acids (EETs) through epoxygenase (Ep-ase) activity

epithelial cells [23, 24] leading to a positive feedback effect on the downstream growth-promoting signalling. PGE_2 can act both in an autocrine and paracrine manner to stimulate aromatase expression in breast carcinoma and in normal tissue [25], consequently up-regulating the production of the most biologically active oestrogen 17-β-oestradiol (E_2) and the subsequent stimulation of proliferative signalling pathways. The up-regulation of COX-2 expression in malignant breast tissue correlated with an increase in aromatase activity [26].

Diverse lines of scientific investigation have found that AA and its metabolites contribute to breast carcinoma development and progression. Clinical, epidemiological and molecular evidence have linked COX-2 expression/activity and PGE_2 production to breast cancer progression (reviewed in [27]). Patient cohort and case control clinical studies have found that women receiving nonsteroidal anti-inflammatory therapies that suppress PG production exhibit a reduction in breast carcinoma risk [28–30]. In addition the expression of 5-LOX and 12-LOX is elevated in breast carcinoma [31], and the LOX antagonist suppresses *N*-methyl-*N*-nitrosurea-induced tumorigenesis is a rat mammary carcinoma model [32]. AA contributes to

carcinogenesis by promoting proliferation in breast cancer cells [33, 34] and has also been implicated in the stimulation of inflammation [35] and angiogenesis [36]. 15-LOX is hypoxia-induced, a state often arising within tumours and catalyses AA conversion to 15(S)-HETE. 15-LOX antagonism suppresses spheroid formation by MCF-7 breast carcinoma cells in vitro and also reduced their metastatic capacity in xenograft models. These experimental data were backed up by immuno-histochemical data supporting a role for 15-LOX and its products in lymphatic invasion [37]. 12-LOX was identified as a potential therapeutic target in the treatment of breast carcinoma, and a peptide antagonist has been evaluated and found to have promise in a mouse xenograft model [38].

6.3 The Coupling of Phospholipase A to Oestrogen Signalling

Inherited genetic factors, predominantly mutation of the *BRCA1* and *BRCA2* genes, account for 10 % of all cases of breast cancer and 25–40 % of breast cancers among younger women [39]. Sporadic incidences account for the vast majority of cases and are associated with diverse risk factors that have a biological or social basis. These factors include age; familial history; diet and the living environment. Endocrine factors and certain reproductive behaviours are associated with an increased risk of developing breast carcinoma. These individual factors influence the lifetime exposure to oestrogens and include early age at menarche and late age at menopause. Nulliparity, later age at first pregnancy and brevity of the breast-feeding term also contribute to increased risk of hormone-dependent proliferation of mammary epithelial cells. The pharmacological elevation of oestrogen levels by oral contraceptives and the extended duration of hormone replacement therapy also contribute to the risk of breast carcinoma development [40]. In recent years concern has been raised over the possibility of an increased breast carcinoma risk associated with environmental exposure to artificial oestrogen-mimicking compounds (xenoestrogens) such as the plasticizer bisphenol A. The first link between ovarian function and breast cancer progression was made in 1896 [41]; over subsequent decades considerable epidemiological and clinical evidence has been found to support a significant link between sustained oestrogen exposure and the increased risk of developing breast carcinoma. The actions of oestrogens on breast carcinoma cells include the stimulation of cell proliferation through ER-dependent up-regulation of proliferative signalling intermediates. The increased rate of cell proliferation under hormonal influence increases the probability of genetic mutations arising that accumulate in the genomes of daughter cells that may ultimately result in carcinogenesis. A second mechanism by which oestrogen contribute to malignancy is through direct, receptor-independent genotoxic effects stimulated by the release of reactive intermediates that are generated through the metabolic activity of aromatase and cytochrome P450. These intermediates increase the rate of genetic mutation such that oestrogen can promote the development of mammary tumours in ERα knockout

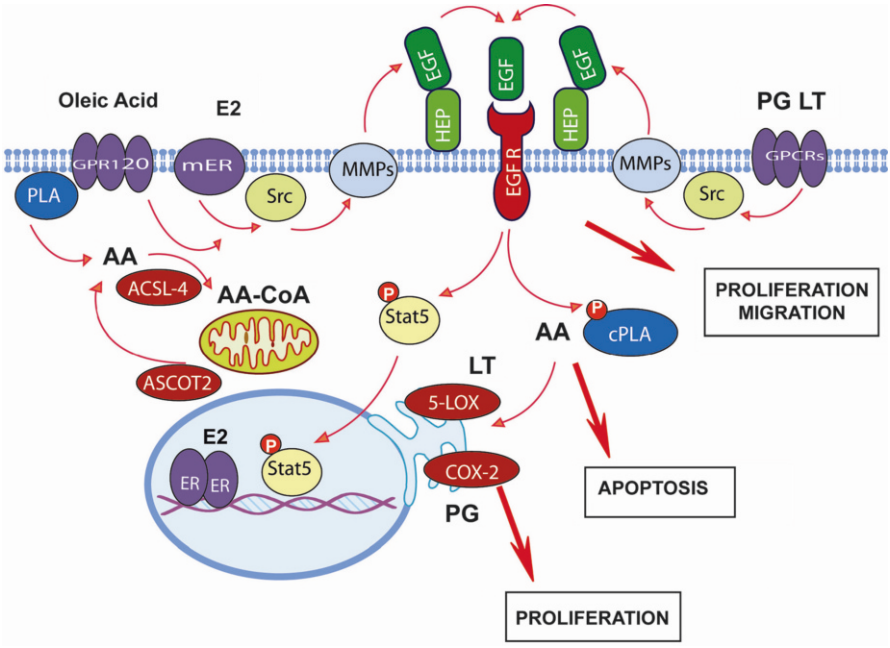

Fig. 6.2 Dietary fatty acids can be metabolised by breast carcinoma cells to form arachidonic acid (AA) or stimulate phospholipase A_2 (PLA_2) activity to liberate AA from membrane phospholipids. Additional lipid mediators such as oestrogen (E2), prostaglandins (PG)s and leukotrienes (LT)s acting through specific membrane receptors can also stimulate PLA_2 activity through the trans-activation of epidermal growth factor (EGF) receptor, downstream of matrix metalloproteinase cascades that liberate heparin (HEP) bound EGF from the cell surface. Free AA is sequestered as AA-CoA by acyl-coenzyme A synthetase (ACSL-4) and stored in the mitochondria to maintain low intracellular concentrations or is metabolised to produce PGs or LTs that not only feed into a positive feedback loop for eicosanoid signally but also participate if the stimulation of tumour promoting proliferation, migration and angiogenesis. Cyclooxygenase-2 (COX-2) is an E2-inducible gene in breast carcinoma that contributes to the production of growth-stimulating PGs, so directing the AA-producing activity of PLA_2 towards PG biosynthesis, mean while the trans-activation of EGF receptor contributes to the activation of other growth-promoting transcription factors such as STAT5

mice [42]. A third mechanism of oestrogen-induced carcinogenesis is believed to involve suppression of the chromosome repair system, leading to the accumulation of genetic lesions including the specific locus deletions in chromosomes 9 and 4 that are required for tumorigenesis [43].

Oestrogen can initiate rapid signalling actions at the cell membrane in advance of the transcriptional actions of nuclear ER (Fig. 6.2). The rapid activation of $cPLA_2\alpha$ by oestrogen contributes to the proliferative effects of the hormone in breast carcinoma cells [20, 44, 45]. Oestrogen-induced $cPLA_2\alpha$ activation is mediated by trans-activation of EGFR/HER2 heterodimers in the cell membrane signalling through ERK1/2 mitogen-activated protein kinase (MAPK) [41]. The result is to

stimulate proliferative signals in ER-positive and ER-negative breast carcinoma cells that express the membrane ER (mER) GPR30/GPER. A role for EGFR/HER2-coupled signalling in promoting oestrogen-independent tumour growth and in the development of resistance to endocrine therapy has been documented [46]. Histological data indicates that over-expression of EGFR and HER2, which is detected in 50 % and 30 % of breast carcinomas, respectively, also correlates with decreased sensitivity to endocrine therapy and with worse patient outcome [47]. The over-expression of HER2 receptors and enhancement of related signalling cascades is a predictor of the specific loss of ER expression, the progression to an ER-negative more invasive phenotype and for the development of resistance to selective ER modulator (SERM)-based therapy [48]. Over recent decades, HER2 expression has become an important prognostic indicator and intervention target for breast carcinoma. The role of EGFR/HER2 trans-activation in oestrogen-induced $cPLA_2\alpha$ activation in breast carcinoma cell lines suggests that $cPLA_2\alpha$ activity and expression may be coupled with HER2 over-expression in tumour cells. Previous investigations found a correlation between the expression of intermediates in the eicosanoid signalling pathway, particularly COX-2, and the abundance of HER2 in breast carcinomas [49–52]. There was a correlation between $cPLA_2\alpha$ expression and HER2 abundance in a small number of breast carcinoma cell lines [41]. A subsequent study of breast carcinoma mRNA expression profiles found that $cPLA_2\alpha$ expression correlated with worse prognosis indicators which also characterise more invasive tumours of the HER2-positive and basal-like subtype [53]. Elevated $cPLA_2\alpha$ expression was associated with decreased survival in patients with luminal breast cancers, and also correlated with a reduced efficacy of endocrine therapy. This study found that $cPLA_2\alpha$ expression was an independent predictive marker for poor response to endocrine therapy over the first 5 years of posttreatment follow-up.

PLA_2 can also mediate carcinogenesis by producing lysophospholipids following AA release, which can induce cell growth via their metabolism to lysophosphatidic acid (LPA) [54]. The heterologous over-expression of the LPA receptor, LPA_1, in the MDA-BO2 breast carcinoma cell line augmented the mitogenic effect of LPA on these cells [55]. In a mouse xenograft model, cells over-expressing LPA_1 had enhanced subcutaneous growth and displayed enhanced bone metastasis. The authors found that LPA was not endogenously synthesised by the MDA-BO2 cells but rather that the tumour cells stimulated LPA release from circulating platelets. This observation emphasises the importance of the biochemical interactions between different cell types in affecting the progression of malignancy. The antagonism of platelet activation reduced metastatic potential of these cells and also suppressed the progression of osteolytic lesions generated by an ovarian tumour cell line. The authors concluded that the release of LPA by tumour cell-stimulated platelets stimulated tumour growth and enhanced cytokine-dependent bone destruction at sites of metastasis. This observation is consistent with data from other investigators showing that PLA_2 is in fact under-expressed in breast carcinoma cells compared to normal mammary epithelium [56]. Furthermore, Boyan et al. found that E_2 treatment of ER(−) and ER(+) breast cancer cell lines did not result in PLA_2 activation [57]. Prior investigations had indicated that membrane-associated PLA_2 expression was a good

predictive indicator for metastatic potential [58] and breast carcinoma survival [51]. AA is itself a promoter of apoptotic signalling and it has been suggested that the increased abundance of COX-2 that is detected in many breast carcinomas not only increases PG release but also reduces cytoplasmic AA abundance; this suggestion is strengthened by the observation that COX-2 becomes associated with the mitochondria of malignant cells as does $iPLA_2$ [59].

PLA_2 activity is the rate-limiting reaction in the release of AA from cellular membranes and is tightly regulated to maintain a low intracellular abundance of AA in resting cells. Dysregulation of PLA_2 activity and the subsequent metabolic imbalance that can also result from an induction of downstream AA metabolising enzymes such as COX-2 leads to high levels of proliferative eicosanoids including PGE_2 [10]. COX expression is elevated in many malignancies including carcinomas of the colon, pancreas, prostate, lung, skin, liver and breast [22, 59, 60]. COX antagonism suppresses cell growth and exacerbates chemotherapy-induced apoptosis in breast carcinoma cells [61]. Collectively these investigations indicate a role of COX inhibitors in suppressing tumour formation in vivo; this is supported by the correlation between the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and growth reduction in breast carcinoma [22]. NSAIDs have recently been used as novel chemotherapeutics in the treatment of different types of malignancy [62]; however, critical side effects have been linked to their use. At present more interest is given to the development of highly specific COX-2 or PGES antagonists [63].

EGFR/HER1 is over-expressed in approximately 50 % of mammary carcinomas [45], and this increased abundance correlates with the development of resistance to hormonal therapy [46, 64]. In such tumours there is crosstalk between ER and EGFR/HER2-coupled pathways that results in a positive feedback cycle of cell survival stimulatory signalling. It is critical to suppress this crosstalk in the clinical setting by blocking both signalling cascades. Studies in immuno-compromised nude mice that support xenografts of human breast carcinoma cells over-expressing HER2 found that Gefitinib (Irresa), an EGFR inhibitor, and oestrogen deprivation in combination is more effective at inhibiting ER + breast cancer growth than either intervention alone [65]. Synergistic interactions have been identified between Gefitinib and Trastuzumab in breast carcinoma cells [66] and EGFR signalling is implicated in the regulation of $cPLA_2\alpha$ expression and activity. EGFR activation also induces COX-2 expression in human breast carcinoma cells [10] and the modulation of COX-2 expression by HER2 has also described in breast cancer [67]. Lanza-Jacoby et al. found a synergistic effect of EGFR and COX-2 antagonists in breast carcinoma cells, suggesting a potential link between the two signalling pathways [68].

6.4 Arachidonic Acid and Signalling Cascades

AA acid acts on signalling pathways directly or through its downstream metabolites to modulate the behaviour of carcinoma cells and other interacting cell types in the tumour. The release of AA can be stimulated through the activation of $cPLA_2$

isoforms and AA can promote Ca^{2+} entry in breast tumour-derived endothelial cells through a protein kinase A-mediated pathway to activate the TRPV4 Ca^{2+} channel [69, 70]. The expression of a subset of endothelial cell proteins has been linked to the Ca^{2+} response including the metastasis suppressor gene NM23A; reduced expression of this gene is associated with lymph node and distant metastasis. Antagonism of the AA-induced Ca^{2+} influx resulted in a 1.8-fold induction in the expression of this protein [71]. The importance of AA metabolism in modulating gene expression in carcinoma cells is also evident from the suggestion that AA promotes epithelial to mesenchymal transition in MCF10A cells [72].

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that participates in the phosphoinositol 3-kinase/Akt signalling cascade. The contribution of this cascade to modulating cell growth, survival and metabolism means that its activity is critical to the biology of tumour cells. At the molecular level, mTOR is integrated into two distinct signalling complexes. mTOR complex 1 (mTORC1) participates in translation initiation by phosphorylating p70 S6 kinase 1 and 4E-BP1 [73, 74]. The mTORC2 complex recruits a different cohort of binding partners and participates in the phosphorylation of Akt. In breast cancer tumours there is a positive correlation between the abundance of cPLA2, Akt phosphorylation and VEGF release [36]. AA treatment of MCF-7 cells stimulated the signalling activity of both the mTORC1 and mTORC2 signalling complexes; however, it is the activation of the mTORC1 pathway that impacts upon angiogenesis. The stimulation of proliferation by AA in the MCF-7 cells and angiogenesis in a chick chorioallantoic membrane model was inhibited by rapamycin and the LOX inhibitor nordihydroguaiaretic acid (NDGA), but not by the COX-2 inhibitor NS389. This study revealed the importance of LOX metabolites in angiogenesis.

6.5 Arachidonic Acid and Breast Carcinoma Cell Metabolism

The cytotoxic properties of AA mean that it is rapidly metabolised by enzymes such as COX and LOX to produce growth-promoting metabolites. Recent evidence also points to the sequestration of AA into the mitochondria of malignant cells. AA is esterified by acyl-CoA synthetase 4 (ACSL4) to produce arachidonoyl-CoA, so reducing the intracellular concentration of AA. ACSL4 is highly expressed in steroid hormone-producing tissues, but is poorly expressed in other tissues. The abundance of ACSL4 is, however, increased in various malignancies including carcinoma of the breast [75]. This study also found that ACSL4 expression was linked to COX-2 expression and PGE2 production that in turn were linked to a more aggressive tumour cell phenotype. ACSL4 can regulate COX2 expression indirectly through the 5-HETE metabolite leukotriene B4 and represents functional integration between the various arms of the AA metabolic network [76].

A recent study found that AA and PGE2 production was more active in ER– breast carcinoma and was linked to the up-regulation of delta-6 desaturase (D6D), the rate-limiting enzyme in the conversion of linoleic acid to AA [77]. Linoleic acid is a

C18, n-6 polyunsaturated fatty acid that is obtained through the diet. AA abundance is elevated in tumour vs. non-tumour tissue; however, linoleic acid abundance shows not significant difference [78]. Even though linoleic acid is essential for development, a dietary excess has the potential to result in overproduction of active AA metabolites and contribute to tumour growth. This appears to be most pronounced in more aggressive ER− breast carcinoma, but does open the opportunity for novel therapeutic interventions in the treatment of tamoxifen-resistant disease. Oleic acid, the most abundant monounsaturated fatty acid in the diet, also promotes breast cancer cell migration, proliferation and invasion. Part of this action is achieved through the activation of Stat5, a member of the signal transducers and activators of transcription (STAT) family of transcription factors [79]. STAT 5 plays a critical role in mammary gland growth and differentiation in pregnancy, but its constitutive activation in breast cancer promotes proliferation [80]. This action of oleic acid is dependent on its metabolism to AA and is coupled to EGFR trans-activation in a similar way to oestrogen activation of $cPLA_2$ [41, 79].

β-1,4-Galactosyltransferase I (GalT-1) can be localised to the cell surface or to the *trans*-Golgi apparatus and catalysis the transfer of galactose from UDP-galactose to terminal *N*-acetylglucosamine residues on oligosaccharide chains. In addition to its catalytic activity, surface GalT-1 acts as a membrane receptor for extracellular matrix proteins and cell to cell interactions [81]. As a result GalT-1 participates in a number of cellular processes associated with enhanced malignancy including cell growth and migration. AA induces the expression and surface exposure of GalT-1 in MDA-MB-231 breast carcinoma cells providing another mechanism by which PLA_2 activity can impact upon the invasive capacity of breast carcinoma cells [82].

6.6 Conclusions

The elevated expression of COX-2 in breast carcinoma and its induction by oestrogen identified a role for eicosanoid signal in the progression of this malignancy. The contribution of PLA_2 in providing AA, the substrate for COX-2 activity, has become apparent, while the overstimulation of PLA_2 activity must be coupled to elevated COX-2 or LOX activity in order to counter the cytotoxic effects of AA. The requirement to balance PLA_2 activity with the metabolism of its products may be responsible for some inconsistencies in published data regarding whether PLA_2 supports or suppresses breast carcinoma progression. The role of LOX signalling in proliferation, metastatic invasion and angiogenesis is emerging. The balance between COX and LOX activity in determining the nature of the AA metabolites produced is not only important establishing their respective and interacting role in breast carcinoma progression, but also in the targeting of novel therapeutic interventions.

Acknowledgments The Author is supported by funding from Science Found Ireland (12/TIDA/I2372).

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Chapter 7
Pathophysiological Aspects of Lipoprotein-Associated Phospholipase A_2: A Brief Overview

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Abstract Macrophages are known to produce significant amount of lipoprotein-associated phospholipase A_2 (Lp-PLA_2). In human plasma Lp-PLA_2 circulates in association with low- and high-density lipoproteins (LDL and HDL), where LDL-associated Lp-PLA_2 was found to be associated with atherosclerosis lesions. Studies have also suggested that LDL and the modified forms of LDL such as oxidized LDL (oxLDL) and glycated LDL (gLDL), and also apolipoprotein E (apoE) isoforms, are also found to be associated with Lp-PLA_2 for initiation and progression of vascular lesions. Additionally, *Chlamydia pneumoniae* infection can increase Lp-PLA_2 activity in the macrophages of atherosclerotic plaque. In adolescents, Lp-PLA_2 changes occur with obesity and it shows important association with markers of cardiovascular disorder. Lp-PLA_2 levels can be lowered by two main pharmacologic interventions—indirectly, by lowering LDL, or directly, by lowering Lp-PLA_2 activity. Notably, darapladib (a product of GlaxoSmithKline) is now considered as an important therapeutic agent to inhibit Lp-PLA_2 activity. However, some studies are still in progress to determine its pharmacokinetics and to prove it as a safe drug.

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_7,

Keywords Phospholipase A_2 • Lipoprotein-associated phospholipase A_2 • Oxidized LDL • Glycated LDL • Coronary artery disease • Diabetes • Carotid artery • Darapladib

7.1 Introduction

Several PLA_2s have been identified based on their nucleotide gene sequences. They are mainly of four types: cytosolic PLA_2, intracellular PLA_2, secretory PLA_2, and lipoprotein-associated PLA_2 (Lp-PLA_2 a.k.a. platelet-activating factor acetyl hydrolase, PAF-AH) [1–4].

The source of the plasma form of lipoprotein phospholipase A_2 (Lp-PLA_2) was initially determined through biochemical studies, which revealed that macrophages produce large amounts of the enzyme [5–8]. Lp-PLA_2 circulates in plasma in association with low- and high-density lipoproteins (LDL and HDL, respectively), where ~80 % of the total activity is present as a complex with LDL and that of ~20 % is associated with HDL [9, 10].

Studies conducted on more than 100,000 participants in about 32 trials demonstrated that serum Lp-PLA_2 correlates positively with an increased risk of heart disease and stroke [11–17]. In atherosclerotic lesions, Lp-PLA_2 was shown to be produced by the inflammatory cells such as macrophages, mast cells, and platelets found in atherosclerotic plaques [13, 18, 19]. Products of Lp-PLA_2 can upregulate expression of adhesion molecules, which can activate leukocytes and recruit macrophages into the inflammatory areas [13, 20–22].

Lp-PLA_2 is known to catalyze hydrolysis of the Sn-2 position of glycerophospholipids to liberate arachidonic acid. Radical peroxidation of arachidonic acid results in a family of prostaglandin F2 isomers, for example, 8-epi-PGF2, a sensitive and independent risk marker for coronary heart disease, which is released into biological fluids through the phospholipase-mediated pathway and consequently excreted in urine [23, 24]. Importantly, plasma samples from Lp-PLA_2-deficient subjects do not excrete F2-isoprostanes. In view of the above, Kim et al. [25] suggested a positive correlation between Lp-PLA_2 activity and urinary excretion of 8-epi-PGF2 in both controls and coronary heart disease cases, which supports the possibility that this enzyme may be modulated by oxidant stress.

7.2 Atherosclerosis and Lp-PLA_2

7.2.1 Lp-PLA_2 Association with LDL and HDL

Epidemiological studies revealed that oxidized P-lipids and lipoprotein-a [Lp(a)] have a close relationship with cardiovascular events, which can be further accentuated by elevated levels of Lp-PLA_2 activity [26]. Lp-PLA_2 has been shown to be

preferentially associated with this apo-B containing lipoprotein [27, 28]. Blencowe et al. [29] showed that association between Lp-PLA$_2$ and Lp(a) does not involve direct binding of apo(a), but requires participation of apoB, and presumably involves apoB-100 [30]. The associations of Lp-PLA$_2$ with HDL and LDL are of physiological and pathological significance and in some cases the distribution changes according to the presence of lipoprotein-a, Lp(a).

Lp-PLA$_2$ has been shown to be redistributed from apolipoprotein B containing lipoproteins between HDL and LDL in dyslipidemic subjects with increased risk for atherosclerotic cardiovascular disease (ASCVD) [31]. Circulating Lp-PLA$_2$ is predominantly bound to LDL, where it exerts proatherogenic properties [32]. However, a small fraction of Lp-PLA$_2$ is associated with HDL and that produces anti-atherogenic potential [27, 33, 34]. Clinically, an increase in the ratio of LDL and Lp-PLA$_2$ is associated with an increased risk of coronary artery disease (CAD) [27]. The mechanism by which HDL is associated with Lp-PLA$_2$ and acts as a potent anti-atherogenic agent is currently not clear and requires further investigation.

7.2.2 ApoE4 and Lp-PLA$_2$

Apo-E has been implicated as a risk factor for CAD. ApoE may modulate immune response and inflammatory properties in vascular cells and tissues [35]. ApoE isoforms are found to be associated with Lp-PLA$_2$, a marker of vascular inflammation. Genetic variability at the apoE locus has been shown to be associated with risk of cardiovascular disease (CVD) [36]. The apoE alleles (ε2, ε3, ε4) code for three common protein isoforms (E2, E3, and E4), resulting in six different genotypes (E2/2, E3/2, E4/2, E3/3, E4/3, and E4/4) [36]. In many studies, the presence of apoε4 allele was found to be positively associated with higher LDL cholesterol levels with risk of CVDs, while the apoε2 allele showed cardiovascular protective characteristics [35, 37].

Genetic variation at the apoε locus has been demonstrated to have a strong impact on CVD, and that the frequency of apoε allele varies considerably across geographical areas and ethnic groups [38]. Higher apoε4 frequencies observed among African or Northern European descent, which suggests a potentially adverse metabolic and inflammatory factor, manifest among the ethnic apoE4 carriers, who are prone to development of CVDs [35, 39].

An association was observed between apoE genotypes and the Lp-PLA$_2$ index, where apoE4 carriers had a higher index [35]. The ability of Lp-PLA$_2$, a marker of vascular inflammation, to predict CVDs has been demonstrated in multiple studies. Lp-PLA$_2$ is produced by inflammatory cells and circulates upon binding with LDL and other lipoproteins and it resides at the crucial position of lipid metabolism to elicit inflammatory response [40, 41].

Interestingly, Murphy et al. [42] suggested that apoE can act as a control switch between lipid and inflammatory risk in the progression of atherosclerosis. The Lp-PLA$_2$ index, a measure of Lp-PLA$_2$ mass and activity, was found to be higher in

apo E4 carriers irrespective of ethnicity [35, 43]. Taken together, these findings underscore the importance of assessing the relationship between genetic predisposition of apoE and subsequent phenotypic characteristics, for example, inflammation in the assessment of cardiovascular risk. Thus, apoE4 acts as a proinflammatory mediator that regulates atherosclerosis by modulating Lp-PLA_2 activity.

7.3 Inflammation, Atherosclerosis, and Lp-PLA_2

Inflammation and oxidative stress are key elements that are known to be involved in the pathophysiology of cardiovascular complications, for example, atherosclerosis [44]. The key role of inflammation is revealed by a number of epidemiological studies to indicate the existence of inflammatory cells, especially macrophages, in the cap of atherosclerotic plaques [45, 46]. In some studies, an association was observed between circulating inflammatory markers such as C-reactive protein (CRP) and interleukin-6 with atherosclerosis [14]. Kolodgie et al. [45] demonstrated that macrophages play a pivotal role in the fibrous cap progression and necrotic core expansion along with Lp-PLA_2 expression in the fibrous cap region, which indicated that Lp-PLA_2 is involved in plaque vulnerability especially in the fibroatheromas formation to plaque rupture. Recently, the non-traditional risk factors have increasingly gained attention among biomedical researchers. These include inflammatory markers such as CRP, tumour necrosis factor-alpha (TNF-α), cell adhesion molecules, and oxidative stress markers such as oxidized low-density lipoprotein (oxLDL) and Lp-PLA_2 [7, 47].

Lp-PLA_2 exhibits both proatherogenic and anti-atherogenic properties. The anti-inflammatory properties of Lp-PLA_2 were related to its ability to hydrolyze the inflammatory mediators, for example, PAF [7, 48]. This along with HDL-mediated effects could, at least partly, explain the effect of Lp-PLA_2 in attenuating myocardial ischemia-reperfusion injury. On the other hand, Lp-PLA_2 may exert proinflammatory effects such as stimulation of chemotaxis and tissue accumulation of macrophages [19, 48] by generating oxidized nonesterified fatty acids and lysophosphatidyl choline from free fatty acids [49]. Additionally, Lp-PLA_2 may cause negative effects on endothelial cell viability and function (Fig. 7.1), which initiates progression of atherosclerosis [34, 50].

7.4 Coronary Calcification and Lp-PLA_2 Among Different Ethnic Groups

The prevalence of coronary artery calcification (CAC) has been found to be substantially lower among Japanese than Americans despite less favourable profile of many traditional risk factors among Japanese [51]. Identification of racial differences in the association of Lp-PLA_2 with CAC in CAD may help to define populations in order to reduce future cardiovascular events by inhibiting Lp-PLA_2 activity.

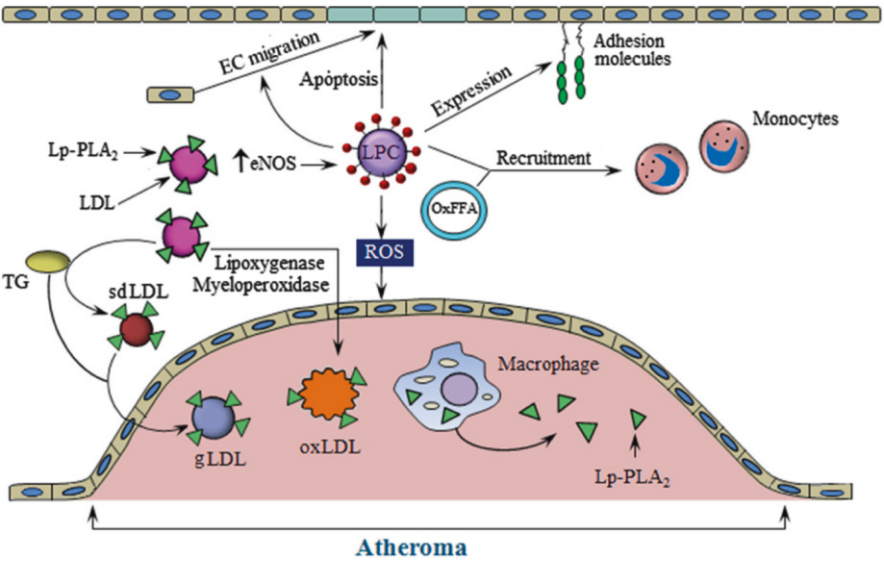

Fig. 7.1 In the circulation lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is bound predominantly to sdLDL and gLDL, the modified forms of LDL is carried to the vessel wall, which subsequently stimulates Lp-PLA_2 activity and yields lysophospholipids, for example, lysophosphatidylcholine (lysoPC) and oxidized fatty acids (oxFFA) leading to endothelial dysfunction. LPC and oxFFA have a role in the progression of atheroma by expressing adhesion molecules and recruitment of macrophages, which in turn increases Lp-PLA_2 activity. *EC* endothelial cell, *eNOS* endothelial nitric oxide synthase, *ROS* reactive oxygen species, *oxLDL* oxidized LDL, *sdLDL* small dense LDL, *gLDL* glycated LDL. Modified form of Fig. 3 of [50] of the text is taken with permission

Inherited deficiency of Lp-PLA_2 was found to be associated with CAD and stroke among Japanese [52]. Studies on some Caucacian populations revealed that Lp-PLA_2 may be considered as an independent risk factor for CAD and stroke [53]. Assessing the relationship of CAC with Lp-PLA_2 among Caucacians and the Japanese, the Caucacians have higher CAD compared to the Japanese [51]. These findings were apparently difficult to explain considering genetic differences between the Japanese and Americans as rates of CAD among Japanese Americans living in the USA are much higher than among the Japanese living in Japan [54]. This indicates that environmental factors such as diet and lifestyle are important for the formation of CAC among Americans and also other ethnic populations living in the USA who adopted Western lifestyle.

7.5 Stable and Unstable Angina and Lp-PLA_2

Studies by Kolodgie et al. [45], Packard et al. [16], and Dulaart et al. [55] revealed that Lp-PLA_2 concentration has been found to be significantly increased in patients with unstable angina (UA) and stable angina (SA), and that Lp-PLA_2 level was

higher in the UA group. This observation indicated that higher levels of Lp-PLA_2 are related with some morphologic parameters associated with vulnerable plaques, and that the larger atherosclerotic plaque burden may be useful for recognizing the high-risk patients [56]. Intravascular ultrasound (IVUS) study indicated that plaque area, remodelling index, and eccentricity index were large among patients in UA group than those of the SA group. Additionally, fibrous cap were found to be thicker in SA patients than the UA patients [16, 55]. Thus, positive remodelling was more frequent in UA group than in SA group; while negative remodelling was less in UA patients [55]. Taken together, these observations suggest that higher concentrations of Lp-PLA_2 imply more serious coronary atherosclerosis, which may elicit vulnerable coronary plaques in UA patients. Kolodgie et al. [45] supported the above by histopathologic studies and suggested that the Lp-PLA_2 expression in macrophages proceeds in the fibrous cap region, which subsequently produced plaque vulnerability. Virmani et al. [57] also observed that Lp-PLA_2 is released into circulation during atherosclerosis owing to the vulnerability of atherosclerotic plaque. Thus, the unstable plaque due to its vulnerability appeared to be a major factor that contributes to acute coronary syndrome with pathologic manifestations. The main components of most vulnerable plaques are usually thin fibrous cap, large lipid pools, and active inflammation. Overall, these findings validate the diagnostic value of Lp-PLA_2 as a specific biomarker to differentiate the patients with unstable angina from the stable angina group.

7.6 Endothelium and Lp-PLA_2

Endothelial dysfunction either in the coronary or the peripheral circulation has been suggested to be a predictor of cardiovascular events [50]. Mobilization of LDL into the arterial endothelial wall negatively affects vascular biology functions [58]. The activity of Lp-PLA_2 correlates with the presence of atherogenic small dense LDL (sdLDL) particles which are considered to be even more atherogenic than regular LDL particles [14]. Lavi et al. [50] have demonstrated that circulating PLA_2 levels are higher and independently associated with coronary endothelial function in patients with CAD. In these patients, an increase in coronary production of Lp-PLA_2 was found to be directly related to the extent of atheroma [50]. An association between oxidative stress and endothelial dysfunction has been established in animal studies, where Lp-PLA_2 can release F2 isoprostanes from esterified phospholipids [24]. Local generation of lysophosphatidyl choline during oxidant stress was found to be higher in patients with early atherosclerosis and correlated directly with apical coronary endothelial function [59, 60]. The role of Lp-PLA_2 in atherosclerosis progression is supported by the observation that vulnerable coronary artery plaques exhibited Lp-PLA_2 accumulation, especially in the macrophage-rich necrotic cores in the fibrous cap. Taken together, these observations indicated a link between inflammation, oxidative stress, and atherosclerotic disease progression.

7.7 Carotid Artery Plaque and Lp-PLA$_2$

ASCVD is a process by which obstruction of the vascular lumen leads to chronic and acute clinical presentation in different vascular territories and that eventually leads to CAD [61]. This is supported by the clinical observation that even a significant number of patients with peripheral arterial disease (PAD) as evidenced by potentially unstable carotid artery plaques and with unstable angina eventually cause high mortality. On the basis of these and other observations, the concept of plaque vulnerability in individuals with ASCVD has evolved over past few years. Carotid artery plaque expression of lipoprotein-associated PLA$_2$ predicts cardiac events [61]. Lp-PLA$_2$ expression in carotid artery plaques is a predictor of long-term cardiac outcome [62].

Systematic research in the recent past revealed that not only the traditional biomarkers, but also lysophosphatidylcholine (lysoPC) content was higher during carotid plaque formation indicating that not only the expression, but also the activity of Lp-PLA$_2$ is of prognostic significance [63, 64]. LysoPC is generated by Lp-PLA$_2$ upon acting on oxidized lipids, for example, oxidized LDL and that contributes to tissue accumulation of macrophages, the main cellular source of Lp-PLA$_2$ in atherosclerotic plaque. Lp-PLA$_2$ is currently known to cause tissue inflammation. Notably, low concentrations of lysoPC are antiapoptotic, but lysoPC at higher concentrations evoke apoptosis of endothelial and vascular smooth muscle cells [65, 66]. Lp-PLA$_2$ and lysoPC expression were correlated with MMP-2 and MMP-9 expression [67]. This is consistent with the study of Herrmann et al. [62] that relates Lp-PLA$_2$ with the induction of MMP production by lysoPC. The finding that lysoPC content was also higher in carotid artery plaques of cardiac patients suggested that not only expression but also the activity of Lp-PLA$_2$ is pathophysiologically important [68]. Thus, expression and activity of Lp-PLA$_2$ in carotid artery plaques suggested to be a predictor of future cardiac events independent of a number of other well-defined predictors including smoking and previous stroke [62].

7.8 IgE-Mediated Response and Lp-PLA$_2$

PAF plays a prominent role in the pathogenesis of IgE-mediated allergic inflammation and anaphylaxis [69]. Because of the PAF catalyzing activity, inhibition of Lp-PLA$_2$ indicates the possibility of an increased predisposition to allergic inflammation or anaphylaxis [70]. Although the direct evidence to support this concern is limited, there are clinical associations reported between low PAF-AH/Lp-PLA$_2$ and high plasma PAF with increased incidents and severity of asthma [70] and anaphylaxis [71]. Jiang et al. [69] reported that PAF-AH/Lp-PLA$_2$ deficiency in allergen-induced IgE-mediated airway inflammation indicates that lack of the enzyme in the serum of knock-out mice or pharmacologic inhibition of the enzyme significantly decreases its PAF hydrolyzing activity.

Polymorphism of val-279-phe by a single nucleotide in the PAF-AH/Lp-PLA_2 gene with its functional deficiency was observed in a group of Japanese population [72]. Studies on the Japanese groups revealed that PAF-AH/Lp-PLA_2 deficiency was predominant in asthmatics in comparison with healthy subjects with marked asthma severity observed among PAF-AH/Lp-PLA_2-deficient subjects [73]. Exogenous administration of PAF-AH/Lp-PLA_2 could reduce the mortality [74] and over-expression of the enzyme attenuated inflammation in mouse models of sepsis, suggesting that the enzyme may ameliorate inflammatory mechanisms involving PAF. This suggestion was questioned by Satoh et al. [75] in their clinical studies. They found no difference in the allele frequency between asthmatic patients and healthy controls and the V279F mutant allele prevalence was unaltered [75]. A bronchoprovocation test carried with PAF on Japanese patients showed no apparent alteration in airway responsiveness irrespective of the presence of V279F mutant allele or not [76]. Additional studies on treatment of human subjects with recombinant PAF-AH/Lp-PLA_2 showed no discernible effect on patients with asthma or sepsis [77]. PAF inhalation-enhanced LPS caused airway inflammation in wild-type (WT) and Lp-PLA_2–/– mice to a similar extent [69]. Wild-type and Lp-PLA_2–/– mice responded to passive or active allergic sensitization produced equal airway inflammation and hyperresponsiveness after allergen challenge and showed no discernible difference. Additionally, there were no difference in the amount of total IgE levels in the allergen-sensitized WT and Lp-PLA_2–/– mice [69]. Thus, Lp-PLA_2 deficiency did not increase local cell-mediated allergic immune responses or airway hyperresponsiveness to these models. In view of these controversial data, further studies are needed to determine whether low circulating PAF-AH/Lp-PLA_2 could induce inflammation and IgE-mediated allergic immune responses.

7.9 *Chlamydia pneumoniae* and Atherogenic Response

Chlamydia pneumoniae is a ubiquitous pathogen that frequently causes upper and lower respiratory tract infection [78]. The organism is thought to infect pulmonary macrophages, which are then transported and localize in arteries where infection can spread [79]. Studies in murine and rabbit models indicated that *C. pneumoniae* can target the vasculature, induce inflammation, and initiate or promote the development of atherosclerosis [80, 81]. More than half of the patients with atherosclerosis have evidence for *C. pneumoniae* infection based on a number of studies using detection methods such as immunohistochemistry (IHC) and electron microscopy of the plaque [82].

C. pneumoniae infection in carotid plaques was determined to be associated with plaque interleukin IL-6, serum IL-6, and CRP, suggesting that infected plaques contribute to systemic inflammatory markers in patients with stroke risk [83]. A prominent association of plaque Lp-PLA_2 with plaque macrophages and *C. pneumoniae* indicates an interactive role of the bacteria in the progression of inflammation in atherosclerosis [82, 84]. *C. pneumoniae* conceivably elicits its role in the atherogenic process by infecting macrophages that induce Lp-PLA_2 generation, leading to

stimulation of the production of inflammatory mediators in the plaque [82, 85]. Additional research is needed to determine the exact mechanism specific for *C. pneumoniae* and Lp-PLA$_2$ interactions in carotid plaque progression, one of the important risk factors for ischemic stroke.

7.10 Pre- and Postmenopausal Women and Lp-PLA$_2$

Paik et al. [86] have undertaken a study aimed to determine association of Lp-PLA$_2$ activity in circulation and peripheral blood mononuclear cells (PBMCs) with inflammatory and oxidative stress markers in non-obese women having pre- and postmenopausal status. Postmenopausal women showed higher circulating levels of oxLDL and IL-6 as well as TNF-α, IL-1β in PBMCs than premenopausal women. In premenopausal women, the plasma Lp-PLA$_2$ activity was positively correlated with IL-6, TNF-α, and IL-1β levels in PBMCs [86]. In postmenopausal women, plasma oxLDL positively correlated with the cytokines generated by PBMCs. However, there is a lack of relationship between Lp-PLA$_2$ activity in plasma and in the PBMCs [86], which indicated that sources of circulating Lp-PLA$_2$ activity other than PBMCs exists in postmenopausal women. This study also indicated that circulating Lp-PLA$_2$ and PBMCs-secreted Lp-PLA$_2$ associate differently with markers of oxidative stress and subclinical inflammation in non-obese women according to the menopausal states [86].

7.11 Diabetes and Lp-PLA$_2$

7.11.1 Type 2 Diabetes

Atherosclerosis-associated diseases are one of the major causes of mortality and morbidity in type-2 diabetes. Plasma lipid profile, including hypertriglycerides, LDL, HDL cholesterol, and apolipoprotein B levels are frequently abnormal in the diabetic patients and that are pivotal for the high prevalence of cardiovascular complications [87].

Barzilay et al. [88] demonstrated that glucose disorders are associated with the increased prevalence of CAD. Kuller et al. [89] also reported that the risk of cardiovascular events was found to be higher for participants with a history of diabetes compared to those with newly diagnosed diabetes at base line in the CAD. Lp-PLA$_2$ activity could, at least, partially explain the greater incidence of CAD outcomes associated with type 2 diabetes [90].

Hyperglycemia and increased oxidative stress, which are hallmarks of diabetes, affect circulating LDL. Diabetes by altering lipoprotein function favour the formation of modified forms of LDL such as glycosylated LDL (gLDL), oxidized LDL (oxLDL), and electronegative LDL (LDL (−)). An increase in oxLDL and gLDL

levels observed in diabetic patients could be attributed to several mechanisms. The sdLDL is known to have an impaired plasma clearance, which could lead to an increase in residence time in blood and favour further modifications. This could be associated with the high susceptibility to oxidation and subsequent non-enzymatic glycosylation that has been demonstrated in sdLDL particles [91]. Younis et al. [92] reported that sdLDL level is an important determinant of LDL glycation. These modified LDLs have a high content of inflammatory lipid metabolites including lysophospholipids, whose concentrations are increased in diabetes [90]. The type-2 diabetic patients who have predominance of sdLDL and gLDL are having higher risk of CVDs. However, the diabetic patients who have large buyont LDL particles are of relatively lower risk of CAD [90, 93].

Diabetes has been demonstrated to change the normal characteristics of HDL fraction. The anti-atherogenic characteristics of HDL such as its role in reversing cholesterol transport and antioxidant properties are perturbed in these patients [94]. These characteristics of HDL are modified due to changes in the relative composition of lipids and proteins and also the enzymatic activities such as paraoxonase (PON-I) and Lp-PLA_2 are associated with it. PON-I is mainly bound to HDL, and upon deactivating lipid peroxides, it exhibits antioxidant function by altering activities of peroxides. In contrast to PON-I, LP-PLA_2 is predominantly associated with only LDL and VLDL, while only marginally with HDL [94, 95].

A decrease of apoA-1 caused lower antioxidant capacity of HDL [93]. Likewise, the higher lipid/protein ratio has been shown to elicit an impairment of the antioxidant properties of HDL, since the HDL3 subfraction (with lower lipid/protein ratio) has a stronger antioxidant capacity than the HDL2 subfraction (with higher lipid/protein ratio) [96]. The attenuation of HDL-3 from patients with type 2 diabetes also point to a decreased ability to promote reverse cholesterol transport [93, 96]. These alterations along with an increase in triglyceride level were observed in type-2 diabetic patients. Although the concentrations of oxLDL and gLDL were found to be increased in type-2 diabetic patients, the relative content of LDL (−) was affected predominantly by the sdLDL component. Thus, although oxidation and glycosylation could be partially involved in the generation of LDL (−), loading of nonesterified fatty acids (NEFA) also play important role for increase in the level of LDL (−) in type 2 diabetes [93].

Importantly, a high concentration of oxLDL and gLDL and a high content of Lp-PLA_2 in apoB containing lipoproteins are present in the diabetic patients [97]. Thus, alterations in the qualitative characteristics of LDL and HDL vis-à-vis Lp-PLA_2 in patients with type 2 diabetes appear to be an important mechanism for cardiovascular disorders.

7.11.2 Type 1 Diabetes

Increasing evidence from epidemiological studies in humans suggests that Lp-PLA_2 is independently associated with the risk of CAD. Patients with type 1 diabetes (T1D) are at increased risk of developing CVD.

In type 1 diabetes the leading cause of death is CAD and that occurs early in life and produces dramatically higher mortality rate. Type 1 diabetes is characterized as a proinflammatory state. Proinflammatory cytokines are expressed in animal models of type 1 diabetes [98]. There is a strong relationship between inflammatory markers (e.g. IL-6, TNF-α) and CVD in type 1 diabetes [99]. Kardys et al. [100] observed an increase in coronary artery Ca^{2+} prevalence in patients with type 1 diabetes. Factors related to coronary Ca^{2+} in type 1 diabetes include inflammatory markers such as CRP and the cytokines such as IL-6 and TNF-α. Progression of coronary calcification with an increased level of Lp-PLA$_2$ has been shown to predict clinical coronary disease events in individuals with type 1 diabetes compared with those without diabetes [101, 102].

Polymorphism of the haptoglobulin gene predisposes CVD risk among patients with diabetes [103]. Haptoglobulin genotype has been suggested to be associated with CAD in the Pittsburgh Epidemiology of Diabetes Complications Study of type 1 diabetes. In the study, type-1 diabetic patients with proteinurea, elevated CRP and Lp-PLA$_2$ levels are associated with an increased risk of CAD [102, 104]. Lp-PLA$_2$ activity alone may add to the prediction of CAD in type-1 diabetic patients, who have a lower haptoglobulin gene expression with genetic predisposition of CAD [103].

7.12 Kidney Disease and Lp-PLA$_2$

An association of Lp-PLA$_2$ with kidney function decline among participants of the Cardiovascular Health Study (CHS) was determined. Lp-PLA$_2$ level was found to be associated with the kidney function decline among elderly without chronic kidney disease (CKD), which was independent of the involvement of CRP and IL-6 [105].

Proposed mechanisms to explain the association between Lp-PLA$_2$ and kidney function decline include increased inflammation or oxidative stress associated with vascular damage, where the associations of inflammatory markers and kidney function are modest [13, 31, 105]. Lp-PLA$_2$ has been demonstrated to be associated with the development of endothelial dysfunction and inflammation and disruption of the arterial intima by oxLDL and gLDL. Lp-PLA$_2$ is also thought to promote endothelial dysfunction and increased arterial stiffness, which are associated with kidney dysfunction; Lp-PLA$_2$ may be a plausible pathway associated with kidney dysfunction [105, 106].

7.13 Obesity and Lp-PLA$_2$

Obesity is developing vary fast in all age groups, but the growth rate has been much more acute in children and adolescents [107]. The metabolic imbalance in obesity supports inflammatory insulin resistance and oxidation of LDL, which favour the early occurrence of atherosclerosis for adolescents who are predisposed with the

diseases like diabetes and abnormal lipid levels [25]. In adolescents, Lp-PLA_2 changes in function of obesity and shows important association with markers of cardiovascular risk, especially with glucose level, and HDL/LDL and ApoB/ApoA ratios. This supports the hypothesis that Lp-PLA_2 may be considered as a biomarker of cardiovascular risk in adolescents [25, 108]. Among the obese, Lp-PLA_2 activity level was found to be higher for men in comparison to women. The lower level of Lp-PLA_2 activity among women could possibly be due to oestrogen-mediated down-regulation of the enzyme [108, 109].

Celik et al. [110] proposed that the incidence of obesity in adolescent may be the first step for atherosclerosis outcome in adults. The monitoring of Lp-PLA_2 in children and adolescents described in literature is scanty and inconclusive. Further studies are required to clearly ascertain whether obesity is associated with change in Lp-PLA_2 in adolescents.

7.14 Pharmacological Inhibition of Lp-PLA_2

Studies in the recent past have shown that various hypolipidemic drugs, e.g. statins, fenofibrate, and ezetimide, decrease plasma Lp-PLA_2 activity due to lowering of LDL-C without appreciably affecting expression of the enzyme [48, 50].

Lp-PLA_2 levels may be lowered by two main pharmacologic interventions—indirectly, by lowering LDL, or directly, by inhibiting Lp-PLA_2 activity. In contrast to the non-lipid-lowering agents used for treatment of cardiovascular disorders, reducing Lp-PLA_2 activity in parallel with reduction in LDL levels has currently gained attention. In a comparison study, rosuvastatin exhibited the most potent effect, whereas fenofibrate only modestly increased HDL-Lp-PLA_2 activity, thereby potentially enhancing the anti-atherogenic effect of HDL [34]. Statin treatment alone has been associated with approximately 20 % reduction in the measurements of Lp-PLA_2 activity in stable CV patients [50, 111]. Similarly, other lipid-modifying drugs such as ezetimide and fenofibrate have only modestly lowered Lp-PLA_2 activity [111]. Although many studies have shown favourable effects of lipid-lowering drugs and statins on cardiovascular outcome, it is currently unknown about the underlying mechanisms associated with the beneficial effects of these drugs on Lp-PLA_2 [34].

Drugs that target Lp-PLA_2 are being developed and its inhibitors are currently being studied in clinical trials by different companies, for example, darapladib (GlaxoSmithKline). Their roles in ameliorating cardiovascular risk and evaluating their pharmacokinetics, safety, and tolerability in patients with CAD are currently being evaluated [34, 112, 113].

Recent study showed that the Lp-PLA_2 inhibitor, darapladib, produces substantial additional reductions in Lp-PLA_2 activity when added to intensive atorvastatin therapy (up to 66 %). This effect was largely independent of atorvastatin doses [34, 112]. Wilensky et al. [113] evaluated the effects of darapladib on atherosclerotic lesions area, composition, and gene expression in diabetic/hypercholesterolemic

(DM-HC) swine. Diabetes mellitus was induced in pigs with intravenous injection of streptozotocin. Subsequently, atherosclerotic lesions were prominent in the control group compared with the darapladib treatment group, where controls demonstrated high risk features. The main necrotic core area was significantly smaller in darapladib treatment group compared with the control group [112]. A study by Mohler et al. [114] also demonstrated the ability of darapladib to produce sustained inhibition of plasma Lp-PLA$_2$ activity in patients with stable CAD or CAD risk equivalents while receiving concomitant atrovastatin therapy.

Lp-PLA$_2$, an emerging biomarker of CV risk, can be pharmacologically modified, for example, by darapladib. Thus, Lp-PLA$_2$ is a potentially important component to focus on the mechanisms associated with CAD. Long-term administration of darapladib decreases chronic heart diseases and also reduces the risk of heart diseases. The effect of darapladib was shown to be independent of base line LDL-C and HDL-C levels [23]. In regard to safety, no adverse effects of darapladib were observed and no major concern on platelet activity has emerged from any study.

7.15 Conclusions

Lp-PLA$_2$ has both proatherogenic and anti-atherogenic properties. The anti-inflammatory properties are related to the ability of Lp-PLA$_2$ to hydrolyze the inflammatory mediator, PAF. This as well as HDL-linked actions may explain the attenuating effect of Lp-PLA$_2$ on myocardial ischemia-reperfusion injury. On the contrary, Lp-PLA$_2$ can exert proinflammatory effects by generating oxidized nonesterified fatty acids and lysophospholipids from phospholipids. The proinflammatory effects include stimulation of chemotaxis and tissue accumulation of macrophages. Furthermore, Lp-PLA$_2$ may negatively affect endothelial cell viability and function, which could be of pivotal significance for the overall pathophysiologic role in atherogenesis.

High tissue levels of Lp-PLA$_2$ have been detected in vulnerable and ruptured plaques, whereas plasma Lp-PLA$_2$ activity was found to be elevated to those artery patients in whom plaque rupture could be established by IVUS. Lp-PLA$_2$ derived from ruptured coronary plaques may contribute to higher circulating Lp-PLA$_2$ levels in early phases of CAD. ApoE genotype has been shown to be associated with CAD. Lp-PLA$_2$ level has also been found to be higher in apoE4 carriers. However, familial factors, to some extent, could explain the variance of Lp-PLA$_2$ activity since few genetic determinants of Lp-PLA$_2$ related to LDL level have been identified. Further studies are needed to clearly ascertain genetic control of Lp-PLA$_2$.

The association of plaque Lp-PLA$_2$ with plaque macrophages and *C. pneumoniae* suggests an interactive role in accelerating inflammation in atherosclerosis. A possible mechanism for *C. pneumoniae* in the atherogenic process may involve infection of macrophages that induce Lp-PLA$_2$ production leading to up-regulation of inflammatory mediators in plaque tissue. Additional research is needed to advance the present understanding of *C. pneumoniae* and Lp-PLA$_2$ interaction in atherosclerosis.

Type-2 diabetic patients with elevated levels of Lp-PLA_2 activity are more likely to develop CVDs than those without elevated levels of the enzyme. However, it is possible that Lp-PLA_2 is not sufficiently sensitive as a single marker, given all the other factors that contribute to CVD risks, for example, in diabetic patients. Further research is needed to ascertain the clinical significance of Lp-PLA_2 as a marker for CVD risk among different populations.

Acknowledgments Thanks are due to the University Grants Commission, New Delhi and Indian Council of Medical Research, New Delhi for partly financing our research.

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Chapter 8
Phospholipase A_2 Activity Exhibited by a Bacterial Virulence Protein That Enters and Operates Within a Variety of Host Cells

Bryan P. Hurley

Abstract Phospholipase A_2 (PLA_2) is a pivotal enzyme in human health and disease. With approximately 30 isoforms exhibiting PLA_2 activity expressed within or secreted by a variety of cell and tissue types, the functional significance of this enzyme is multifaceted. Mammalian PLA_2s are known contributors during host–pathogen interactions during infectious disease processes. Interestingly, several bacterial pathogens themselves express PLA_2 enzymes that exhibit a patatin domain and have sequence similarity to cytosolic and calcium-independent PLA_2s. The most prominent example termed ExoU is expressed by *Pseudomonas aeruginosa* and operates exclusively in eukaryotic host cells. ExoU serves as a potent cytotoxin expressed by *P. aeruginosa* clinical isolates most associated with severe acute pneumonia and microbial keratitis. The PLA_2 activity of ExoU is responsible for this potent toxicity and is also capable of mediating host production of eicosanoids and stimulating cytokine and chemokine production in a variety of cell types. Efforts are underway to better understand and potentially neutralize these potent microbial PLA_2 virulence factors.

Keywords Phospholipase A_2 • ExoU • *Pseudomonas aeruginosa* • Eicosanoids

8.1 Introduction

There are approximately 30 individual enzymes expressed in mammalian cells that possess phospholipase A_2 (PLA_2) activity [1, 2]. These enzymes participate in a wide array of cellular processes in a variety of distinct tissues and exhibit numerous

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_8,

redundant as well as non-redundant functions [1, 2]. Individual PLA_2 enzymes can be distinguished from one another based on several criteria including whether they are secreted or operate within the cell. The main classes of mammalian PLA_2s include secretory PLA_2s (sPLA_2s), cytoplasmic PLA_2s (cPLA_2s), calcium-independent PLA_2s (iPLA_2s), lysosomal PLA_2s (L-PLA_2s), and platelet-activating factor acetylhydrolases (PAF-AH) [1, 2]. Critical physiological processes are ascribed to PLA_2s acting both inside the cell and within the extracellular environment of tissues to promote proper functioning of multiple organs including the heart, lung, and brain [1, 2]. As such, aberrant activity of PLA_2s has been widely reported in several different disease states that manifest within multiple organ systems [1, 2].

Disease resulting from infection from various microbial pathogens is also known to involve PLA_2s [1, 2]. Several isoforms from each of the classes of PLA_2 have been demonstrated to participate in the host response to infection. Members of the sPLA_2 group, particularly group IIA, possess antibacterial activity capable of causing damage to Gram-positive and Gram-negative bacterial organisms by virtue of their ability to cleave bacterial membrane phospholipids [1–3]. Intracellular PLA_2s, particularly c$PLA_2\alpha$, are activated upon cellular sensing of pathogen presence by macrophages, fibroblasts, and epithelial cells through MAP kinase-mediated phosphorylation resulting in the production of inflammatory modulating eicosanoids [2, 4–7]. For example, it has been demonstrated that *Pseudomonas aeruginosa* is capable of stimulating c$PLA_2\alpha$ phosphorylation and translocation to the membrane in epithelial cells whereby eicosanoids such as prostaglandin E_2 (PGE_2) are readily produced and serve as immune modulating factors [4, 5, 7]. Interference with PLA_2 activity can thus have a range of effects on the ability of the host to respond to infection depending on the context as well as the specific isoform(s) involved.

In addition to these diverse host PLA_2s participating in the infectious process, it has also become appreciated that bacterial pathogens themselves possess enzymes exhibiting PLA_2 activity [2, 8–10]. These bacterial enzymes have the ability to exert a multitude of effects on various cells of the infected host [2, 8–10]. This theme of bacterial-derived PLA_2s will be highlighted in this chapter by reviewing the current state of knowledge of the widely investigated *Pseudomonas aeruginosa* exotoxin known as ExoU [8, 9]. ExoU is a potent toxin produced by a subset of *Pseudomonas aeruginosa* clinical isolates that can dramatically enhance the virulence of this organism [8, 9]. As will be discussed in detail, ExoU is a functional PLA_2 and this enzymatic activity underlies its cell-associated toxicity as well as its enhanced virulence in animal models and in the clinical settings [8, 9]. The enhanced virulence is likely due not only to the cell-associated toxicity but also to other more subtle PLA_2-mediated activities on various cell types that serve to drive the disease process [2, 8, 9]. Thus, ExoU represents an important example of a pathogen-derived PLA_2 that bears a profound impact on the infectious disease process.

8.2 Disease Caused by Infection with *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is considered an opportunistic pathogen that can infect a range of tissues from injured or immune-compromised hosts leading to significant morbidity and mortality [11, 12]. *Pseudomonas aeruginosa* is a common microbe in the environment and displays a propensity for being resistant to a myriad of antibiotics reinforcing it as a significant contributor to nosocomial infections [13]. A major site of infection for *P. aeruginosa* is the lung. *P. aeruginosa* can instigate an acute pneumonia in immune-compromised individuals or it can establish a chronic infection by virtue of adopting a mucoid phenotype that promotes cell to cell association forming a complex intractable biofilm that persists within the lungs of individuals with cystic fibrosis [11]. Cystic fibrosis is a genetic condition involving the inheritance of a defective chloride channel known as the cystic fibrosis transmembrane conductance regulator (CFTR) expressed on the apical surface of epithelial cells. Epithelial cells lining the airway that express defective CFTR are marked by production of dehydrated mucus and dysfunctional cilia that fail to clear microbes, particularly *P. aeruginosa* resulting in chronic infection, high levels of inflammation, and eventually organ failure [11].

In addition to targeting the lung, *P. aeruginosa* is also well known to infect vulnerable sites exposed due to injury [8, 12, 14]. Patients with severe wounds and burns are often infected with *P. aeruginosa*, further compromising these damaged tissues [8]. *P. aeruginosa* is a major cause of microbial keratitis that can manifest following injury to the cornea or as a complication of contact lens wear [12, 14]. Microbial keratitis causes major damage to the cornea, which can result in the loss of vision [12, 14]. Clearly a better understanding of this problematic, yet ubiquitous, pathogen and how it drives inflammation and disease would greatly assist the medical community.

8.3 Mechanisms of *Pseudomonas aeruginosa*-Mediated Virulence

P. aeruginosa is an extremely versatile organism, capable of thriving in a diversity of niches and resisting a range of potentially toxic compounds [15]. An enormous amount of the *P. aeruginosa* genome (almost 10 %) is tasked with coding for regulatory proteins that serve to rapidly sense and respond to changing environmental conditions endowing this organism with an unusually enhanced capacity for adapting [16]. Several virulence factors expressed by *P. aeruginosa* have been identified and their roles have been characterized during infection [8, 15]. Through the cooperation of a multitude of proteins, *P. aeruginosa* wield a flagellum, which facilitates

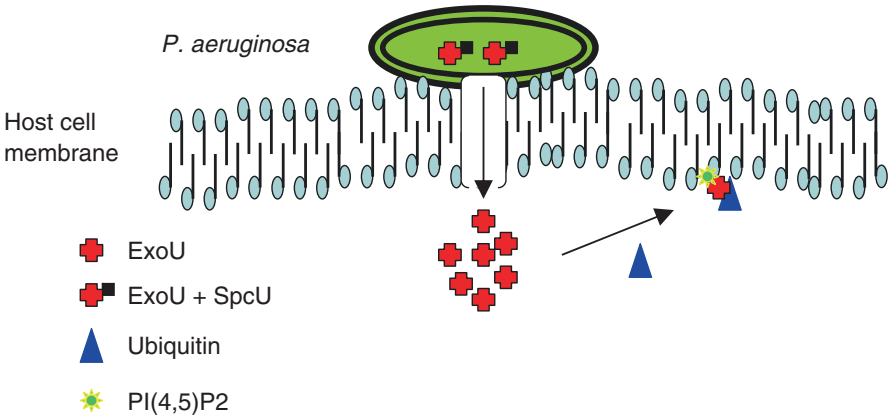

Fig. 8.1 ExoU produced by *Pseudomonas aeruginosa* utilizes the type three secretion system (TTSS) in association with its bacterial-encoded chaperone SpcU. ExoU is delivered by the TTSS directly from the bacterial cytoplasm to the cytosol of the infected host cell. This is accomplished through construction of a needle-like appendage by multiple proteins that span multiple lipid bilayers from bacterial and host membranes. Once inside the cell, ExoU locates to the plasma membrane and interacts with the eukaryotic-encoded cofactors ubiquitin and PI(4,5)P2 enabling phospholipase activity towards membrane lipid substrates. At present it is unclear whether ExoU encounters ubiquitin or ubiquitinylated proteins in the cytosol prior to PI(4,5)P2 and membrane association or whether ubiquitin associates with ExoU after membrane association

motility and contributes towards colonization of susceptible hosts [17]. Flagellar proteins are also involved in cross talk between pathogen and host [18, 19]. A key structural protein of the flagellum known as flagellin engages the host immune system by associating with pattern recognition receptors and triggering the production of chemokines and cytokines [19]. *P. aeruginosa* possess pili on their surface, which also contribute to bacterial motility as well as mediating attachment to biotic and abiotic surfaces [18, 20]. Components of pili can also stimulate host innate immune responses [18, 20]. Other surface constituents of *P. aeruginosa* include exopolysaccharides such as LPS and alginate that can protect *P. aeruginosa* from an onslaught of antibiotics or antimicrobial substances elicited by recruited host immune cells such as neutrophils [11, 21, 22]. These exopolysaccharides assist individual bacteria in forming complex protective biofilms [23]. Similar to flagella and pili, these molecules are also capable of alerting the host immune system [11, 18, 21, 22].

Despite their direct roles in mediating disease, attributes of *P. aeruginosa* such as pili, flagella, and exopolysaccharides are shared by pathogens and non-pathogens alike. *P. aeruginosa* also possess a secretion system believed to be unique to pathogenic organisms that is capable of delivering toxins directly into host cells [8]. This multi-protein complex is known as the type three secretion system (TTSS) and is shared by a variety of Gram-negative pathogens [8]. A needle-like structure spans both the bacterial and host cell membranes providing a conduit for bacterial-encoded toxins to access the cytosol of host cells without being exposed to the extracellular environment (Fig. 8.1) [8]. In the case of *P. aeruginosa*, four toxins have been

described that leverage the TTSS for direct access to the host cell cytosol and these toxins include ExoS, ExoT, ExoY, and ExoU [8]. ExoS and ExoT are dual functioning enzymes with N-terminal GTPase activating protein activity and C-terminal ADP ribosylase activity that each acts upon host proteins to alter host cell behavior [8]. ExoY exhibits adenylate cyclase activity [8]. ExoU was initially described as a potent cytotoxin that rapidly kills multiple cell types and subsequently discovered to be a PLA_2 that operates from inside of host cells, as will be described in detail below [8].

8.4 Discovery of ExoU as a Potent Cytotoxin

Over a decade ago, it became appreciated that a sizable subset of *P. aeruginosa* isolates induced rapid cytotoxicity towards mammalian host cells [24–26]. Curiously, the previously identified *P. aeruginosa* toxin ExoS appeared to be absent from these acutely cytotoxic strains [24–26]. The cytotoxic phenotype could not be explained by the presence of another known *P. aeruginosa* toxin ExoT; however, the TTSS responsible for injecting various exotoxins into host cells did appear to be critical for endowing these strains with the ability to rapidly kill infected cells [24–26]. This phenotype was ultimately determined to arise from the actions of a distinct 70-kDa TTSS effector protein termed ExoU [24, 26]. Not only is ExoU exquisitely toxic towards cells cultured in vitro, but ExoU was also demonstrated to significantly enhance lethality during acute pneumonia in rats and mice as well as facilitate bacterial dissemination and sepsis in rabbits [27, 28]. The N-terminus of this toxin has similarity to ExoS and ExoT and promotes interaction with the TTSS allowing injection into host cells [29]. Sequence downstream from the initial 100 amino acids was subsequently identified to be responsible for the cytotoxic phenotype [30]. A chaperone protein termed SpcU is transcribed within the same operon as ExoU and facilitates ExoU secretion (Fig. 8.1) [31]. Once injected into cells through the TTSS, ExoU has the capacity to rapidly kill a variety of mammalian cells including epithelial cells, endothelial cells, fibroblasts, neutrophils, and macrophages (Fig. 8.2) [24, 32–36]. In addition to targeting mammalian host cells, ExoU also exhibits cytotoxicity towards single-celled amoeba (*Dictyostelium discoideum*) and yeast (*Saccharomyces cerevisiae*), demonstrating a wide and diverse range of potentially susceptible target cells (Fig. 8.2) [35, 37].

ExoU is detected in approximately one third of *P. aeruginosa* clinical isolates and its presence tends to be associated with worse clinical outcomes [8, 10, 38, 39]. Within the population of *P. aeruginosa* isolates analyzed in the context of severe disease, an estimated 90 % are ExoU-positive strains [40]. Interestingly, expression of ExoU and ExoS is mutually exclusive, with about two thirds of the clinical isolates being ExoS positive/ExoU negative [8]. ExoU is thus considered a marker for highly virulent strains in the context of acute infections such as nosocomial pneumonia, wound infections, and microbial keratitis but appears to be underrepresented in chronic infections such as those associated with cystic fibrosis [8, 11]. Clinical isolates containing ExoU have a greater propensity to facilitate bacterial

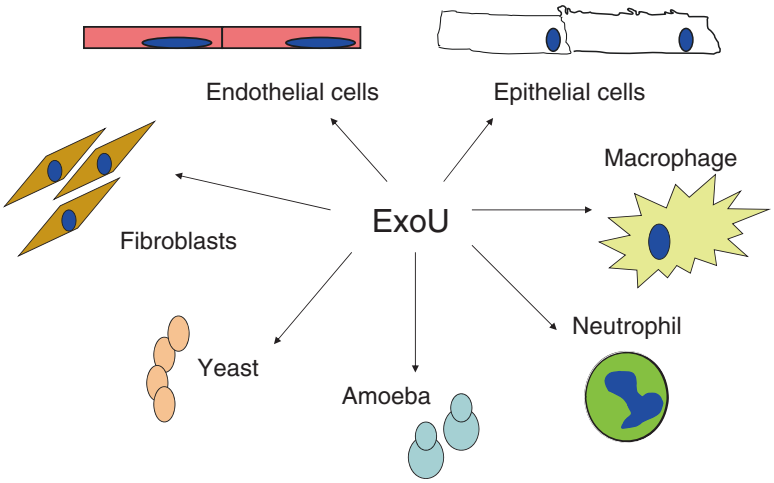

Fig. 8.2 A diverse array of cell types are susceptible to ExoU-mediated killing including fibroblasts, epithelial cells, endothelial cells, and macrophages of mammalian tissue as well as unicellular eukaryotic species such as *Dictyostelium discoideum* (amoeba) and *Saccharomyces cerevisiae* (yeast)

dissemination leading to sepsis [8, 9, 28]. Studies employing both in vitro and in vivo experimental models as well as investigations characterizing *P. aeruginosa-mediated* human diseases all strongly point to the notion that ExoU represents a highly potent cytotoxin that serves as a major contributor to disease pathogenesis, yet it was initially unclear the mechanism by which ExoU evoked such lethality and cell death.

8.5 ExoU Identified as a Phospholipase A_2

An early clue regarding the enzymatic activity that underlies the toxicity of ExoU was the observation from amino acid sequence analysis that this bacterial toxin featured a patatin domain [35]. Enzymes that possess a patatin domain exhibit a catalytic dyad distinct from the catalytic triad of classical lipases [1, 2]. Patatin, an abundant potato tuber protein, is the original enzyme described to possess this domain; however, it is now appreciated that several mammalian enzymes exhibiting PLA_2 activity encode this key domain [1, 2]. These mammalian enzymes include $cPLA_2\alpha$ and $iPLA_2\beta$ described above [1, 2]. Inhibitors of $cPLA_2\alpha$ and $iPLA_2\beta$ (catalytic serine), but not $sPLA_2$s (catalytic histidine), are capable of preventing ExoU-mediated cytotoxicity [40, 41]. Key features of the patatin domain present in the sequence of ExoU include a glycine-rich nucleotide binding motif G-X-G-X-X-G/A, a serine hydrolase motif G-X-S-X-G at ser-142, and the conserved active site aspartate residue D-X-G/A at asp-344 (Fig. 8.3) [9, 41]. Through mutational analysis, it was concluded that the ser-142 and asp-344 are essential to the ability of ExoU to

Fig. 8.3 Primary amino acid sequence of ExoU highlighted with key residues including the patatin domain (*highlighted in black*) and the ubiquitination site (*underlined in red*)

cause epithelial cell damage and acute lung injury [34]. Further it has been determined that these residues, endowing ExoU with PLA_2 activity, are also critical for promoting *P. aeruginosa* colonization and corneal disease-related pathology in a corneal scratch mouse model of infection [12].

ExoU has broad substrate specificity acting on phospholipids and neutral lipids and, like $cPLA_2$ and $iPLA_2$ isoforms, also exhibits lysophospholipase activity [42]. ExoU is incapable of exerting cytotoxicity from outside the cell and must be present within the cell where it is able to interact with cytosolic cofactors and access substrate at the cytosolic face of the plasma membrane [9]. The interaction of ExoU with lipid substrates at the plasma membrane is thought to disturb lipid metabolism and compromise cell membrane integrity leading to death of the cell [9]. Initially, evidence pointed to superoxide dismutase 1 (SOD1) as the eukaryotic cofactor that interacts with ExoU [43]. It was later determined that the SOD1 mediated enhancement of ExoU activity as a consequence of ubiquitinylated SOD1 and it was truly ubiquitin and ubiquitinylated proteins that activate ExoU by associating with ExoU and altering its conformation facilitating ExoU interactions with substrate within the plasma membrane (Fig. 8.1) [44, 45]. The C-terminal region of ExoU appears to be important in this regard [46, 47]. ExoU also associates with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), which serves as an additional cofactor that works in conjunction with ubiquitin to enhance ExoU activity (Fig. 8.1) [48]. Interestingly, PI(4,5)P2 is capable of binding to the mammalian $cPLA_2\alpha$, as well as ExoU, serving a similar role in terms of enhancing membrane association and enzymatic activity [49]. Requirement of eukaryotic cofactors such as ubiquitin, ubiquitinylated proteins, and PI(4,5)P2 for the functioning of potent toxins such as ExoU is likely to focus the activity of ExoU at the intended target and to prevent ExoU from directly acting upon and harming the very bacteria that produce it [9]. In addition to association with ubiquitin as a key eukaryotic cofactor to facilitate activation, ExoU itself can also be ubiquitinated within the host environment. As a consequence of

membrane localization, ExoU is ubiquitinated at lys-178 (Fig. 8.3) [50]. Thus it is clear that the *P. aeruginosa* toxin ExoU has an intricate relationship with the host cell. This relationship can result in disease through rapid host cell death as a consequence of the PLA_2 activity of ExoU that is directed towards lipids in the plasma membrane. Mammalian PLA_2 enzymes, which are diverse and abundant within a variety of cell types, are not meant to be toxic towards the cells that produce them [1, 2]. These enzymes, including $cPLA_2\alpha$, exert less dramatic but critically important effects on cellular processes [1, 2]. Interestingly, several investigators have observed that in certain circumstances, ExoU can also have a more subtle influence on host cells and these effects have an impact on the disease process.

8.6 Role of ExoU Beyond Cytotoxicity During Disease

Since ExoU is a highly lethal toxin to many cells, it has been naturally assumed that the phospholipase activity exhibited by this enzyme is primarily tasked with facilitating the destruction of cells by dismantling cell membranes [27, 34, 35, 38, 40–42]. Mammalian-encoded PLA_2s participate in a wide array of cellular activity through their ability to generate arachidonic acid, which is the substrate of an important class of diverse inflammatory and anti-inflammatory lipid mediators known as eicosanoids [1, 2]. Indeed, these functions have also been ascribed to ExoU, suggesting that, under certain circumstances, a pathogenic approach is adopted by ExoU producing *P. aeruginosa* that is based on manipulating host cellular process rather than directly attacking the host cell. Endothelial cells were demonstrated to produce the eicosanoids PGE_2 and prostacyclin (PGI_2) as a result of infection with *P. aeruginosa* harboring ExoU [36]. Enhanced release of the eicosanoid precursor and PLA_2 enzymatic product arachidonic acid was observed to require ExoU with functional PLA_2 activity [36, 51]. In addition, ExoU expression was associated with increased PGE_2 release in the airspace of mice during acute *P. aeruginosa* pneumonia [36, 51]. These lipid mediators produced as a consequence of PLA_2 activity from ExoU may contribute to increased bacterial dissemination and septic shock during infection [36, 51]. The presence of ExoU within airway epithelial cells also can result in the overproduction of PGE_2, which may impact the inflammatory response during infection [52]. Whether ExoU is capable of releasing arachidonic acid in host cells leading to enhanced production of other eicosanoids with distinct functions such as leukotrienes, lipoxins, or hepoxilins is unclear. The ability of ExoU to direct the production of lipid mediators through PLA_2-mediated arachidonic acid release is likely balanced with ExoU-mediated membrane disruption and cell death. This balance likely depends on the intracellular ExoU localization, level of ExoU intoxication, and cell types harboring ExoU, each aspect guiding the *P. aeruginosa* infectious process.

In addition to directly impacting eicosanoid synthesis through PLA_2-mediated release of arachidonic acid, ExoU is also capable of stimulating the production of cytokines, chemokines, and adhesion molecules involved in inflammation and

cellular recruitment [10, 53]. ExoU was shown to mediate IL-8 release in airway epithelial cells through the c-Jun NH_2-terminal kinase pathway and the NFκB pathway [54]. Activation of this pathway was dependent on the PLA_2 activity, but not on cell death. Modulation of intracellular adhesion molecule 1 (ICAM-1) on endothelial cells by specifically reducing the membrane-bound form and increasing the soluble form was observed to occur in an ExoU-dependent fashion [53]. ExoU has also been shown to be capable of directly impacting the inflammasome [55]. *P. aeruginosa* strains that lack ExoU are capable of killing macrophages through stimulation of the IPAF/NLRC4 inflammasome leading to caspase-1 activation and IL-1β release. In contrast, *P. aeruginosa* expressing ExoU destroy macrophages through a caspase-1-independent pathway whereby ExoU inhibits caspase-1 activation in a PLA_2 activity-dependent manner [55]. Clearly the PLA_2 activity exhibited by ExoU is capable of manipulating host cells in ways far beyond simply destroying the phospholipid membrane and causing host cell death.

8.7 Other Microbial Factors with Similarity to ExoU

There is emerging evidence that other pathogenic bacteria possess virulence factors with similarity to ExoU [56, 57]. *Legionella pneumoniae* is a Gram-negative lung pathogen that causes a severe pneumonia commonly referred to as Legionnaires' disease [57]. A virulence factor named VipD has been identified that is injected into host macrophages by a secretion system distinct from type III known as the type IV secretion system [57]. VipD shares significant sequence similarity with ExoU including a conserved phospholipase domain. VipD demonstrates a mild toxicity when expressed in yeast, which is partially abrogated when PLA_2 active site serine and aspartate are replaced with alanine [57]. Like ExoU, VipD exhibits PLA_2 activity and this activity contributes to the mild toxicity observed. Recent evidence suggests that VipD targets the mitochondrial membrane resulting in the hydrolysis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) [58]. As a consequence of VipD hydrolysis of membrane PE and PC, free fatty acids and 2-lysophospholipids are released. These lipid mediators contribute to cytochrome c disassociation from the mitochondrial membrane, which most likely leads to activation of caspase-3 [58].

ExoU homologues have also been identified in *Rickettsia* species [56, 59]. *Rickettsia* species such as *R. prowazekii* and *R. typhi* are Gram-negative obligate intracellular pathogens and certain *Rickettsia* species represent serious human pathogens [56, 59]. The presence of PLA_2 activity amongst *Rickettsia* species has been appreciated for several years and thought to potentially facilitate host cell entry, lysis of host cell vacuoles, and/or lysis of host cells [56, 59]. Recently genes that possess PLA_2 activity have been identified and these genes have considerable sequence similarity to ExoU including a patatin domain with an active site serine and aspartate. Proteins encoded by these genes termed pat1 and pat2 are released into host cell cytoplasm and likely play a role in the infectious process [56, 59].

8.8 Conclusions

Enzymes that exhibit PLA_2 activity are diverse, abundant, and serve major roles in countless cellular functions. For this reason, impairment of PLA_2s or altered behavior manifests as the underpinnings of a variety of diseases. It is thus not surprising that pathogenic bacteria have adopted strategies to co-opt this enzymatic activity to facilitate survival and spread in a eukaryotic host cell environment. ExoU is a grand example of this phenomenon and clearly has direct and independent impact on a variety of disease processes through its PLA_2 enzymatic activity. Other pathogens seem to have evolved this strategy, as newly emerging bacterial genes that possess patatin domains are being discovered. Elucidation of the multifaceted roles of ExoU upon intoxication of cells following *P. aeruginosa* colonization of different tissue and cell types will ultimately provide the critical knowledge needed to effectively combat these serious and intractable human infections.

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Chapter 9
Expression and Role of Phospholipase A_2 in Central Nervous System Injury and Disease

Samuel David and Rubèn Lòpez-Vales

Abstract Phospholipase A_2 (PLA_2) enzymes hydrolyze membrane phospholipids to generate a free fatty acid and a lysophospholipid. These products of PLA_2 can generate about two dozen different bioactive lipid mediators that bind to specific receptors to regulate a wide variety of responses that modulate inflammation. They also play roles in normal physiological functions in the nervous system, which for the most part still remain to be fully elucidated. PLA_2s are therefore at the apex of a pyramid, downstream of which are a large number of other enzymes that give rise to an even larger number of mediators. Modulating the activity of PLA_2s can therefore influence a number of downstream pathways and may serve as a focal point for therapies. There are about two dozen mammalian PLA_2s but only some members of this superfamily have so far been reported to be expressed in the nervous system. In this chapter, we will review the evidence for the expression and role of PLA_2s in the nervous system. The main focus, however, will be on the work we have done on their role in two neurological conditions—spinal cord injury and experimental autoimmune encephalomyelitis.

Keywords Phospholipase A_2 • Spinal cord injury • Multiple sclerosis • Experimental autoimmune encephalomyelitis • CNS inflammation • Demyelination

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_9,

9.1 Introduction

Although much work has been done on the role of phospholipase A_2 (PLA_2) in various non-neural tissues, the involvement of PLA_2 in the nervous system is still an emerging field. Of the 16,740 publications of PLA_2s in the past 45 years, only 414 of them relate to the nervous system. Given the large number of PLA_2s and their diverse functions in mediating a wide variety of cell and tissue responses, we are likely to see more evidence of their involvement in both the functioning of the normal nervous system and neurological disorders. In this chapter, we will focus attention on the involvement of PLA_2s in two very different models of central nervous system (CNS) inflammation—that following spinal cord injury (SCI) and in experimental autoimmune encephalomyelitis (EAE) a widely used model for multiple sclerosis.

Injury to the CNS triggers an inflammatory response that is initiated within minutes. This injury-induced inflammation in CNS tissue is maximal during the first 2 weeks and extends for several weeks thereafter [1]. Although the inflammatory response to any tissue injury is meant to restore tissue homeostasis, fight infections, and initiate wound healing, some aspects of this response can cause unwanted tissue damage and scarring. This can cause major problems in the CNS which unlike many other tissues has a very limited capacity for tissue regeneration, replacement of damaged neurons and oligodendrocytes, and axon regeneration. This is in sharp contrast to peripheral nerves in which the inflammatory response after injury plays an important role in facilitating axonal regeneration [2, 3]. We will focus here on our studies on SCI in which inflammation has been shown to mediate secondary tissue damage that includes loss of neurons and myelin, and loss of tissue integrity that leads to further loss of axons [4, 5]. Preventing or reducing inflammation-induced secondary damage after SCI ameliorates functional loss and can optimize the tissue environment for repair. Inflammation in the injured spinal cord immediately after injury is mainly an innate immune response involving macrophages, microglia, and neutrophils, while at later times some aspects of the adaptive immune response are also observed, that involves T and B lymphocytes and antibody production [6–8]. Inflammation after SCI therefore involves many cell types, including resident CNS cells and immune cells from the periphery; and a variety of extracellular immune mediators and intracellular signaling pathways [5]. Multiple pathways contribute to different aspects of this inflammatory response. Immune mediators from which several pathways emerge and influence different aspects of the inflammatory response are therefore likely to be ideal therapeutic targets. The PLA_2 superfamily and its downstream mediators are one such multifunctional system which regulates various aspects of the inflammatory response and is additionally relevant in CNS injury because one of the major products of these enzymes, lysophosphatidylcholine (LPC), is a potent demyelinating agent. This is relevant, as demyelination of intact axons is thought to be an important factor contributing to functional loss after SCI [9–11]. The other CNS condition we will discuss is the role of PLA_2 in EAE. The neuropathology of EAE is characterized by multiple focal inflammatory lesions in the spinal cord that contain areas of demyelination and

axon loss [12]. Unlike the mainly innate immune response in SCI, EAE is a CNS autoimmune disease. It is generated by immunizing mice with a myelin antigen and adjuvants, which trigger a Th1, Th17 CD4 T cell response. This adaptive immune response also involves CD8 T cells, as well as activation of macrophages and microglia, and a range of pro-inflammatory chemokines and cytokines [12]. Following immunization, T cells become activated in the peripheral lymph nodes, and subsequently leave the lymphoid tissue to enter the CNS via the circulation. On entering the CNS, these cells become reactivated, leading to the influx into the CNS of macrophages from the peripheral circulation. The entry of these immune cells from the periphery also triggers activation of resident glial cells including microglia and astrocytes that then set up an inflammatory environment that leads to myelin loss and damage to oligodendrocytes, as well as axons and neurons. As with SCI, multiple pathways are involved in triggering the immune response in EAE. We found that the PLA_2 superfamily is involved in the pathogenesis of EAE. Importantly, we see striking differences in the role of different members of the PLA_2 superfamily in SCI and EAE. In this chapter, we will compare these differences and discuss the potential relevance of these findings in pathogenesis and as therapeutic targets. We will first provide a brief overview of PLA_2s and some of their downstream products that mediate diverse pro-inflammatory and pro-resolution responses.

9.2 Phospholipase A_2

PLA_2s hydrolyze the acyl bond at the *sn*-2 position in membrane phospholipids resulting in the release of a free fatty acid and the formation of a lysophospholipid [13, 14]. If the fatty acid released is arachidonic acid (AA) it can via the cyclooxygenase 1 and 2 (COX-1, COX-2) enzymes give rise to prostaglandins (PGs) (e.g., PGE2, PGD2, 15dPGJ2, PGI2) and thromboxanes, or via lipoxygenase (LOX) enzymes produce leukotrienes (LT) (LTB4, LTC4, LTD4, LTE4). These eicosanoids have diverse effects in triggering inflammation. If omega-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are released instead of AA (an omega-6 fatty acid), they can give rise to pro-resolution mediators via the actions of 5-LOX and 12/15-LOX. These pro-resolution mediators called resolvins of the D or E series, and maresins, work actively to switch off inflammation [15]. In addition, if the lysophospholipid that is formed is LPC, it can give rise to platelet activating factor, and lysophosphatidic acid. LPC itself is a potent demyelinating agent that can induce rapid demyelination in the adult mammalian CNS [16]. LPC can also induce the expression of pro-inflammatory chemokines and cytokines including MCP-1, MIP-1α, GM-CSF, and TNF-α in the CNS [17]. PLA_2s therefore give rise to a number of bioactive lipid mediators that produce a wide range of responses that contribute to inflammation.

The PLA_2 superfamily consists of secreted and intracellular forms that include about 21 different mammalian forms—12 secreted PLA_2s ($sPLA_2$) ($sPLA_2$ GIB, IIA, IIC, IID, IIE, IIF, III, V, VII, X, XIIA, and XIIB), and 9 intracellular forms that

are grouped into 6 calcium-dependent PLA_2s (cPLA$_2$ GIVA, IVB, IVC, IVD, IVE, IVF) and 3 calcium-independent PLA_2s (iPLA$_2$ GVIA, VIB, VIC). Only some of these have been reported to be expressed in nervous system. sPLA$_2$ IIA and V are expressed in various types of inflammation in the CNS [18–22]. sPLA$_2$ has also been shown to be involved in pain [23]. There is also evidence that sPLA$_2$ group X is expressed by subsets of neurons in the dorsal root ganglia and mediates pain responses, as well as neurite growth in vitro [24, 25]. sPLA$_2$ group X is also expressed by parasympathetic neurons and influences autonomic responses [25, 26]. cPLA$_2$ has been shown to be involved in stroke, EAE, SCI, and Wallerian degeneration in peripheral nerve [3, 19, 21, 27] and also shown to mediate pain responses [28]. Recent work has identified mutations in human iPLA$_2$ GVIA (*PLA2G6*) in infantile neuroaxonal dystrophy, neurodegeneration with brain iron accumulation (NBIA) [29, 30]. iPLA$_2$ GVIA null mice [31, 32] as well as *Pla2g6* mutations generated in mice by *N*-ethyl-*N*-nitrosourea also show similar axonal degeneration pathology [33]. Increase in sPLA$_2$ activity was detected in the CSF of Alzheimer's patients [34], while intracellular PLA$_2$ (cPLA$_2$ GIV and iPLA$_2$ GVI) activity is reduced [35]. We will focus our discussion here on the expression and role of various members of the PLA$_2$ superfamily in SCI and EAE.

9.3 PLA$_2$ in Spinal Cord Injury

In the last decade several studies have highlighted the importance of PLA$_2$ enzymes and their downstream products in the onset and resolution phase of the inflammatory response [36]. As previously mentioned, AA produced by the action of the PLA$_2$ is metabolized by COX-1/2 and lipooxygenase enzymes to generate eicosanoids. COX-2, the rate limiting enzyme for the production of prostaglandins, is up-regulated in SCI. COX-2 is increased within hours after SCI and remains elevated for several days thereafter [37–39], while increased COX-1 expression is seen for several weeks after SCI in rats and for several months in human brain injury [40, 41] suggesting that there might be continued production of prostanoids that sustain inflammation for prolonged periods after CNS injury. Levels of PGE2 are significantly augmented in the spinal cord over the first 72 h after injury [38]. However, recent data shows increased levels of PGE2 in the contused spinal cord at 9 months following injury, although at lower levels as compared to 24 h after lesion [42]. We have reported that PGD2 synthesis is not induced in the contused spinal cord over the first 5 days post-lesion, but increases threefold at day 14 [43]. It is not known whether it remains elevated beyond this time point. Leukotrienes, which are generated from AA via 5-LOX, are also important regulators of the inflammatory response and vascular changes [36]. Although the expression of LOX enzymes has not been characterized in SCI yet, LTC4 and LTB4 levels are increased rapidly post-injury [44, 45], and LTB4 remains at high levels in the spinal cord at 9 months after injury [42].

The production of eicosanoid during the acute phase of SCI appears to be an important contributor to secondary damage as pharmacological inhibition of COX-2

Table 9.1 Cellular localization and role of the three PLA2s that are up-regulated in SCI and EAE

Type	Localization			Role	
	Naive	SCI	EAE	SCI	EAE
$cPLA_2$ GIVA	Neurons	Neurons	Neurons	Protective	Detrimental
$iPLA_2$ GVIA	Oligodendrocytes	Oligodendrocytes	Oligodendrocytes	Detrimental	Detrimental
$sPLA_2$ GIIA	Oligodendrocytes	Oligodendrocytes	Macrophages	Detrimental	Unknown
	Not expressed	Astrocytes	T cells		
		Neurons	Oligodendrocytes		
		Microglia/ macrophages	Macrophages		
		Oligodendrocytes	T cells		
		Astrocytes	Astrocytes		
		Neurons	Oligodendrocytes		
		Microglia/ macrophages	Macrophages		
			T cells		

[38, 46, 47] and LOX [48], as well as gene deletion of 5-LOX [49], results in greater locomotor recovery and neuroprotection. In addition, administration of AA in rats induces a stronger inflammatory response and leads to greater cell death and functional deficits [50]. However, the production of eicosanoids that is seen in chronic phase of SCI is not likely to contribute to secondary tissue damage, as much of the tissue degeneration occurs over the first few days and weeks following lesion. Interestingly, a recent study demonstrates that administration of licofelone, a dual inhibitor of COX/5-LOX enzymes, between 8 and 9 months after SCI, reduces mechanical hypersensitivity in rats [42], suggesting an involvement of eicosanoids in the chronic phase of SCI with the development of neuropathic pain. These studies provide direct evidence that several of the downstream products of PLA_2, namely AA-derived lipid mediators, play a role in inflammation, secondary damage, and neuropathic pain in SCI. Several recent studies have assessed the role of PLA_2s in SCI [20–22, 51].

Various forms of mammalian PLA_2s that include $sPLA_2$ IB, IIC, and V, $cPLA_2$ GIVA, and $iPLA_2$ GVIA are constitutively expressed in the rat and mouse spinal cord [20–22]. Among 14 mammalian PLA_2s studied so far, we have reported that only $cPLA_2$ GIVA, $iPLA_2$ GVIA, and $sPLA_2$ GIIA are significantly up-regulated at the mRNA and protein levels after SCI (Table 9.1) [21]. Earlier experiments revealed that intraspinal injection of bee venom $sPLA_2$ group III into the normal uninjured spinal cord induces focal demyelination and functional deficits [20, 52]. The first direct evidence for the role of mammalian forms of PLA_2s in secondary damage following SCI was provided by experiments in which rats with SCI were treated with arachidonyl trifluoromethyl ketone (AACOCF3), a nonselective inhibitor that blocks all members of intracellular PLA_2s ($cPLA_2$ and $iPLA_2$) [51, 53]. Animals treated with AACOCF3 exhibit greater survival of neurons and oligodendrocytes at 7 days following compression injury (maximum time point assessed), and small, but significant, enhancement of locomotor performance [51]. More recently we

characterized in more detail the expression of 22 members of the PLA_2 superfamily in SCI in mice and used a panel of small molecule inhibitors to dissect out the roles of different PLA_2s in SCI including that of $cPLA_2$ and $iPLA_2$ [21].

We found that the protein levels of the active (phosphorylated) form of $cPLA_2$ GIVA are up-regulated from 3 to 28 days after spinal cord contusion injury in mice [21]. It is expressed in neurons and oligodendrocytes in the uninjured and contused spinal cord [20, 21]. Interesting, although previous studies revealed that $cPLA_2$ GIVA exerts detrimental effects in other experimental mouse models of neurological disorders, such as EAE and brain ischemia, mice treated with a selective inhibitor of $cPLA_2$, as well as $cPLA_2$ GIVA null mice, develop greater locomotor deficits, and greater neuronal and myelin loss after spinal cord contusion [21]. This suggests a protective role for $cPLA_2$ GIVA in SCI [21]. The $cPLA_2$ inhibitor and $cPLA_2$-null mice line used in this study were also used in EAE [19] and cerebral ischemia [54], highlighting the striking difference in the role of $cPLA_2$ in SCI as compared to other CNS disorders. This unexpected finding with regard to potential protective role of $cPLA_2$ in SCI may have to do with the loss of its normal physiological function in motor neurons and oligodendrocytes, possibly related to membrane turnover or some other functions.

$iPLA_2$ GVIA is also up-regulated after SCI, peaking at day 14 after injury. $iPLA_2$ GVIA is constitutively expressed at low levels in oligodendrocytes, but its expression increases in this cell type after SCI [21]. It is also induced in astrocytes and a very small proportion of neurons and microglial/macrophages [21]. $iPLA_2$ appears to contribute to some secondary damage after SCI, as treatment with a selective and potent $iPLA_2$ inhibitor (FKGK11) results in some tissue and myelin sparing, with only very slight recovery of the finer aspects of locomotor function [21]. Blocking both $iPLA_2$ GVIA and $cPLA_2$ GIVA simultaneously may therefore yield only a small effect, as observed earlier with the AACOCF3 inhibitor [51].

Unlike $cPLA_2$ GIVA and $iPLA_2$ GVIA, $sPLA_2$ GII is not constitutively expressed in the uninjured spinal cord [21]. $sPLA_2$ GIIA protein levels, however, are increased between 3 and 7 days post-injury [21]. Oligodendrocytes and astrocytes are the main cell types expressing $sPLA_2$ GIIA in the contused spinal cord, although a small proportion of neurons and microglia/macrophage also express this enzyme [21]. Mice with SCI treated with a selective small molecule inhibitor of $sPLA_2$ (GK115) showed improved locomotor recovery and significant prevention of myelin loss [21]. The harmful role of $sPLA_2$ GIIA on oligodendrocytes is in agreement with previous in vitro studies showing that recombinant human $sPLA_2$ GIIA causes a dose-dependent cytotoxicity in differentiated adult oligodendrocytes, but not in primary astrocytes or Schwann cells [22]. In addition, treatment with an $sPLA_2$ inhibitor (S3319) protects oligodendrocytes from cell death mediated by hydrogen peroxide or IL-1β and TNF-α in vitro [22].

Collectively, these data suggest that $cPLA_2$ GIVA mediates beneficial effects in SCI whereas $sPLA_2$ GIIA and $iPLA_2$ GVIA exert harmful effects (Table 9.1). However, the greatest functional and histological improvement after SCI was achieved with the use of a weak pan-PLA_2 inhibitor (AX115) that blocks about 50 % of the activity of all three PLA_2s [21], suggesting that some of the normal

physiological functions of these PLA_2s need to be retained for recovery to be optimal. Interestingly, we found that AX115 also induces an increase in the expression of $cPLA_2$ GIVA and its downstream pathway COX-2, mPGEs-1, and EP1 receptor. Treatment with an EP1 antagonist was able to abrogate the effects of AX115 suggesting an important role for this pathway in recovery from SCI [21].

The divergent effects exerted by the distinct PLA_2s in SCI are likely due to their preference to generate different fatty acids and lysophospholipids. For instance, $cPLA_2$ GIVA shows the stronger preference to AA found at *sn*-2 position and, thus, facilitates eicosanoid formation [14]. Although, eicosanoids are commonly seen as harmful mediators of inflammation and tissue degeneration, recent insights suggest that some bioactive lipids derived from AA are anti-inflammatory. 15dPGJ2, an anti-inflammatory prostaglandin generated from PGD2 via a series of nonenzymatic dehydration steps, improves locomotor recovery and reduces motor neuron loss, microglial/macrophage activation, and chemokine/cytokine expression after SCI [55]. Similarly, administration of iloprost, a synthetic analog of PGI2, reduces inflammation and functional deficits in SCI [56] suggesting a protective role for PGI2 in spinal cord trauma. Another lipid metabolite derived from AA by LOX action, known as lipoxin A4 (LXA4), has potent anti-inflammatory features and triggers the activation of the resolution programs of inflammation [57]. Although the role of LXA4 has not been assessed in SCI yet, its production following trauma may help to contain the activation of the inflammatory response. Apart from AA, PLA_2 enzymes also generate the omega-3 PUFAs DHA and EPA. Metabolites derived from these PUFAs by the action of LOX enzymes, known as resolvins, protectins, and maresin, have potent anti-inflammatory and pro-resolution properties [57, 58]. In addition, omega-3 fatty acids also have anti-oxidative features [59, 60]. Administration of DHA and EPA after SCI reduces inflammation and enhances functional and histological outcomes, suggesting a beneficial effect of omega-3 fatty acids in spinal cord trauma [50, 51]. Similar protective effects are observed with the administration of fenretinide, a semisynthetic analog of retinoid which increases DHA and reduces AA levels in the injured spinal cord [61].

9.4 PLA_2 in Experimental Autoimmune Encephalomyelitis

As was seen in SCI, increases in mRNA expression in EAE were seen in only 4 out of 14 PLA_2s examined. These include $sPLA_2$ GIIA, $sPLA_2$ GV, $cPLA_2$ GIVA, and $iPLA_2$ GVIA, which showed differences in their mRNA expression in the spinal cord at different stages of EAE. $cPLA_2$ GIVA expression is increased mainly at the onset, while $iPLA_2$ GVIA is increased at the onset and peak of disease. In contrast, $sPLA_2$ GIIA mRNA is increased at the peak stage of disease, while $sPLA_2$ GV is increased in the peak and remission stages. Interestingly, in the spleen, $cPLA_2$ GIVA expression is highest (fourfold) at the onset, $iPLA_2$ GVIA highest (threefold) at the peak, and $sPLA_2$ GIIA highest (twofold) at the peak and remission stages. These findings suggest that these PLA_2s are likely to play differing roles in different

stages of the disease in the spinal cord and spleen. Fluorescence-activated cell sorting (FACS) analysis of the protein expression in immune cell populations showed that about 40 % of macrophages in the EAE spinal cord expressed $cPLA_2$ GIVA, $iPLA_2$ GVIA, and $sPLA_2$ GIIA at the onset of disease. The next highest expression was seen in 10–20 % of CD4+ T cells in the spinal cord at the peak of disease. Immunofluorescence staining of spinal cord tissue sections showed staining of both $sPLA_2$s (GIIA and GV) in astrocytes and oligodendrocytes at the peak and remission stages, while $cPLA_2$ GIVA was expressed in astrocytes and oligodendrocytes at the peak of EAE. $iPLA_2$ GVIA was only seen in infiltrating immune cells. The timing of the expression pattern of these PLA_2s in EAE and SCI shows interesting differences. While $cPLA_2$ GIVA is expressed mainly at the onset of EAE, in SCI it is expressed throughout in the early, mid, and late time periods after injury (3–28 days); $iPLA_2$ GVIA is expressed at the onset and peak of EAE, and in SCI is increased at the mid and late periods after injury (7–28 days); while $sPLA_2$ GIIA is expressed at the peak and remission phase of EAE, it is expressed only in the acute phase (3–7 days) after SCI.

In our earlier work on EAE induced in C57BL/6 mice by immunization with myelin-oligodendrocyte glycoprotein (MOG), we tested the effects of AACOCF3, which blocks both intracellular forms of PLA_2 ($cPLA_2$ and $iPLA_2$) [53]. These experiments showed that blocking $cPLA_2$ and $iPLA_2$ with AACOCF3 was able to completely prevent disease when treated from the day of immunization to day 24 [62]. Furthermore, a brief treatment for 7 days after the peak of disease (7–20 days) prevented subsequent relapses and reduced the chronic disability score remarkably [62]. In our subsequent work on EAE generated in SJL/J mice by immunizing with proteolipid protein (PLP), we dissected out the effects of $cPLA_2$ and $iPLA_2$ in EAE using the highly selective, potent inhibitors (AX059 and FKGK11, respectively) [19] which we also used in SCI [21]. Unlike SCI in which the $iPLA_2$ inhibitor (FKGK11) had very little effect, in EAE it was remarkably effective when treated either before or after onset of EAE [19]. The treated mice only showed mild symptoms of the disease (tail weakness) [19]. However, blocking $cPLA_2$ with AX059 was effective in EAE when treatment was given during the acute onset phase of the disease [19], and effective only while the treatment lasted. The symptoms (paralysis) appeared as soon as the treatment was withdrawn [19]. This data indicates that inhibition of $cPLA_2$ needs to be maintained throughout the course of the disease for it to be effective. This is corroborated in EAE studies on mice lacking $cPLA_2$ GIVA ($cPLA_2$ null mice) in which $cPLA_2$ null mice are resistant to EAE [63]. $cPLA_2$ GIVA was also shown to play a role in Th1 and Th17 T cell differentiation in EAE [63, 64]. Our data also suggests strongly that the remarkable effects of AACOCF3 in reducing onset and progression of EAE [62] were likely due to its effects in blocking $iPLA_2$ [19]. These findings reveal the striking difference in the role of $iPLA_2$ in SCI and EAE. Furthermore, unlike the SCI experiments in which the weak pan-PLA2 inhibitor AX115 that blocks all three PLA_2s to about 50 % level is the most effect, AX115 has no effect in EAE when treatment is begun on the day of immunization, and surprisingly worsens disease when treatment is given during the peak to remission period (i.e., the period when $sPLA_2$ GIIA expression is maximal) [19].

Our studies on EAE indicate that both $cPLA_2$ GIVA and $iPLA_2$ GVIA are detrimental, while the precise roles of $sPLA_2$s are still not clearly understood (Table 9.1). Of the two intracellular PLA_2s, blocking $iPLA_2$ appears to have a profound effect in reducing the progression of EAE even after the treatment is stopped. Furthermore, our work also points out that the role of $cPLA_2$ appears to be strikingly different in SCI and EAE (Table 9.1).

9.5 Conclusions

It is interesting to note that of all the members of the PLA_2 superfamily, only three appear to be up-regulated and play roles in the two neurological conditions we have examined. These include $cPLA_2$ GIVA, $iPLA_2$ GVIA, and $sPLA_2$ GIIA (Table 9.1). In addition, the expression of $sPLA_2$ GV also appeared to be increased in EAE. Since the inhibitors tested block both these forms of $sPLA_2$, it is not possible so far to distinguish between the roles of these $sPLA_2$s. There is evidence from work done by several other groups that $sPLA_2$ and $cPLA_2$ play a role in SCI, EAE, and cerebral ischemia. In addition, a striking illustration of the role of $iPLA_2$ in the CNS is the development of axonal and neuronal degeneration in mice and humans lacking this enzyme. Much more work is needed to explore the normal physiological roles of other forms of PLA_2 in the nervous system, and their contribution to inflammation and other neurological conditions.

Acknowledgments The work done in S.D.'s laboratory was supported by grants from the Canadian Institutes of Health Research, Wings for Life Spinal Cord Research Foundation, and the Multiple Sclerosis Society of Canada. Work in R.L.V.'s laboratory was supported by grants from Ministerio de Economía y Competividad of Spain, Wings for Life Spinal Cord Research Foundation, Marie-Curie International Reintegration Grant, and International Foundation for Research in Paraplegia, and by funds from the Fondo de Investigación Sanitaria of Spain (TERCEL and CIBERNED).

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Chapter 10
Cytosolic Phospholipase A2 and Autotaxin Inhibitors as Potential Radiosensitizers

Dinesh Thotala, Andrei Laszlo, and Dennis E. Hallahan

Abstract Several classes of lipid mediators are initially derived through the action of phospholipase A2 (PLA_2) enzymes on phospholipids that release fatty acids and lysophospholipids. Both fatty acids and lysophospholipids have biological functions relevant to cancer progression. Fatty acids are metabolized to prostanoids by cyclooxygenase and leukotrienes by lipoxygenase, while lysophospholipids are metabolized to lysophosphatidic acid (LPA) by autotaxin (ATX). These metabolites modulate cellular differentiation, proliferation, apoptosis, and senescence, thereby contributing to the homeostatic control of tissue growth, remodeling, and vascularization. Tumor cells subvert these cellular functions to grow locally and to metastasize to distant sites. The deregulation of cyclooxygenase and lipoxygenase in various cancers supports role for the aberration of these two pathways in tumorigenesis. Pharmacological studies in humans have demonstrated the benefits of eicosanoid pathway intervention in certain cancers. In recent years the importance of the tumor microenvironment (TME) has become increasingly salient with respect to both tumorigenesis and response to therapy. We have focused on characterizing the role of the lysophospholipid molecules generated by PLA_2, such as lysophosphatidylcholine

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_10,

(LPC) and its further metabolites, such as LPA, in the response of the vascular system to ionizing radiation. In normal endothelial cells, ionizing radiation rapidly induces $cPLA_2$ activity, leading to the activation of pro-survival pathways like Akt and ERK. Inhibition of $cPLA_2$ leads to radiosensitization and inhibition of endothelial cell-specific functions, such as cell migration, cell invasion, and tubule formation. Combined treatment with radiation and $cPLA_2$ inhibitors resulted in delayed tumor growth. Inhibition of the extracellular enzyme involved in the generation of LPA from LPC, ATX, also inhibits endothelial-specific functions and leads to tumor radiosensitization. Finally, experiments conducted with $cPLA_2$ knockout mice indicated that the $cPLA_2$ deficiency within the host component resulted in delayed tumor growth and impaired tumor vascularization. The $cPLA_2$ from the normal TME is an important mediator of tumor development and progression. Radiation-induced modifications in the TME that result in increased angiogenesis are novel targets for inhibiting pro-survival pathways in the tumor. The concurrent inhibition of inflammatory responses in normal tissues would lead to significant therapeutic gain.

Keywords Tumor microenvironment • Phospholipase A2 • Autotaxin • Radiosensitization • Tumor vasculature • Radiotherapy

10.1 Introduction

It is estimated that in 2013 there will be 1,660,290 new cases of cancer and 580,350 deaths due to cancer in the United States [1]. The main arsenal available for fighting this disease includes surgery, chemotherapy, and radiation. Currently, definitive radiotherapy (RT) is a widely used therapeutic modality and it is estimated that as much as 50 % of all cancer patients receive some form of radiotherapy [2]. Outcome of RT depends on accurate delineation of both the tumor area and the involved lymph nodes [3]. Failure to do so will lead to local recurrences and/or result in increased normal tissue damage. New technologies, such as three-dimensional conformal planning, the use of multi-leaf collimators, four-dimensional planning CT scans, intensity-modulated radiation therapy (IMRT), and image-guided radiation therapy (IGRT), have significantly improved the targeting of tumor volume [4, 5]. Furthermore, the new radiation delivery techniques have resulted in significant dose escalation without concurrent increases in normal tissue toxicity [4, 5]. Despite these improvements in therapeutic regimens, local recurrence of some malignancies, including lung cancer and glioblastoma, remains persistent problems [3]. These tumor types are highly angiogenic and resistant to radiation. Despite aggressive treatment, most patients with unresectable glioblastoma have a median survival of approximately 1 year, while patients with unresectable non-small-cell lung cancer (NSCLC) have a similarly poor prognosis with median survivals of approximately 18 months [3]. There is, therefore, an acute need to develop new approaches for the treatment of these difficult-to-cure cancers with RT.

10.2 Radiation-Induced Signaling

In the last 25 years, ionizing radiation (IR) has been shown to trigger the activation of an intricate network of intracellular signaling events, in addition to inducing DNA damage in the nucleus [6, 7]. This network includes transient activation of pro-survival pathways involving receptor tyrosine kinase (RTK) pathways, such as the epidermal growth factor receptor (EGFR) pathway and the downstream Ras and phosphoinositide 3-kinase/atypical kinase (PI3K/Akt) signaling pathway. Radiation also activates several transcription factors and upregulates the levels of numerous cytokines [8]. The radiation-induced multifaceted signaling network orchestrates postirradiation cell survival responses [9]. The traditional view of intrinsic tumor radiosensitivity holds that it reflects the balance between radiation-induced DNA damage and its repair [10]. However, the cascade of radiation-induced cytoplasmic signaling events also plays a vital role in tumor radiosensitivity [11].

In the nucleus, IR elicits a DNA damage response (DDR), which coordinates DNA repair, cell cycle checkpoints, and cell death pathways [12]. The DDR involves molecules that sense the DNA damage, eliciting the participation of several mediators, such as ATM, which, in turn, recruit an army of proteins that perform key roles in pivotal cellular processes, as enumerated above.

In the cytoplasm, the induction of reactive oxygen species (ROS) from radiation-generated ionizing events in water molecules is amplified in the mitochondria, in a Ca^{2+}-dependent manner, resulting in the generation of large amounts of ROS and reactive nitrogen species (RNS) [13]. The resulting redox imbalance leads to the inhibition of protein tyrosine phosphatase (PTPase) activities [14] that are sensitive to oxidation or nitrosylation of key cysteine residues in their active site, resulting in increased tyrosine phosphorylation of multiple proteins [15]. This, in turn, results in the activation of RTK and non-RTK and the activation of downstream signal transduction pathways.

High doses (>10 Gy) of radiation activate acidic sphingomyelinase and increase the production of ceramide. Ceramide is generated in endothelial cells within minutes after exposure to 15–20 Gy radiation that later results in apoptosis [16]. However, endothelial cell viability is not affected by low doses of radiation (2–5 Gy), pointing to involvement of the activation of pro-survival phosphatidylinositol 3-kinase (PI3K)/Akt signaling [17, 18]. Radiation-induced ceramide has been shown to promote membrane-associated receptor activation by facilitating the clustering of receptors within lipid rafts [19, 20]. Radiation also induces the eicosanoid inflammatory pathway by inducing increased activity of cytosolic phospholipase A2 (cPLA$_2$), resulting in increased levels of arachidonic acid that is metabolized through cyclooxygenase-2 (COX-2), into various forms of prostaglandins [21].

10.3 Tumor Microenvironment

The increasingly accepted importance of the tumor microenvironment (TME) concept is associated with the notion that cancer cells are not the only players in tumors; they also conscript and corrupt resident and recruited normal cell types to serve as contributing members to the outlaw society of cells that form a tumor [22]. This community of various cell types has been referred to as the TME. While the role of the stromain tumor angiogenesis and the remodeling of the extracellular matrix (ECM) have long been appreciated [23–25], the larger impact of the TME on tumor growth and progression has become increasingly evident only recently. The different cellular constituents of the stroma in tumors make various functional contributions toward cancer phenotypes, by contributing to both the core and emergent hallmarks of cancer [26, 27]. Furthermore, infiltrating cells of the immune system are increasingly accepted to be constituents of tumors [27].

Thus, tumors are increasingly being recognized as organs whose complexity approaches and may even exceed that of normal healthy tissues. Much of the cellular heterogeneity within tumors is found in their stromal compartments. Collaborative interactions between neoplastic cancer cells and their supporting stroma unite into the chronically proliferating organ-like structures that typify most human cancers, in the form of tumors, local invasions and metastases. Driver oncogenic mutations that generate chronic proliferative signals still play essential roles in most human cancers. On the other hand, most stromal cells also have the capacity to support hyperproliferation of cancer cells in some contexts. The proliferation signals supplied by stromal cells maybe play a role in the biology of different tumor types at any stage of tumorigenesis and progression, ranging from the initiation of abnormal proliferation to the development of resistance to therapies targeting the driving oncogenic signals [22].

The cells of the tumor-associated vasculature are prominent among the stromal constituents. The development, differentiation, and homeostasis of the endothelial cells that form the arteries, veins, and capillaries are probably the most important processes associated with the TME. The activation of quiescent endothelial cells leads to the initiation of a program that directs them to construct new blood vessels, through a network of interconnected signaling pathways [28–31].

10.4 Radiation-Induced Signaling in Membranes

Immediate signal transduction initiated at the cell membrane by radiation is less well characterized than the kinase networks and cytokines. Biologically active lipids and proteins, such as phospholipases, lipid kinases, and phosphatases, which regulate the production of lipid second messengers, can initiate pro-survival signal transduction [19]. The response of the TME to radiation is important for the outcome of RT. Several studies have shown that the effectiveness of RT is limited by the response of

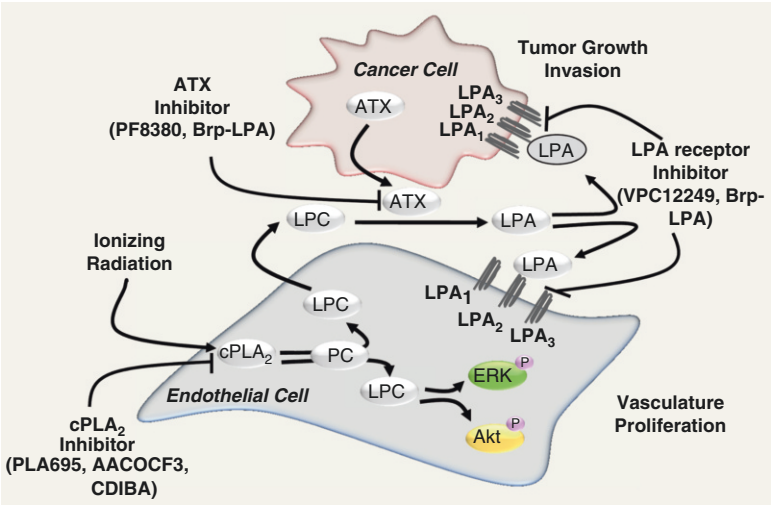

Fig. 10.1 Phospholipase A signaling in response to ionizing radiation. Ionizing radiation activates cytosolic phospholipase A2 (cPLA$_2$) which cleaves phosphatidylcholine (PC) to yield lysophosphatidylcholine (LPC). Autotaxin that possess lysophospholipase D (lysoPLD) activity catalyzes the reaction by cleaving the headgroup of LPC to form lysophosphatidic acid (LPA). LPA can then bind to lysophosphatidic acid receptors (LPA$_{1–3}$). LPA$_{1–3}$ belong to the endothelial differentiation gene (EDG) family

the tumor vascular endothelium [32, 33]. Tyrosine kinase inhibitors have been shown to attenuate the response of the vascular endothelium to radiation [18]. Due to the rapidity of such responses, it has recently become of interest to determine the role of radiation-induced membrane alterations in postirradiation survival. Recent reports have demonstrated that increased viability of vascular endothelial cells in response to low doses of ionizing radiation is due to the activation of pro-survival signaling pathways [17, 34]. A systematic characterization of lipids isolated from the membranes of irradiated cells revealed changes in abundance as a consequence of irradiation. Levels of lysophospholipids (lysophosphatidylcholine, LPC) increased rapidly after irradiation, indicating increased PLA$_2$ activity (Hallahan et al. unpublished results), that also lead to increased levels of arachidonic acid which are metabolized through COX-2, into various forms of prostaglandins [21].

PLA$_2$ are enzymes that catalyze the hydrolysis of membrane phospholipids at the sn-2 position to release lipid second messengers that play a vital role in cancer [35]. Ionizing radiation triggers the activation of cytosolic phospholipase A2 (cPLA$_2$) that cleaves phosphatidylcholine (PC) to yield LPC (Fig. 10.1).

cPLA$_2$-α (the most frequent isoform of cPLA$_2$) is an 85 kDa monomeric protein consisting of 749 amino acids, which is highly conserved, with 95 % homology between human and mouse. cPLA$_2$-α has an N-terminal C2 domain and a C-terminal catalytic domain that are linked by a short and flexible peptide domain [36]. Calcium binding to the C2 domain through Asp and Asn residues promotes association of

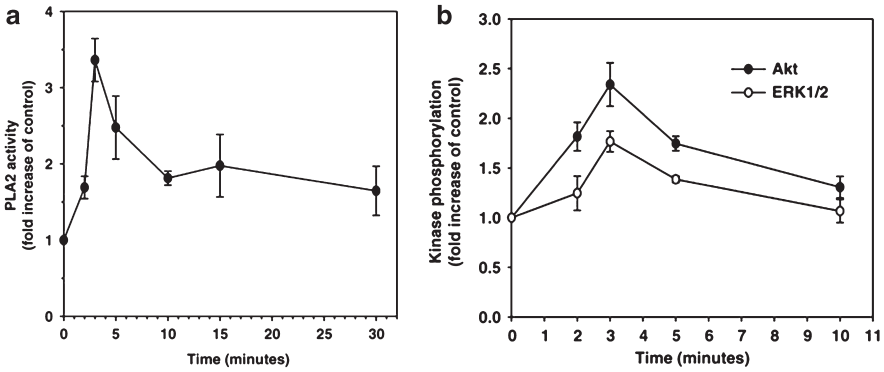

Fig. 10.2 (**a**) Irradiation with 3 Gy leads to a rapid increase in phospholipase A2 activity in human umbilical vein endothelial cells. (**b**) Irradiation with 3 Gy induces the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and Akt with similar kinetics in human umbilical vein endothelial cells

hydrophobic residues with membrane phosphatidylcholine, causing penetration of the enzyme into the membrane bilayer. Alterations of the amino acid sequence of the calcium binding regions modify the membrane targeting specificity. The association with the membrane bilayer activates the enzymatic activity of cPLA$_2$-α. The conserved active site is a dyad and composed of a serine (Ser^{228}) and aspartic acid (Asp^{549}). The Ser/Asp active site of cPLA$_2$-α is in a deep funnel lined by hydrophobic residues. Changes in amino acid residues in the funnel in other cPLA$_2$s are thought to account for decreased AA specificity and differences in sensitivity to various cPLA$_2$ inhibitors [37, 38]. cPLA$_2$-α phosphorylation also regulates its enzymatic activity. cPLA$_2$-α is phosphorylated at Ser^{505} and activated by p42-MAP kinase and PKC [39, 40]. The increased Ser^{505} phosphorylation by MAP kinase has been implicated in the activation of cPLA$_2$-α in response to various cellular stimuli [39, 41–43].

Although the loss of cPLA$_2$-α gene does not result in sterile mice, it does impair embryo implantation and impaired luteolysis during parturition [44]. cPLA$_2$-α-deficient mice are resistant to ischemia reperfusion injury, anaphylactic responses, acute respiratory distress syndrome, chemical-induced lung inflammation, and collagen-induced autoimmune arthritis. These results support the unequivocal role of cPLA$_2$-α as a mediator of inflammation. cPLA$_2$-α has also been implicated in carcinogenesis [45]. cPLA$_2$-α expression is increased by pro-inflammatory cytokines and growth factors and repressed by glucocorticoids [46]. Recently, a homeodomain-interacting protein kinase-2 (HIPK2), also a corepressor for homeodomain transcriptional factors, has been found to restrain cPLA$_2$-α gene expression through interacting with histone deacetylase-1 [47].

Irradiation of human umbilical vein endothelial cells (HUVECs) with 3 Gy leads to the rapid activation of PLA$_2$ activity, with a maximum at 3 min postirradiation, followed by a gradual decay returning to baseline by 30 min postirradiation [34] (Fig. 10.2a). Analysis of the activity of pro-survival kinases such as Akt and

extracellular signal-regulated kinase (ERK) revealed a rapid increase in their phosphorylation (indicative of their activation), postirradiation, with kinetics mirroring the activation of PLA_2 activity (Fig. 10.2b). Screening for subtypes of the PLA_2 family activated by radiation using specific inhibitors of the various forms of PLA_2 indicated that the cytosolic isoform of PLA_2, $cPLA_2$, was the major PLA_2 subtype activated by low doses of ionizing radiation. Inhibitors of $cPLA_2$, but not of $sPLA_2$ or $iPLA_2$, markedly decreased radiation-induced activation of Akt and ERK1/2, demonstrating that the radiation-induced $cPLA_2$ activity contributes to the radiation-induced activation of these pro-survival kinases.

Genetic manipulation of $cPLA_2$ activity with specific siRNAs or knockout of the $cPLA_2$-α gene both led to significant decreases in the activation of Akt and ERK, providing direct proof for the involvement of the radiation-induced $cPLA_2$ in this event. Exogenously added LPC also led to the rapid phosphorylation of ERK and Akt. Inhibition of $cPLA_2$ significantly abolished endothelial functions, including cell migration and endothelial cell tubule formation. Pretreatment with specific inhibitors of $cPLA_2$ led to radiosensitization. Such radiosensitization was due to mitotic catastrophe induced by the inhibition of $cPLA_2$-dependent pro-survival signaling in irradiated cells. Mitotic catastrophe evolves to cell death through apoptosis; this was found to be the case in cells irradiated following a preincubation with $cPLA_2$-specific inhibitors. While vascular endothelial cells were radiosensitized by $cPLA_2$ inhibitors, both mouse and human non small cell lung carcinoma (NSCLC) were not [48]. However, treatment of heterotopic mouse and human NSCLC tumor models with a combination of $cPLA_2$ inhibitors and radiation led to a significantly greater tumor growth delay when compared to radiation alone. The tumor growth delay was associated with increased apoptosis, decreased Akt phosphorylation, and increased tumor vasculature destruction. Tumor blood flow and the tumor vascular index were lowest in the tumors treated with a combination of the $cPLA_2$ inhibitor and irradiation (Fig. 10.3). These observations indicate that inhibition of $cPLA_2$ disrupts the biological functions of the tumor vasculature, enhances destruction of tumor blood vessels (Fig. 10.4), and suppresses tumor growth and, thus, it is an effective radiosensitizer for lung tumor models in mice.

The studies described so far have used $cPLA_2$ inhibitors unsuited for translation to the clinic due to their toxicity [32, 34, 37]. Therefore, the effects of PLA-695, a $cPLA_2$ inhibitor that has already been tested in clinical trials, were studied. The phase I study (NCT00366262) evaluating the safety of PLA-695 compared to placebo and naproxen has been completed (clinical trials.gov). A subsequent phase II clinical trial (NCT00396955) compared four dose regimens of PLA-695, naproxen, and placebo in subjects with osteoarthritis of the knee (clinical trials.gov).

Treatment of mouse and human NSCLC cells with PLA-695 attenuated radiation-induced increases of phospho-ERK and phospho-Akt in endothelial cells [49]. PLA-695 pretreatment radiosensitized endothelial cells but not NSCLC cells. On the other hand, NSCLC cells co-cultured with endothelial cells and pre-treated with PLA-695 became radiosensitive, indicating the importance of the TME in the outcome of such experiments. The combination of PLA-695 with irradiation significantly reduced migration and proliferation of endothelial cells and induced cell

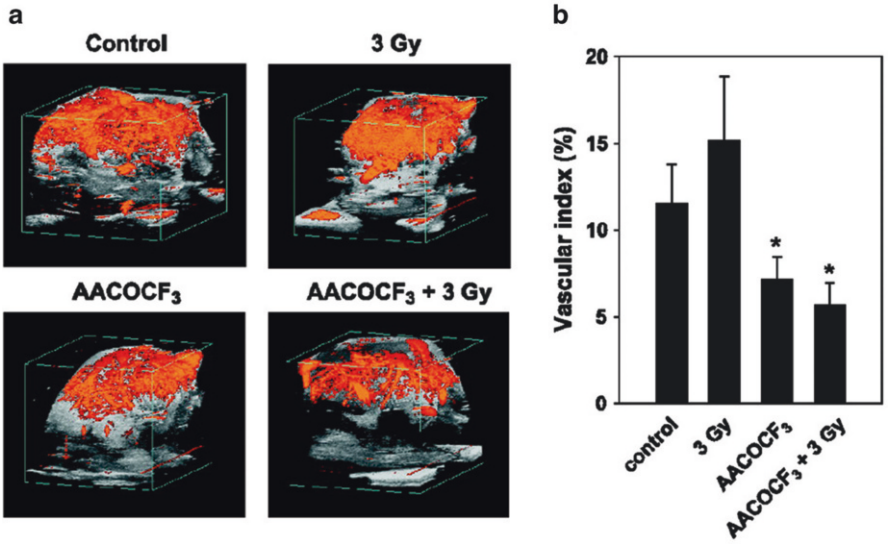

Fig. 10.3 Treatment with the cPLA$_2$ inhibitor AACOCF3 attenuates vascularity in irradiated tumors. C57/BL6 mice with LLC tumors received i.p. injections of vehicle or 10 mg/kg AACOCF3 30 min prior to irradiation with 3 Gy. Treatment was repeated for 5 consecutive days. Twenty-four hours after the final treatment, tumor blood flow was analyzed by three-dimensional power Doppler sonography (**a**) and the vascular index was calculated (**b**)

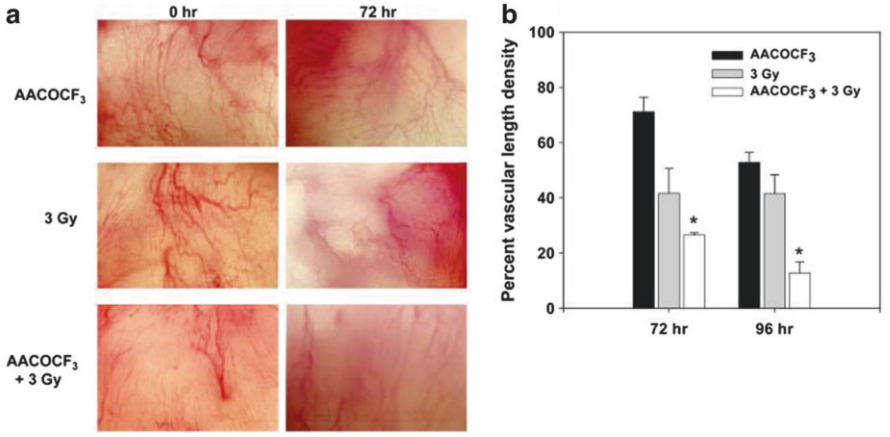

Fig. 10.4 Tumor vascular window model and vascular length density analysis. Mouse Lewis lung carcinoma cells were implanted into the dorsal skinfold window in C57/BL6 mice. (**a**) Representative micrographs of LLC tumor vascular window models at 0 and 72 h after treatment. (**b**) Changes in the quantity of blood vessels over time were compared with that observed at 0 h. Shown is a bar graph of the percent vascular length density 72 and 96 h after treatment of implanted tumors

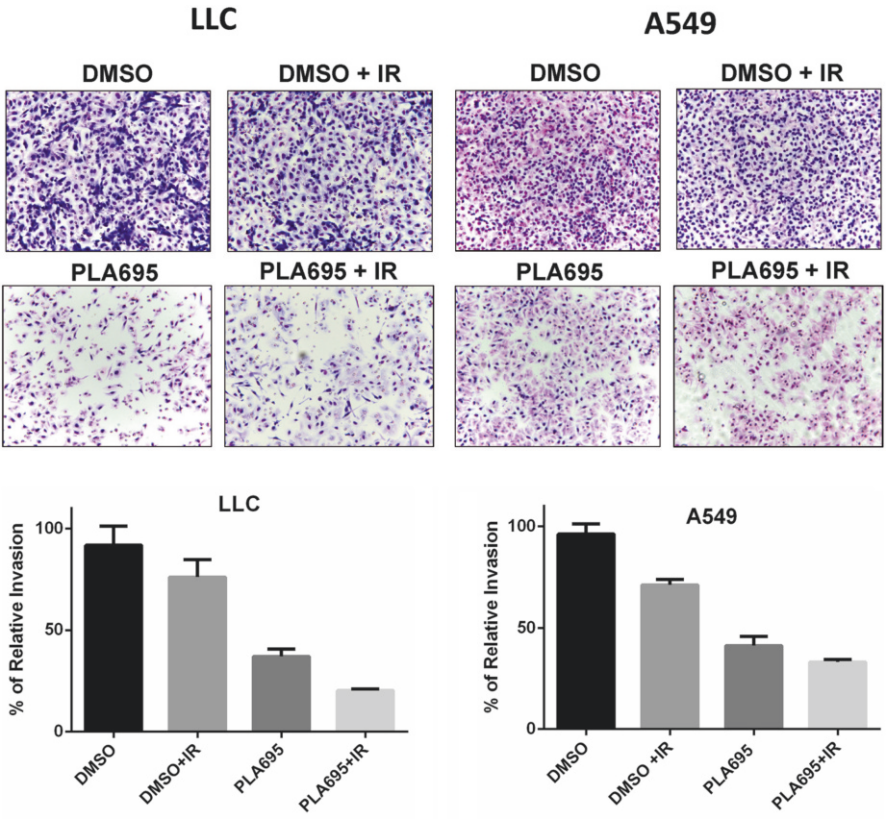

Fig. 10.5 Treatment with PLA-695 reduces tumor cell invasion after radiation. LLC and A549 cells were added to a simplified Boyden Chamber and treated with 300 nM PLA-695 or DMSO for 45 min prior to 3 Gy irradiation. Cells were allowed to invade/migrate from the top chamber through the coated filter pores to the complete medium at the bottom of the inserts for 48 h. Cells were then fixed, stained, and the number of cells that invaded through the membrane was quantitated by counting the number of cells per HPF. Shown are representative photomicrographs and bar graphs representing the number of invasive cells

death and attenuated invasion by tumor cells (Fig. 10.5). In heterotopic tumor models, the combination of PLA-695 and radiation delayed growth in both Lewis lung carcinoma (LLC) and A549 tumors. The tumors treated with a combination of PLA-695 and radiation displayed reduced tumor vasculature. In a dorsal skinfold model of LLC tumors, irradiation in combination with PLA-695 led to enhanced destruction of tumor blood vessels. The anti-angiogenic effects of PLA-695, an orally available and clinical trial-tested cPLA$_2$ inhibitor, and its enhancement of the efficacy of radiotherapy in mouse models of NSCLC all suggest that clinical trials for its capacity to improve radiotherapy outcomes are warranted.

The role of cPLA$_2$ in angiogenesis and tumor progression was further examined by monitoring tumor growth from lung tumor cells or glioblastoma cells that were

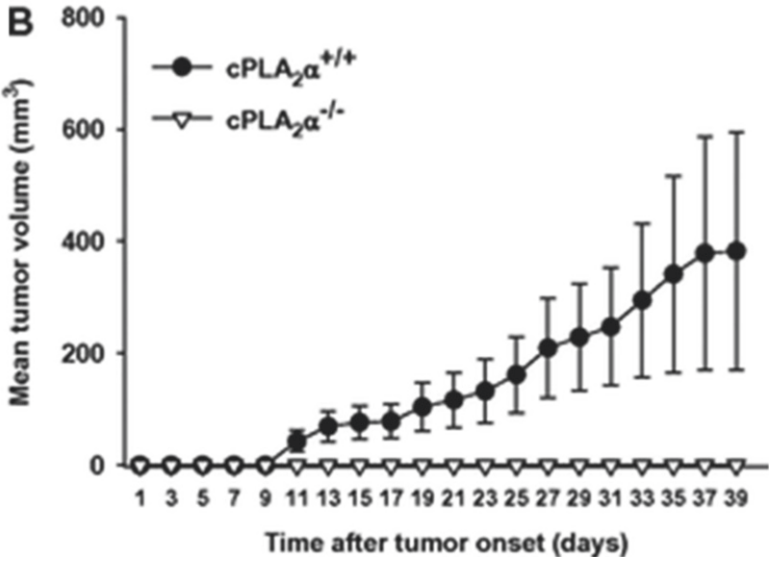

Fig. 10.6 Tumor growth in $cPLA_2$-α-deficient mice. GL261 cells were injected subcutaneously into the hind limbs of $cPLA_2$-$\alpha^{+/+}$ or $cPLA_2$-$\alpha^{-/-}$ C57/BL6 mice. Tumor volume was measured using power Doppler sonography at 48-h intervals, beginning 1 week after injection and ending when tumors reached a volume of 700 mm^3

injected subcutaneously into the hind limbs of $cPLA_2$-$\alpha^{+/+}$ or $cPLA_2$-$\alpha^{-/-}$ mice [50]. Despite an initial tumor take rate of 100 % and progression to tumor volumes in the range of 100–200 mm^3, by 14 days after tumor cell injection in both groups of mice, complete spontaneous LLC (lung) tumor regression was observed in 50 % of the $cPLA_2$-$\alpha^{-/-}$ mice but in none of the $cPLA_2$-$\alpha^{+/+}$ mice. Furthermore, tumor volume measurements from day 16 onward revealed a statistically significant reduction in mean tumor volume in the remaining tumors in $cPLA_2$-$\alpha^{-/-}$ mice compared with tumors from $cPLA_2$-$\alpha^{+/+}$ mice. The effects of $cPLA_2$ deficiency on tumor growth were even more pronounced in the glioblastoma (GL261) tumor model. Whereas $cPLA_2$-$\alpha^{+/+}$ mice exhibited gradual tumor growth progression (tumor take = 100 %), GL261 tumor formation in $cPLA_2$-$\alpha^{-/-}$ mice remained undetectable 1 month after the injection of tumor cells (Fig. 10.6).

The effects of $cPLA_2$ deficiency on tumor vascularity were ascertained by immunohistochemical staining for von Willebrand factor (vWF), an established vascular endothelial cell marker [50]. Tumors from $cPLA_2$-$\alpha^{-/-}$ mice had statistically significant fewer vessels per HPF when compared with tumors from wild-type mice. Hematoxylin–eosin staining of tumor sections revealed multiple necrotic areas in tumors from $cPLA_2$-$\alpha^{-/-}$ mice, but only minimal necrosis in tumors from $cPLA_2$-$\alpha^{+/+}$ mice, suggesting that $cPLA_2$-α is an important factor for tumor formation, growth, and maintenance (Fig. 10.7).

The role of $cPLA_2$-α in tumor blood vessel maturation was determined by co-staining of tumor sections with antibodies against vWF and α-smooth muscle

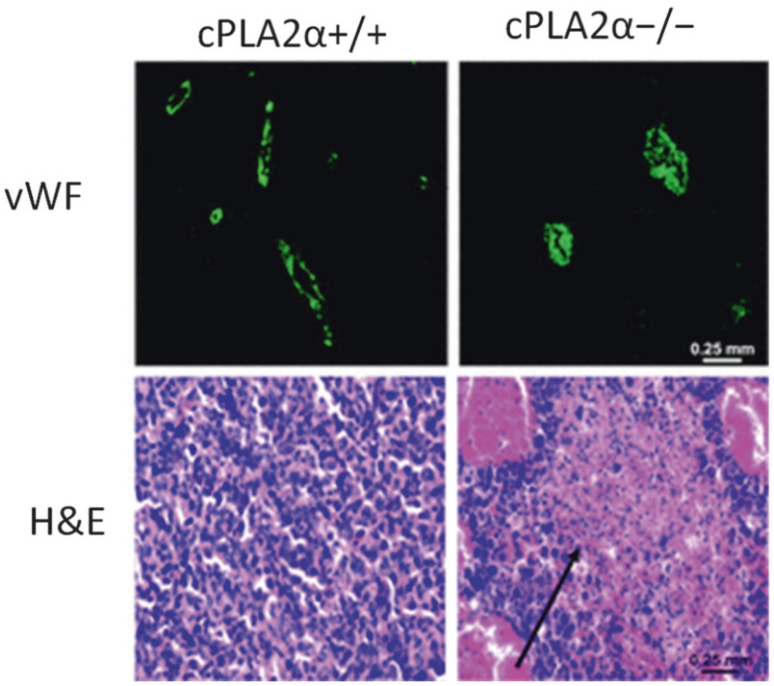

Fig. 10.7 Vascularity and necrosis from tumors in cPLA$_2$-α$^{+/+}$ or cPLA$_2$-α$^{-/-}$ mice. Lewis lung carcinoma (LLC) tumors were stained with an antibody against von Willebrand factor (vWF) (an endothelial cell marker) or hematoxylin and eosin. The *black arrow* indicates necrotic regions

actin (α-SMA) or desmin, which are all expressed by pericytes (cells that surround small blood vessels) [51]. Substantial pericyte coverage of the tumor vasculature in LLC tumors from cPLA$_2$-α$^{+/+}$ mice was found, while in tumors from cPLA$_2$-α$^{-/-}$ mice, vessel-encircling pericytes were undetectable. Staining with an antibody against desmin yielded similar results.

These outcomes strongly support the notion that cPLA$_2$-α and lysophospholipids play a key role in the invasive migration, proliferation, and capillary-like tubule formation of vascular endothelial cells. Moreover, in mouse tumor models, cPLA$_2$-α deficiency within the host component resulted in delayed tumor growth and impaired tumor vascularization. Thus, cPLA$_2$-α is an important factor in tumor angiogenesis and cPLA$_2$-α may be a novel molecular target for anti-angiogenic cancer therapy.

Because the experimental system used cPLA$_2$-α-deficient mice bearing tumors derived from tumor cell lines that were not cPLA$_2$-α deficient, the results implicate cPLA$_2$-α from the normal TME as an important mediator of tumor development and progression. Thus radiation-induced modifications in the microenvironment of irradiated tumors, which leads to increased angiogenesis, are putative targets for inhibiting pathways that play a pro-survival in the tumor, and at the same time inhibit inflammatory responses in normal tissues, leading to a significant therapeutic gain.

10.5 Autotaxin

LPC is the most abundant phospholipid in plasma with a concentration of about 200 μM in humans [52]. Autotaxin (ATX) converts extracellular LPC to lysophosphatidic acid (LPA) through its lysophospholipase D activity (lysoPLD) (Fig. 10.1). LPA is a second messenger in many lipid signaling pathways that stimulate endothelial cell survival and proliferation by regulating cytokine synthesis, endothelial growth factor expression, and chemotaxis [53]. ATX is a 120-kDa protein belonging to the ectonucleotide pyrophosphate/phosphodiesterase (ENPP) family and is encoded by the *ENPP2* gene [54]. There are three splice variants of ATX namely α, β, and γ. The predominant form is ATX β, which has 863 amino acids and is identical to plasma lysoPLD. ATX is widely expressed in almost all tissues and has the highest expression in the brain. ATX is the only ENPP family member that has lysoPLD activity. Lipid phosphatases (LPPs) dephosphorylate LPA and degrade it rapidly. LPA has a half-life of about 3 min in the blood and is then rapidly dephosphorylated [55]. The cellular effects of LPA are mediated through the six distinct G-protein-coupled receptors (GPCRs) [56]. The three GPCRs encoded by the endothelial differentiation gene family are known as LPA_1, LPA_2, and LPA_3. The other three belong to the purinergic family and are referred to as LPA_4, LPA_5, and LPA_6. There are reports indicating the role of LPA receptors in metastasis [57] and proliferation [58]. These receptors regulate various aspects of cancer, including proliferation, migration, and metastasis [59, 60]. Recent studies have shown that ATX is not only a lysoPLD enzyme but also a lipid carrier protein that efficiently transports LPA to respective cognate GPCRs [61]. Receptor expression is cell type specific which allows unique cellular responses to LPA depending upon the type of GPCR to which it binds. GPCR mediates cellular effects such as migration and proliferation in cancer [58]. There are indications that ATX binds to integrin receptors on lymphocytes indicating that it could play a role in lymphocyte trafficking [62]. ATX was originally identified as a tumor motility protein and is overexpressed in various human cancers known to contribute to tumor invasiveness [63]. ATX is involved in tubule formation in endothelial cells, indicating a possible role in tumor angiogenesis [64, 65]. There is direct evidence using transgenic mice that ATX and LPA are involved in invasiveness and metastasis of breast cancer [66]. ATX knockout mice are not viable and die in the uterus due to defects in the vasculogenesis. ATX heterozygous mice have reduced levels of LPA compared to their wild-type counterparts [67]. Increased expression of ATX and its receptors LPA_1, LPA_2, and LPA_3 in mammary epithelium of transgenic mice induced estrogen-positive mammary cancer [66]. ATX has been shown to stimulate angiogenesis either by enhancing the expression of vascular endothelial growth factor (VEGF) [68] or by stimulating endothelial cell motility [64] in endothelial cells. It has been shown that in Hodgkin's lymphoma cell motility is dependent on ATX expression and expression of LPA receptors [69].

Small molecule inhibitors of ATX are attractive tools for studying the role of ATX in various physiological processes including disease progression in cancer. There are various reports indicating the potential use of ATX inhibitors for

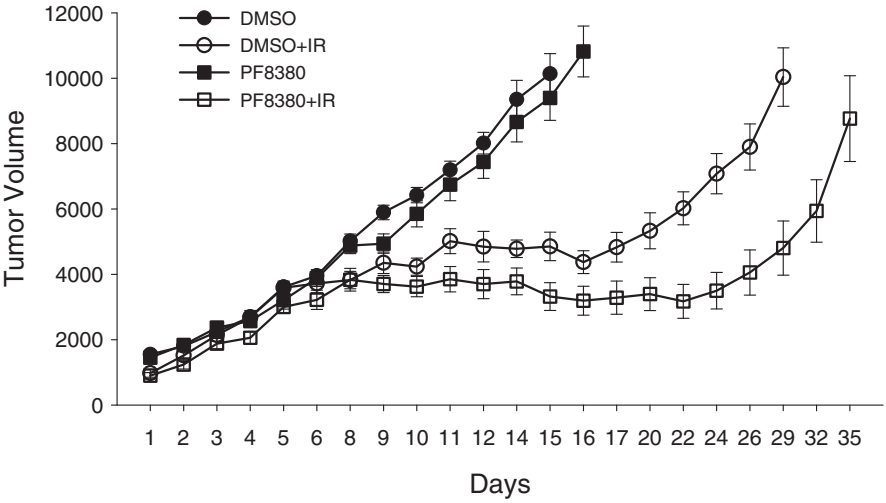

Fig. 10.8 Inhibition of ATX represses tumor growth in irradiated GL261 mouse model. GL261 cells were injected into the hind limbs of nude mice. Tumors were irradiated with 3 Gy for 5 consecutive days for a total of 15 Gy. Mice were treated with 1 mg/kg PF-8380 for 45 min prior to irradiation

anticancer therapy [65]. The most potent inhibitor described so far is PF-8380 with an IC_{50} of 1.7 nM. Recent studies showed that inhibition of ATX by PF-8380 led to decreased invasion and enhanced radiosensitization of glioma cells. Radiation-induced activation of Akt was abrogated by inhibition of ATX with this compound. Furthermore, inhibition of ATX by PF-8380 led to diminished tumor vascularity and delayed tumor growth [70] (Fig. 10.8). The specific ATX inhibitor PF-8380 reduces the LPA levels in the TME and blocks LPA signaling [71]. Another potent inhibitor of ATX is boronic acid derived HA155 with an IC_{50} of 5.7 nM. PF-8380 and HA155 are the only two inhibitors that have lowered the LPA levels in vivo [72]. Crystal structures have shown that HA155 targeted the ATX active site [73]. BrP-LPA, a pan-antagonist of $LPA_{1–4}$ receptors and inhibitor of the lysoPLD activity of ATX, was shown to inhibit cell migration and cell invasion of lung cancer cells [74] and glioblastoma cells [65]. ATX and LPA receptor inhibition enhanced radiation-induced endothelial cell death, disrupted endothelial cell biological function, and reduced glioma cell viability and migration [65] In a 3-D lung cancer xenograft model Brp-LPA inhibited tumor growth and reduced tumor vascularization [74]. Inhibition of ATX and LPA receptors by Brp-LPA diminished the radiation-induced activation of pro-survival kinase Akt. Brp-LPA treatment also enhanced radiation-induced endothelial cell killing, disrupted endothelial cell biological functions, reduced glioma cell viability, and migration [65]. Other inhibitors of ATX inhibitors are described in a recent review [72]. Overall, these findings suggest that ATX represents a novel potential molecular target for the enhancement of the efficacy of radiation therapy.

10.6 Conclusions

Overall, the studies performed in our laboratory link $cPLA_2$-α to the regulation of tumor angiogenesis. $cPLA_2$-α-deficient endothelial cells (either endothelial cells treated with the $cPLA_2$-α inhibitors or pulmonary microvascular endothelial cells derived from $cPLA_2$-α-deficient mice) are defective at replicating, migrating, and invading the ECM and forming a strong tumor vascular bed in mice. It is notable that tumor vessels in $cPLA_2$-α-deficient mice appear to lack pericyte coverage. Pericytes play essential functions in maintaining vessel integrity and thus tumor vessels would be functionally impaired in the absence of pericyte coverage. Such impairment would lead to attenuation of tumor growth [75]. The role of $cPLA_2$-α in regulating pericyte coverage and/or pericyte function remains to be uncovered. Studies conducted in recent years have revealed that pericytes are associated with the neovasculature of most if not all tumors [51, 76]. Recently, mechanistic studies have revealed that pericyte coverage is important for the maintenance of a functional tumor neovasculature [27].

The function of $cPLA_2$ in the arachidonic acid pathway and the potential importance of inhibiting multiple mediators of inflammation have attracted considerable interest as a target for therapeutic intervention [77]. Our studies suggest that $cPLA_2$ inhibitors may be useful anti-angiogenic agents that can potentially target both endothelial cells and pericytes, an approach that has been predicted to be superior to targeting endothelial cells alone [78].

ATX plays an important role in translating the activation of $cPLA_2$ by ionizing radiation into responses observed in the glioblastoma and endothelium. Inhibition of ATX resulted in reduced production of LPA and disruption of downstream targets. LPA-mediated signaling in tumor growth and cell survival identifies ATX as a viable molecular target for the radiosensitization of glioblastoma, and destruction of the tumor vascular network.

As we further define how the TME contributes to tumor progression, we may be able to develop novel therapeutic approaches that target both tumor cells and their TME. Our work has identified $cPLA_2$ and ATX as molecules expressed in normal cells that promote tumor growth in mice; thus, $cPLA_2$ and ATX inhibitors may be useful anticancer drugs and improve the efficacy of RT. Clinical trials with such drugs will be required before any clinical benefit can be determined.

Acknowledgements This work was supported by National Cancer Institute grants 1R01CA140220-02, 5R01CA125757-06, 7R01CA112385-0, Siteman Cancer Research Award, Elizabeth and James McDonnell III Endowment (Dennis E. Hallahan) and Department of Radiation Oncology Startup Funds (Dinesh Thotala).

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Chapter 11
Phospholipase A_2 Enzymes: Potential Targets for Therapy

Janhavi Sharma, John Marentette, and Jane McHowat

Abstract The endothelial cells lining blood vessels control vascular tone and actively participate in the inflammatory process by recruiting circulating cells to the underlying tissue. Activation of endothelial cell phospholipase A_2 (PLA_2) results in enhanced membrane phospholipid hydrolysis, leading to free fatty acid and lysophospholipid production. Arachidonic acid is further metabolized into eicosanoids, and lysophospholipids can be acetylated to form platelet-activating factor (PAF). Endothelial cells can release vasodilator or constrictor prostaglandins depending on the stimulus involved thereby regulating the vascular tone and local blood flow. Recruitment of inflammatory cells is mediated by endothelial cell PAF production. We have determined that endothelial cell PAF production is dependent upon $iPLA_2\beta$-mediated phospholipid hydrolysis and we propose that selective inhibition of this enzyme could be of potential therapeutic value for inflammatory diseases in the future. This review offers a discussion of activation and inhibition of PLA_2 enzymes and further focuses on our more recent studies that highlight endothelial cell $iPLA_2\beta$ activation and the implication for inflammatory disease management.

Keywords Platelet-activating factor • *Gingko biloba* • Inflammation • Metastasis

11.1 Introduction

The endothelial cell membrane consists of a phospholipid bilayer containing integral membrane proteins that regulate active and passive transport and cellular responses to stimuli. Integrity of the membrane is vital for maintaining homeostasis and its

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_11,

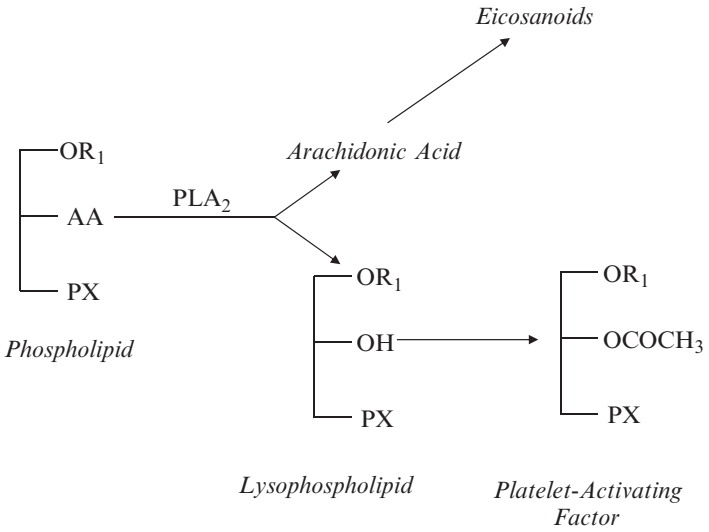

Fig. 11.1 Membrane phospholipids are hydrolyzed at the *sn*-2 position by phospholipase A_2 (PLA_2), resulting in the production of a free fatty acid and a lysophospholipid. Arachidonic acid (AA) can be oxidized to form eicosanoids and lysophospholipids can be acetylated to form platelet-activating factor

disruption can drastically alter the functional characteristics of cells [1, 2]. In addition to maintaining the homeostasis of the cell, membrane phospholipids serve as the substrate for multiple active metabolites that can play a role in physiology and pathology [1, 2]. These metabolites are primarily formed following the action of phospholipases on membrane phospholipids.

Phospholipases A_2 (PLA_2) are a group of enzymes that catalyze the hydrolysis of the *sn*-2 position fatty acid of membrane phospholipids and result in the production of a free fatty acid and a lysophospholipid (Fig. 11.1) [3, 4]. Both of these metabolites can directly alter the properties of the cell membrane and/or can serve as precursors for biologically active metabolites. In particular, PLA_2-catalyzed hydrolysis of arachidonylated phospholipids results in free arachidonic acid that serves as the precursor for eicosanoid generation (Fig. 11.1). The accompanying lysophospholipid can be acetylated at the *sn*-2 position, resulting in platelet-activating factor (PAF) production (Fig. 11.1).

Phospholipase A_2 enzymes are separated into three main classes: secretory, cytosolic and calcium independent [5]. The PLA_2 enzymes within each class have been subsequently divided into groups and sub-groups based on their amino acid sequences [6]. The three types of PLA_2 coexist in mammalian cells and may interact with each other [7]. A brief overview of each PLA_2 class with a discussion of currently available inhibitors follows below.

11.2 Secretory Phospholipase A$_2$

There are several secretory PLA$_2$ (sPLA$_2$) enzymes characterized to date. They all have low molecular weight (14–18 kDa) and include members from groups I, II, III, V, IX, X, XI, XII, XIII, and XIV [4, 6]. They possess a highly conserved calcium-binding loop (XCGXGG), up to eight disulfide bonds and a common catalytic site (DXCCXXHD) [8]. These enzymes require millimolar concentrations of calcium for catalysis and thus act extracellularly. It is important to note that the sPLA$_2$ enzymes are unable to hydrolyze membrane phospholipids until they are modified by additional cellular processes such as the loss of membrane asymmetry or phospholipid peroxidation and secretion [9]. However, after activation, the majority of sPLA$_2$ enzymes display increased activity in the presence of lipid aggregates [10]. Calcium is required for hydrolysis and is bound to the conserved Asp-49 [11]. The enzyme has very little selectivity for the fatty acid at the *sn*-2 position of membrane phospholipids. The majority of sPLA$_2$ enzymes preferentially hydrolyze anionic substrates [12] however, groups I A, V, and X enzymes can also hydrolyze zwitterionic substrates due to the aromatic residues present on the interfacial binding surfaces.

The most potent sPLA$_2$ inhibitors with drug potential in terms of pharmacokinetic profiles are substituted indoles and indolizines. 3-(3-acetamide-1-benzyl-2-ethyl-indolyl-5-oxy) propane phosphonic acid (LY311727) [13] is a widely used and one of the best characterized sPLA$_2$ inhibitors. It inhibits both group II A and group V sPLA$_2$ and resides in the hydrophobic channel, resulting in structural changes that bring the inhibitor in direct contact with the active site. LY315920, an analog of LY311727, displays 40-fold greater selectivity for group II A, non-pancreatic, sPLA$_2$ (IC_{50} = 9 nM) when compared to group I B sPLA$_2$, highlighting the potential for the development of highly selective sPLA$_2$ inhibitors [14].

PGBx compounds such as PX-18 and PX-52 are prostaglandin oligomers with at least two fatty acid moieties and one unsaturated double bond. These compounds selectively inhibit sPLA$_2$ and block arachidonic acid release from neutrophils [15, 16]. It has been shown by our group and others that PX-18 inhibits human sPLA$_2$ with an IC_{50} of <1 μM but does not inhibit recombinant cPLA$_2$ or endothelial cell cPLA$_2$ or iPLA$_2$ [17].

More recently, CHEC-9, a small peptide fragment of diffusible survival evasion peptide (DSEP) has been proposed to be an “uncompetitive” inhibitor of sPLA$_2$, presumably binding to the enzyme-substrate complex with its efficacy dependent on the levels of both enzyme and substrate in the reaction medium. A subcutaneous injection of CHEC-9 promotes anti-inflammatory and neuron survival effects in cerebral cortex lesions in rats by interrupting the inflammatory cascade [18].

11.3 Cytosolic Phospholipase A_2

Cytosolic PLA_2 ($cPLA_2$) enzymes are constitutively expressed in most human tissues and are large molecular weight proteins (61–114 kDa) that comprise group IV PLA_2 [6]. Thus far, four human cytosolic PLA_2 have been cloned α, β, γ, and δ [19]. $cPLA_2$ enzymes demonstrate a preference for choline phospholipids that are arachidonylated at the *sn*-2 position [20]. The $cPLA_2$ isoforms contain two catalytic domains, A and B, and a lipase consensus sequence GXSGS which is located within the catalytic domain A. They cleave membrane phospholipids utilizing a catalytic serine [21]. The nucleophilic Ser 228 targets the *sn*-2 ester linked fatty acid while Asp 549 activates the catalytic center. The catalytic dyad of Ser 228 and Asp 549 is placed at the bottom of the active site channel lined by hydrophobic residues [22]. An increase in intracellular calcium induces translocation of $cPLA_2$ to an intracellular phospholipid bilayer [23], that is regulated by a cationic cluster of lysine groups on the enzyme [24]. The phospholipid substrate molecules bind to the narrow cleft of the active site bringing the *sn*-2 ester bond in close proximity to Ser 228 [23]. The phosphate head group is stabilized by Arg 200, leading to the formation of an enzyme–substrate complex. Asp 549 now removes a proton by nucleophilic attack on the *sn*-2 ester. The serine-acyl intermediate is formed when the proton is transferred to the lysophospholipid. Hydrolysis of the acyl intermediate leads to either dissociation of the enzyme from the membrane interface or binding to another phospholipid molecule, repeating the cycle. This sequential reaction allows for a more targeted delivery of PLA_2 enzyme-bound arachidonate to other downstream elements required for eicosanoid generation, thereby increasing metabolic efficiency [25].

The earliest $cPLA_2$ inhibitors include arachidonyltrifluoromethyl ketone ($AACOCF_3$) [26] and methyl arachidonylfluorophosphonate (MAFP) [27]. These compounds compete with endogenous phospholipid molecules for the active catalytic site. With rigorous testing, these compounds that were originally designed as $cPLA_2$ inhibitors were subsequently found to inhibit $iPLA_2$ at similar concentrations [28] since the structure of the catalytic site in both $iPLA_2$ and $cPLA_2$ is similar. Both inhibitors possess an arachidonyl tail that is coupled to a serine reactive group. $AACOCF_3$ is a tight-binding, reversible inhibitor that forms a stable hemiketal with the active site serine residues in both $cPLA_2$ and $iPLA_2$ enzymes. MAFP binds irreversibly to inhibit both $cPLA_2$ and $iPLA_2$, by phosphorylating the active site serine residues. Neither of these inhibitors has been shown to demonstrate any effect on $sPLA_2$ activity. Further, our group has also demonstrated that pretreatment of endothelial cells with MAFP leads to an increase in basal and thrombin stimulated PAF production as a direct result of inhibition of PAF acetylhydrolase (PAF-AH) activity [29, 30].

Another class of inhibitors of human $cPLA_2\alpha$ and $iPLA_2\beta$ are the 2-oxoamides [31]. These were designed as phospholipid substrate analogs. The 2-oxoamide moiety can act as an electrophilic target for the nucleophilic active site serine. The potencies of the specific $cPLA_2\alpha$ 2-oxoamide inhibitors in animal models of inflammation and pain provide the potential for development of anti-inflammatory therapeutics [32].

The main obstacles to the development of pharmacological inhibitors of cPLA$_2$ are insufficient oral bioavailability, low affinity, and potency in vivo, and insufficient isoenzyme selectivity. 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol (FTY720) might be a drug that overcomes these obstacles since it is highly effective after oral administration, inhibits cPLA$_2$ activity in vitro without affecting sPLA$_2$ or iPLA$_2$, and also inhibits production of arachidonic acid-derived eicosanoids by mast cells [33]. FTY720 is a potent immunomodulator drug that is used to prevent transplant rejection by sequestration of the lymphocytes into secondary lymphoid tissues and preventing their access to graft tissue [34]. It stoichiometrically interacts with recombinant cPLA$_2$α to achieve full inhibition of its enzymatic activity. The half-life for clearance of FTY720 in humans is nearly 8 days, and nanomolar concentrations of FTY720 can inhibit eicosanoid release from mast cells and macrophages within a very short period of treatment. These data suggest that FTY720 may represent a viable cPLA$_2$ inhibitor that can be used in eicosanoid-driven inflammatory disorders.

11.4 Calcium-Independent Phospholipase A$_2$

Calcium-independent PLA$_2$ (iPLA$_2$) is ubiquitously expressed in a wide variety of cells and tissues and is unique in that it can be preferentially distributed in the membrane fraction. Group VI A iPLA$_2$ (iPLA$_2$β) is an 85 kDa protein with eight N-terminal ankyrin repeat sequences. The gene for classic group VI A iPLA$_2$ resides on chromosome 22q13.1 [35]. There are 16 exons which lead to the possibility of formation of several splice variants. The catalytic domain has a consensus lipase motif of GXSXG with Ser465 present in the catalytic center. An additional glycine-rich, nucleotide-binding motif (GXGXXG) occurs just prior to the catalytic site [36]. A calmodulin-binding domain occurs near the C-terminus [37]. The presence of calcium leads to the formation of a calmodulin-iPLA$_2$ complex and subsequently inactivates the enzyme [37]. The N-terminus sequence contains eight ankyrin repeats that are involved in protein–protein interactions and contains several serine and threonine residues [38]. Serine is used for catalysis and, similar to the hydrolytic action of cytosolic PLA$_2$, two sequential nucleophilic displacement reactions are involved leading to the formation of an acyl enzyme intermediate and a lysophospholipid.

Group VI B iPLA$_2$ (iPLA$_2$γ) was identified by screening the human genome for ATP binding and active site motifs [39]. iPLA$_2$β and iPLA$_2$γ comprise the majority of iPLA$_2$ activity in mammalian cells, however, there is little sequence homology between the two enzymes. Group VI B iPLA$_2$ contains a C-terminal peroxisomal localization sequence and a mitochondrial import sequence at the N-terminus, thus this isoform is predominantly membrane-bound [40].

Group VI C (cPLA$_2$γ, neuropathy target esterase-NTE) is expressed in human neurons and its esterase domain slowly hydrolyzes the *sn*-2 position fatty acid of phosphatidylcholine and plays a role in membrane homeostasis [41, 42]. The other

three group VI enzymes (D–F) hydrolyze arachidonic acid at the *sn*-2 position [43]. Additionally they possess a high triacylglycerol lipase and acylglyceroltransacylase activity [43].

The rate limiting step for $iPLA_2$ activity is hydrolysis of the acyl-enzyme intermediate, leading to the production of a fatty acid anion and a proton, both of which become stabilized with solvation by water molecules [44, 45]. This reaction proceeds to completion and is accompanied by the release of a considerable amount of chemical-free energy. Hence the targeted delivery of acyl-enzyme intermediate stabilized by reversible protein–protein interactions would provide a means for the controlled quantitative release of arachidonate product upon hydrolysis providing a much more efficient mechanism for intracellular arachidonate transport [46, 47].

To date, bromoenol lactone (BEL) is the most group-specific inhibitor. It demonstrates 100-fold selectivity for $iPLA_2$ when compared to $cPLA_2$ and $sPLA_2$ isoforms [48, 49]. In addition, Jenkins et al. reported that separation of racemic BEL into its R and S enantiomers demonstrated a tenfold selectivity of (*S*)-BEL for $iPLA_2\beta$ and of (*R*)-BEL for $iPLA_2\gamma$ [50]. This suggests that chiral pharmacologic agents can be used to augment the potency of inhibitors and delineate the $iPLA_2$ isoforms that play a role in cellular responses to various stimulants. However, the selectivity of BEL is not absolute, since it has also been shown to inhibit magnesium-dependent phosphatidate phosphohydrolase, an enzyme that converts phosphatidic acid to diacylglycerol ($IC_{50} = 8$ μM) [51].

11.5 Endothelial Cell Phospholipase A_2 and Membrane Phospholipid Hydrolysis

Activation of endothelial cell PLA_2 can have implications for the production of inflammatory metabolites, inflammatory cell recruitment, motility, signaling, and angiogenesis. Several studies have detected the presence of all three classes of PLA_2 isoenzymes in endothelial cells isolated from different vascular beds [52–60], however the contribution of each to the production of inflammatory mediators remains largely unknown and likely depends on the vascular bed, status of the endothelium and stimulus studied. A recent review [61] on endothelial cell PLA_2 has described the detection of multiple PLA_2 isoforms in endothelial cells from several vascular beds. In addition, relatively few studies have measured the PLA_2 activity directly and rely heavily on the use of PLA_2 inhibitors for data interpretation.

Assay systems have been developed to measure the activity of specific isoforms/classes of enzyme whilst minimizing the contribution from other PLA_2 types. These involve the use of multiple phospholipid substrates, altering calcium concentrations, the inclusion of specific inhibitors, variable incubation times and temperatures, and a range of phospholipid substrate concentrations [62]. We have measured endothelial cell PLA_2 activity using several published assay methods and determined that the majority of endothelial cell PLA_2 activity in cells from several

vascular beds is calcium-independent and membrane-associated [62]. Furthermore, endothelial cell iPLA$_2$ selectively hydrolyzes arachidonylated plasmalogen phospholipids, resulting in accelerated arachidonic acid release.

Activation of endothelial cell PLA$_2$ to release arachidonic acid, and cyclooxygenase (COX)-mediated hydrolysis of arachidonic acid to prostaglandin H$_2$ (PGH$_2$), represent the rate-limiting steps for the prostaglandin biosynthetic pathway. It is thought that different PLA$_2$/COX enzymes are involved in immediate and delayed prostaglandin production [63–67]. The proposed catalytic mechanism for intracellular PLA$_2$ isoforms which allows formation of an acyl enzyme intermediate would support the theory of a direct role for intracellular PLA$_2$ in immediate prostaglandin synthesis. The presence of the long-lived enzyme-acyl intermediate would allow for targeted delivery of enzyme-bound arachidonic acid to downstream enzymes of eicosanoid generation. Although intracellular PLA$_2$ isoforms may be directly coupled to COX within cells, several studies have suggested that immediate eicosanoid production involves both cPLA$_2$ and sPLA$_2$, with cPLA$_2$ being the activator of the response, but sPLA$_2$ providing the bulk of arachidonic acid release [7, 68–70]. There is evidence that cPLA$_2$ is also involved in the delayed production of eicosanoids, although it is likely that its involvement is indirect since intracellular calcium concentrations would be low [68, 71, 72]. It is thought that once cPLA$_2$ is activated, the expression of sPLA$_2$ and COX-2 is upregulated in delayed prostaglandin production. Although the combination of cPLA$_2$/sPLA$_2$/COX has been shown to be responsible for immediate and delayed prostaglandin production in several studies, these studies have primarily been performed in monocyte and macrophage-like cell lines. Whether this scenario holds true for all cell types and under all conditions remains to be elucidated. Several studies have demonstrated that cPLA$_2$ activation may be a result of increased sPLA$_2$ or COX-2 activity, thus the question of directionality or sequence of cPLA$_2$/sPLA$_2$/COX activation is not completely resolved [73–75].

We have measured the increased arachidonic acid release in stimulated endothelial cells from several different vascular beds and determined that this is the direct result of iPLA$_2$ activation [57, 76–82]. Using human coronary artery endothelial cells (HCAEC), we have demonstrated that pretreatment with BEL to inhibit iPLA$_2$ activity resulted in complete inhibition of thrombin- or tryptase-stimulated arachidonic acid and prostaglandin (PGI$_2$ and PGE$_2$) release [17]. These responses were also partially inhibited by PX-18 pretreatment (inhibits sPLA$_2$, see above) suggesting that there may be an interaction between iPLA$_2$ and sPLA$_2$ enzymes in the immediate release of prostaglandins from endothelial cells [17]. Cardiac endothelial cells isolated from wild-type and iPLA$_2$β- or iPLA$_2$γ-knockout mice demonstrated maximal PLA$_2$ activity in the absence of calcium, with the majority of PLA$_2$ activity in unstimulated cells attributable to iPLA$_2$β [77]. When endothelial cells were stimulated with thrombin or tryptase, arachidonic acid and PGI$_2$ release were attenuated in cells isolated from both iPLA$_2$β- or iPLA$_2$γ-knockout mice when compared to wild type, suggesting that both isoforms may contribute to eicosanoid generation [77]. However, endothelial cell PAF production was found to be dependent upon iPLA$_2$β and not iPLA$_2$γ activity [77, 78].

11.6 Role of Endothelial Cell Phospholipase A_2 in Platelet-Activating Factor Production

PAF is a phospholipid metabolite produced by endothelial cells, macrophages, polymorphonuclear leucocytes, eosinophils, basophils, and platelets [83]. The synthesis of PAF in endothelial cells occurs via the remodeling pathway, activated during inflammation and hypersensitivity responses [84]. In thrombin-stimulated endothelial cells, we have shown that the remodeling pathway begins with the activation of $iPLA_2$ and accelerated hydrolysis of plasmenyl ethanolamine (PlsEtn, Fig. 11.2). The resultant lysoplasmenyl ethanolamine (lysoPlsEtn) undergoes a transacylation reaction with plasmanyl choline (PakCho) to form lysoPAF, which is then acetylated by lysoPAF acetyltransferase to form biologically active PAF (Fig. 11.2) [29]. The biological activities of PAF can be rapidly terminated by PAF-acetyl hydrolases (PAF-AH), a family of unique $iPLA_2$ enzymes that hydrolyze the acetyl group at the *sn*-2 position of PAF to generate biologically inactive lyso-PAF and acetate (Fig. 11.2) [85]. This provides an immediate mechanism to prevent, control or terminate the proinflammatory effects elicited by PAF. It follows that the dysregulation of this mechanism would lead to PAF accumulation. Thus, maintaining appropriate PAF-AH activity is essential to suppress the effects of PAF in active inflammation.

Once formed, PAF can bind to its receptor to exert inflammatory affects [86, 87]. Several important endothelial cell functions are regulated by PAF, including impairment of the barrier function, and the adhesion of circulating inflammatory cells to the endothelial monolayer prior to transmigration [84]. PAF also stimulates smooth muscle contraction and changes in the cell cytoskeleton leading to cell retraction

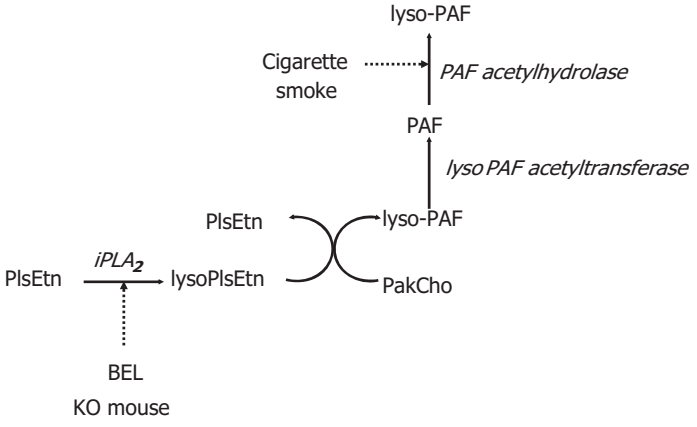

Fig. 11.2 Plasmenylethanolamine (PlsEtn) hydrolysis by endothelial cell $iPLA_2\boldsymbol{\beta}$ is the initial step of endothelial cell PAF production. LysoPlsEtn is transacylated with plasmanyl choline (PakCho) to lyso PAF, which is acetylated to PAF. Inhibition of $iPLA_2$ with bromoenol lactone or in knockout (KO) mice inhibits PAF production, whereas inhibition of PAF acetylhydrolase results in PAF accumulation (see text for details)

and formation of intercellular gaps [88–90]. Changes in the shape of the endothelial cells are associated with the activation of calcium-dependent potassium channels and the hyper-polarization of the cell membranes [91, 92]. PAF stimulation of endothelial cells also induces a dose-dependent synthesis of both prostacyclin and thromboxane A_2, or alternatively the release of plasminogen activator, cleaving plasminogen to plasmin to break up blood clots [93].

During the process of leukocyte adherence to endothelial cells, PAF serves as a spatially-regulated juxtacrine signal between the cells, facilitating adherence and specifically acting at the interface between the endothelium and circulating leukocytes [94]. Newly synthesized PAF remains cell-associated with the endothelial cell monolayer and can directly activate tethered leukocytes [95, 96]. In leukocytes, PAF promotes aggregation, chemotaxis, granule secretion, and oxygen radical generation as well as adherence to the endothelium. PAF is also able to induce the action of β_2-integrins, such as the CD11a/CD18 complex, on activated neutrophils which bind to intracellular adhesion molecule (ICAM) on the endothelial cell surface [97, 98]. Additionally, activation of leukocytes by PAF has been shown to modify the distribution and function of P-selectin glycoprotein ligand-1 (PSGL-1) on the leukocyte surface, possibly leading to the termination of the receptor–ligand bond between the PSGL-1 and P-selectin, allowing movement and transmigration to occur [99]. Studies have demonstrated the ability of PAF receptor antagonists to block the migration of neutrophils across monolayers of cytokine pretreated endothelial cells [100].

11.7 iPLA$_2$ and Cardiovascular Disease

Myocardial infarction and the development of thrombotic coronary artery occlusion are associated with the presence of the serine proteases thrombin and tryptase, both of which can activate iPLA$_2$ in endothelial cells and cardiac myocytes [30, 81, 101, 102]. Activation of myocardial iPLA$_2$ results in the production of lysoplasmenyl choline and arachidonic acid, both of which can change the electrophysiologic properties of the myocardium [103–105]. Gross et al. have also provided evidence that ischemia activates group VI A iPLA$_2$ in myocardium and that group VI A iPLA$_2$-mediated hydrolysis of membrane phospholipids can induce lethal malignant ventricular tachyarrhythmias during acute cardiac ischemia [106].

Thrombin stimulation of HCAEC leads to increased iPLA$_2$ activity. In addition to the resultant increase in PAF and arachidonic acid that can propagate the inflammatory response, release of lysophosphatidyl choline and lysoplasmenyl choline can have direct effects on the myocardium [1, 5]. Both these metabolites have the potential to incorporate into the sarcolemma of cardiac myocytes and cause alterations in the electrophysiological properties [1]. Under hypoxic conditions, accumulation of lysoplasmenyl choline can cause action potential derangements leading to arrythmias [104]. Lysophosphatidyl choline can increase intracellular calcium, change cell shape, and increase creatine kinase release in cardiac myocytes [107, 108]. Accordingly activation of endothelial iPLA$_2$ by thrombin at the sites of vascular

injury or thrombosis can contribute to cardiac dysfunction due to increased choline lysophospholipid generation.

In addition to signal transduction, $iPLA_2$ is involved in membrane phospholipid remodeling [7, 109], particularly when unsaturated fatty acids are oxidized and accumulate in the phospholipid bilayer [110–113]. Polyunsaturated fatty acids are preferentially oxidized and found almost exclusively at the *sn*-2 position of membrane phospholipids [113]. Neither $cPLA_2$ nor $sPLA_2$ display a preference for oxidized phospholipid substrates, suggesting that $iPLA_2$ or PAF-AH enzymes are responsible for the membrane repairs. Release of peroxidized fatty acids from membrane phospholipids is an absolute requirement for glutathione peroxidase to reduce and detoxify fatty acid hydroperoxides in the membrane, indicating that $iPLA_2$ has an essential function in the detoxification of oxidized membrane phospholipids and protection against cell injury and death [114]. It follows that inhibition of $iPLA_2$ may augment oxidant-induced cell injury and we have demonstrated a marked inhibition of myocardial $iPLA_2$ activity with clinical concentrations of doxorubicin, an anticancer drug that is associated with a high incidence of cardiotoxicity, possibly mediated by increased oxygen-free radical formation and phospholipid peroxidation [26, 115–118]. Additionally, inhibition of $iPLA_2$ activity with BEL potentiated doxorubicin-induced cell death, suggesting that $iPLA_2$ serves a protective role possibly via membrane remodeling in response to oxidative stress.

Myocarditis is associated with progressive inflammation and injury of the myocardium [119] and, as such, suggests that management of inflammatory cell recruitment could be beneficial in this disease. However, the underlying cause of the myocarditis must first be identified. Chagas' disease, an infection caused by *T. cruzi*, is the primary cause of myocarditis Worldwide [120–122]. It is a major health problem in Central and South America and an emergent medical problem in the United States and Western Europe. Although there have been no published studies to date that have directly addressed the role of endothelial cell $iPLA_2\beta$ in myocarditis, we propose that modulation of its activity could prove to be a viable therapy to manage recruitment of inflammatory cells and myocardial damage during acute infection. We have shown that *T. cruzi* infection of HCAECs results in a time-dependent increase in PAF production and thus may play a role in inflammatory cell recruitment (Fig. 11.3). Earlier studies have shown that PAF is produced in response to *T. cruzi* and mediates resistance to infection [123]. Taken together with our data, this suggests that inhibition of PAF production as a result of $iPLA_2\beta$ inhibition could result in exacerbation of parasitemia and death. Taken together, the above data suggest that $iPLA_2$ inhibition in cardiovascular disease may be a viable therapy, but must be evaluated with extreme caution.

11.8 Endothelial Cell $iPLA_2\beta$ and Tumor Metastasis

Eicosanoids and PAF in the primary tumor microenvironment have been extensively studied as mediators of inflammation, tumor growth and differentiation, and angiogenesis, but their role in metastases is not well studied. The majority of cancer

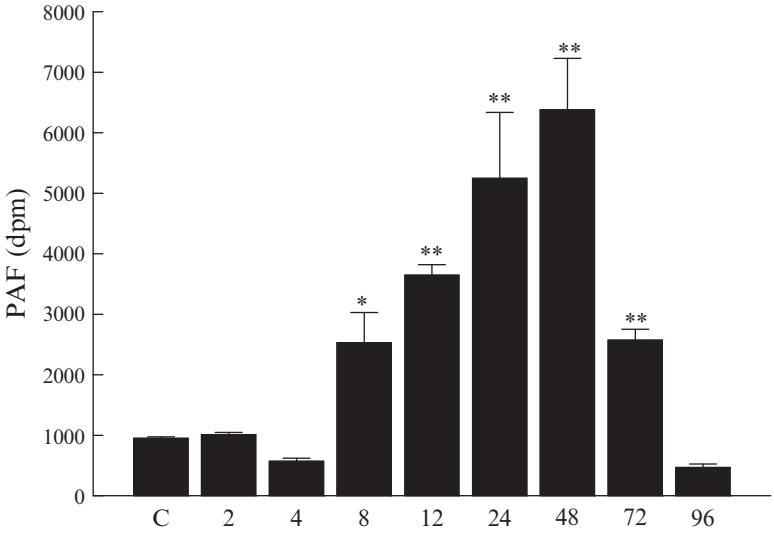

Fig. 11.3 PAF production in human coronary artery endothelial cells in response to *T. cruzi* infection (Brazil strain, MOI 0.2). $^{*}p<0.05$, $^{**}p<0.01$ when compared to uninfected controls. $n=6$

deaths are attributed to the growth of metastases rather than the primary tumor [124] and so control of the metastatic process is vital to managing cancer patients. Spreading of tumor cells from the primary site to distant organs requires transendothelial cell migration to and from the circulation. Transendothelial cell migration is dependent at least in part on the interaction between endothelial cell surface PAF expression and the PAF receptor on tumor cells in a similar manner to that of the circulating inflammatory cells [125, 126].

PAF-receptor antagonists have been shown to interfere with melanoma cell adherence to the endothelium [127] and daily injections of a PAF-receptor antagonist decreased lung metastases following i.v. injection of human melanoma cells into nude mice [126]. In a recent study, we determined the contribution of iPLA$_2$β to lung metastasis development after injection of E0771 breast cancer cells into the mammary pads of wild type and iPLA$_2$β-knockout mice and observed an 11-fold decrease in breast cancer cell number in the lungs of iPLA$_2$β-KO mice when compared to WT [128]. We have also determined that E0771 cells possess the PAF receptor and that they adhere to mouse lung endothelial cells stimulated with thrombin or TNF-α [128]. Adherence to lung endothelial cells isolated from iPLA$_2$β-knockout mice was absent, suggesting that the decrease in lung metastases was at least in part due to the absence of endothelial cell PAF production and decreased endothelial transmigration by the tumor cells. We have demonstrated that TNFα stimulation of human lung microvascular endothelial cells results in enhanced adherence of MDA-MB-231 cells (a highly invasive, estrogen-independent breast cancer cell line) and that adherence is inhibited by pretreating the endothelial cells with (*S*)-BEL to inhibit iPLA$_2$β activity or treating the cancer cells with ginkgolide B

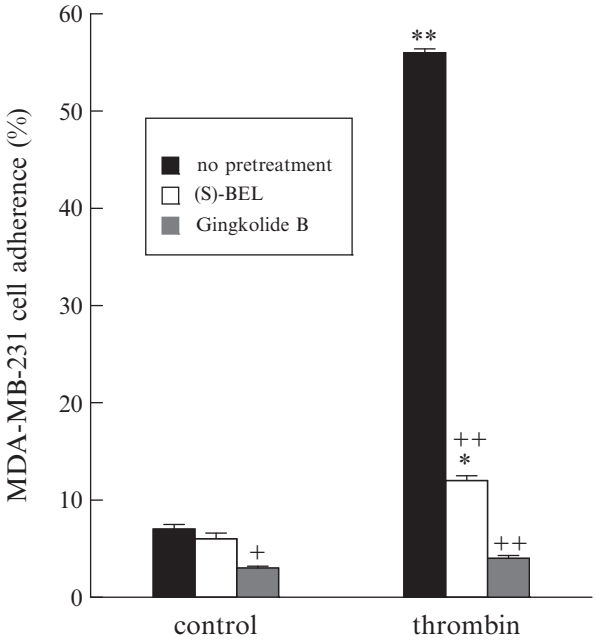

Fig. 11.4 Adherence of MDA-MB-231 cells to human lung microvascular endothelial cells stimulated with thrombin (1 IU/mL, 1 h). Endothelial cells were incubated with (*S*)-BEL (2 μM, 30 min) or tumor cells were incubated with ginkgolide B (10 μM, 30 min). $^{*}p<0.05$, $^{**}p<0.01$ when compared to unstimulated cells. $^{+}p<0.05$, $^{++}p<0.01$ when comparing data with and without inhibitor. $n=8$

to block the PAF receptor (Fig. 11.4). These data highlight the importance of the PAF–PAF receptor interaction in adherence of tumor cells to the endothelium and identify a promising potential for the use of a nutraceutical in cancer management. The seeds and leaves of *Ginkgo biloba* have been used in traditional medicine to treat respiratory diseases, cardiovascular disorders, memory loss, sexual dysfunction, and loss of hearing [129]. In vitro, *Ginkgo biloba* extract exhibits anti-infective, chemopreventive, anticancer, and cytotoxic effects [130–132]. *Ginkgo biloba* has been implicated in reducing the risk of ovarian cancer [133], reducing tumor area in gastric cancer [132] and augmenting 5-fluorouracil treatment of advanced colorectal cancer [134]. Based on our in vitro data, we propose that *Ginkgo biloba* may also be beneficial in reducing the risk of metastasis and represents a novel and exciting aspect of our recent studies.

Another recently published study using knockout mice has demonstrated that iPLA$_2$β in both tumor and host cells participates in epithelial ovarian cancer development and that the absence of this enzyme attenuates tumorigenesis, metastasis, and ascites formation induced by ovarian cancer cells [135]. These studies suggest that iPLA$_2$β inhibition could be of considerable therapeutic value in patients with a variety of cancers.

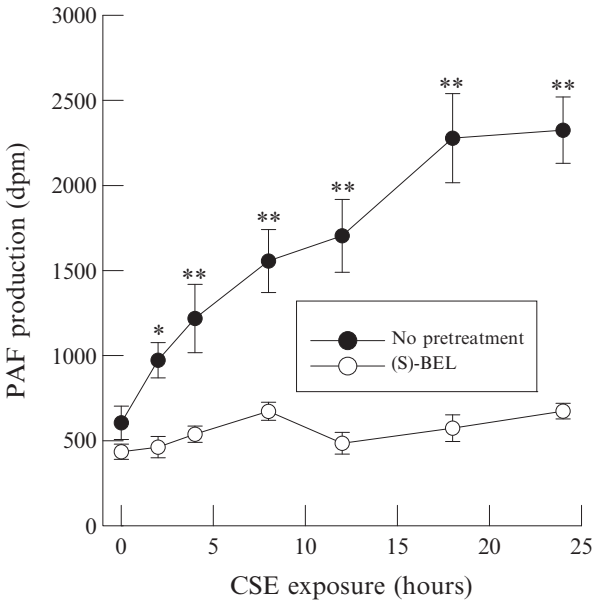

Fig. 11.5 PAF production in human lung endothelial cells exposed to cigarette smoke extract (CSE) with or without pretreatment with (*S*)-bromoenol lactone ((*S*)-BEL, 2 μM, 30 min prior to CSE). $^{*}p<0.05$, $^{**}p<0.01$ when compared to controls. $n=6$

As mentioned previously, inhibition of PAF-AH activity would result in PAF accumulation and propagation of its inflammatory effects. Cigarette smoke has been shown to inhibit circulating PAF-AH [136, 137] and results in increased plasma PAF concentrations in smokers. In a recent study [76], we have shown that cigarette smoke extract inhibits lung endothelial cell PAF-AH activity as shown in Fig. 11.5, resulting in increased PAF production and adherence of polymorphonuclear leukocytes. These studies demonstrate that inhibition of endothelial cell PAF-AH activity alone is sufficient to increase PAF production and highlight a possible therapeutic target in smokers. Inhibition of human lung endothelial cell iPLA$_2$β with (S)-BEL (Fig. 11.4) or the absence of this enzyme in endothelial cells isolated from the iPLA$_2$β-knockout mice resulted in inhibition of the cigarette smoke-induced increase in PAF production, highlighting the importance of this enzyme in endothelial cell PAF production and modulation of its effects. These data have important implications for inflammatory diseases in smokers, including cancer metastasis.

In a small number of studies, cigarette smoking has been associated with pulmonary metastases secondary to cancer, including breast cancer [138–144]. In 2001, Murin & Inciardi demonstrated an association between cigarette smoking and pulmonary metastatic disease in breast cancer patients [139]. Subsequently, Murin et al. demonstrated an increase in pulmonary metastases subsequent to i.v. injection of breast cancer cells in mice exposed to cigarette smoke when compared to non-smoking controls [138]. These studies suggest that there is a cause-and-effect

relationship between cigarette smoke and lung metastases, although no mechanism for this has been elucidated to date. We propose that the increase in metastases in smoking may be a result of increased PAF accumulation in the endothelium, resulting in increased transmigration of cells from the primary tumor to the circulation, from the circulation to secondary sites, or both. Accordingly, modulation of endothelial cell iPLA$_2\beta$ activity may prove to be an exciting development of cancer metastasis in future.

11.9 Conclusions

As discussed previously, since several PLA$_2$ enzymes hydrolyze membrane phospholipids utilizing the same catalytic site on the enzyme, the development of selective pharmacologic inhibitors for a single PLA$_2$ isoform or group has proved difficult. One of the major problems with developing a viable PLA$_2$ inhibitor is separation of the physiological from the pathological properties. PLA$_2$-catalyzed hydrolysis of membrane phospholipids is important in many essential and beneficial processes in both normal and disease states. Hydrolysis of phospholipids by PLA$_2$ is the rate limiting step for multiple cell signaling and biochemical responses and is essential for membrane phospholipid remodeling and repair. However, the development of specific PLA$_2$ inhibitors still has the potential to eliminate or limit the production of several inflammatory mediators either individually or together.

The theoretical advantages of a PLA$_2$ inhibitor over a COX or lipoxygenase inhibitor, for example, include limitation of eicosanoid production in general via a reduction in the arachidonic acid precursor, plus inhibition of PAF production which would minimize inflammatory cell recruitment. On the other hand, limiting inflammatory eicosanoid production and preserving protective eicosanoids is the key to a viable PLA$_2$ inhibitor. Clearly, the key to the development of a therapeutic inhibitor is to optimize maximal efficacy with minimal side effects. Whether specific PLA$_2$ inhibition can be achieved effectively for identified disease states remains unknown, but as more data emerges characterizing PLA$_2$-catalyzed membrane phospholipid hydrolysis, it is hoped that our understanding of this complicated process may lead to new therapeutic avenues. As data from several of our recent studies suggest, the future development of inhibitors may be driven more by a pharmaceutical aspect than a pharmacological one, with targeting inhibitors towards specific tissues or cells or using local rather than systemic administration.

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Part III
Role of Phospholipase C

Chapter 12
The Role of Phospholipase C Isozymes in Cellular Homeostasis

Kiyoko Fukami and Yoshikazu Nakamura

Abstract Phosphoinositide turnover influences various functions such as cell proliferation/differentiation, fertilization, neuronal functions, and cell motility. Phospholipase C (PLC) triggers the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to generate two second messengers, inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), and diacylglycerol (DAG). $Ins(1,4,5)P_3$ releases calcium from intracellular stores, and DAG activates protein kinase C (PKC). $PI(4,5)P_2$ also directly regulates various cellular functions, including cytoskeletal remodeling, endocytosis/exocytosis, and channel activity. Imbalances in these phosphoinositides facilitate the pathogenesis of various human diseases. Therefore, precise regulation of the levels of $PI(4,5)P_2$ by PLC or other interconverting enzymes is indispensable for normal cellular functions. Recently several mouse models with genetic-deficits of PLC isozymes have been generated and these analyses revealed the specific functions of each of these isozymes. Taken together with the genome-based information, specific isozymes were found to have a pivotal role in maintaining cellular homeostasis. Since PLC is an intracellular calcium-regulating enzyme, the PLC knockout (KO) mice often show disruption in the calcium homeostasis. This article reviews the regulation of calcium homeostasis by PLC isozymes in fertilization and neuronal functions. PLCKO mice have abnormal cellular proliferation, differentiation, apoptosis, and development, suggesting that PLC isozyme facilitates the determination of cell fate. These physiological regulations are implicated in several cellular functions and play a very important role, especially in tissues with high metabolic turnover such as the skin, colon, hematopoietic cells, and developing embryo. Therefore, the focus of this review is on the physiological functions of PLC isozymes in these cells and on the diseases that are caused by the dysregulation of PLC isozymes and consequent disruption in calcium- and cellular-homeostasis.

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_12,

Keywords Phospholipase C • Homeostasis • Calcium • Skin • Tumorigenesis • Cell growth • Differentiation

12.1 Introduction

Thirteen phospholipase C (PLC) isozymes have been identified in mammals, and they are classified into six types, β(1−4)-, γ(1,2)-, δ(1,3,4)-, ε-, η(1,2)-, and ζ-type, based on their structural and regulatory mechanisms [1]. Each isozyme is composed of subtype-specific domains and conserved domains. Specific domains contribute to the anchoring of the enzymes to the plasma membrane enabling their activation and facilitating their activities. The regulatory mechanisms of β-type and γ-type PLCs have been analyzed extensively. Association of heterotrimeric G proteins of the Gq family stimulates the activity of β-type PLC, and γ-type isozymes, and are regulated primarily by receptor and cytosolic tyrosine kinases. The δ-, η-, and ζ-type PLC isozymes are calcium sensitive and may be regulated by low calcium concentration. The ε-type PLC was identified as an effector of Ras protein and is regulated by Ras in a GTP-dependent manner [1].

Analyses of genetically engineered mice with disruption of these isozymes and the genome-based information have revealed the specific function of individual isozymes. Isozymes were found to have significant but unique roles in maintaining cellular homeostasis. In this review, we focus on the physiological functions of PLC isozymes and the diseases caused by disrupted homeostasis. This review aims to highlight the regulation of calcium homeostasis and cellular proliferation/differentiation by PLCs, especially PLCδ-type isozyme.

12.2 PLC Regulates Calcium Homeostasis

Calcium as a signal transduction system is a double-edged sword in higher animals. High calcium concentrations are toxic for cells and leads to apoptosis or necrosis. However, an optimal increase in calcium levels positively signals various physiological functions. It has been clarified that the phosphoinositide metabolism has an important role in intracellular calcium mobilization. Increase in calcium levels is especially important in neuronal and cardiac muscle function and in fertilization. Therefore, we first review the PLC isozymes that modulate calcium levels in specific cells or organs that influence fertilization or neuronal function.

12.3 Role of PLCζ and PLCδ4 in the Initial Stages of Fertilization

Transient or oscillating increase in calcium levels is observed in eggs at fertilization, and this calcium increase is critical for the initiation of egg activation [2]. This calcium increase is mediated by an increase in $Ins(1,4,5)P_3$ levels, which releases the

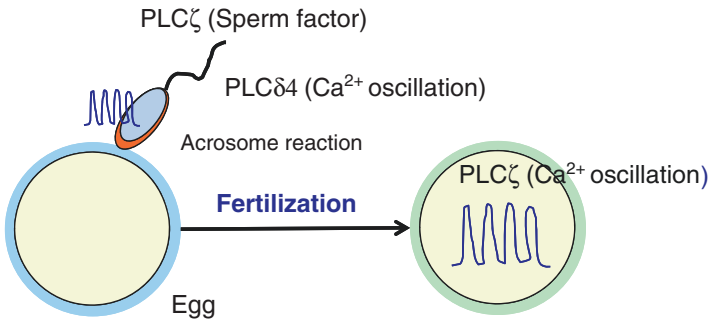

Fig. 12.1 Role of PLCζ and PLCδ4 in fertilization. PLCζ functions as a sperm factor that induces calcium oscillation in eggs at fertilization. PLCδ4 is also implicated in the calcium oscillation in sperm to induce the ZP-induced acrosome reaction

calcium from the intracellular stores in the egg. Microinjection of sperm extracts into eggs trigger fluctuations in calcium levels similar to those observed in the fertilization of mammalian eggs, where an unidentified "sperm factor" has been predicted to elicit a serial increase in calcium levels. Lai et al. made a major breakthrough in these studies when they reported that the sperm-derived PLCζ is the key molecule that causes these calcium oscillations during fertilization [3, 4] (Fig. 12.1). In addition, other studies have also reported that recombinant PLCζ protein can induce calcium oscillation-like patterns during fertilization of mammalian eggs [5]. These studies indicate that PLCζ is a sperm factor that induces calcium oscillation in eggs at fertilization.

On the other hand, calcium has also a primary role in the execution of the acrosome reaction in sperm [6]. In mammalian sperm, the acrosome reaction is initiated in vivo by the binding to the zona pellucida (ZP), and only sperm that have completed this process can penetrate the ZP and fuse with the egg plasma membrane. PLCδ4KO male mice show reduced fertility in vivo [7]. In vitro fertilization studies have shown that insemination with PLCδ4KO sperm results in significantly fewer eggs becoming activated and that the transient fluctuations in calcium levels associated with the fertilization are absent or delayed. Furthermore, the calcium responses observed in a single sperm treated with ZP were not observed in the PLCδ4KO sperm [8], resulting in PLCδ4KO sperm being not able to initiate the acrosome reaction. These results indicate that PLCδ4 has an important role in the calcium responses during the ZP-associated acrosome reaction (Fig. 12.1).

12.4 Different PLC Isozymes Influence Neuronal Function

The importance of calcium in neurons has been widely reported. Calcium plays an essential role in the regulation of axon extension and retraction, guidance of growth cones, synapse formation, and responses to various neurotransmitters. It is worth noting that most PLC isozymes, except for PLCγ2 and PLCζ, exist abundantly in

brain, indicating that PLC participates in the neuronal function by regulating the calcium mobilization. In addition, it is intriguing to note that most PLC isozymes are highly expressed in the retina [1]. This study reflects the correlation of visual responses to these enzymes.

Multiple functional analyses using KO mice revealed that PLCβ-type enzymes have a central role in the neuronal function. PLCβ1KO mice developed epilepsy, whereas PLCβ4KO mice developed ataxia [9]. PLCβ3 was implicated in μ-opioid-mediated responses and in sweet and bitter taste reception [10]. In addition to PLCβ, the expression of PLCη-type enzyme is exclusively restricted to the brain [11, 12]. Interestingly, PLCη2 was specifically expressed in the habenula [13], which regulates emotional and social behaviors such as sexual behavior, circadian rhythms, schizophrenia, and drug dependence [14]. Recently, a link between PLCη2 and mental retardation has been reported. Patients bearing constitutional deletion of a smaller region, 1p36.3, present with a number of features, including mental retardation. Since PLCη2 maps on the 1p36.32 region, PLCη2 might be a putative candidate gene for the neurodevelopmental delay observed in these patients [15].

It has been reported that PLC-γ1 forms an integral part of neural networks that regulate various brain functions such as memory and mood-related behaviors [16–18]. In addition, polymorphisms in the human PLC-γ1 gene have been linked to the pathogenesis of bipolar disorders [19]. Since PLC-γ1KO is lethal at the embryonic stage, generation of conditional KO mice will reveal the implication of PLC-γ1 in the brain function.

12.5 Cell Fate Determination by PLCs

Determination of cell fate such as cell growth, differentiation, and cell death is critical in every mammalian cell for maintaining cellular homeostasis. Accumulating data strongly suggest the potential links of PLCs to the decision-making process in determining the cell fates. Here we focus on PLC isozymes that are related to these phenomena in skin and embryo development.

12.6 Skin Homeostasis and PLCs

The skin is a mechanical and immune barrier between the inner and outer sides of the body. The skin is composed of the epidermis, dermis, hypodermis, and many mini-organs, such as hair follicles and sebaceous glands. Among them, the epidermis is a stratified epithelium composed mainly of keratinocytes, with a single basal layer of proliferating keratinocytes and multiple overlying differentiated layers. PLC potentially regulates keratinocyte differentiation, because downstream signals of PLC, such as increased calcium and PKC activation, are known to regulate keratinocyte differentiation [20]. Although PLCγ1, PLCδ1, and PLCε exist in

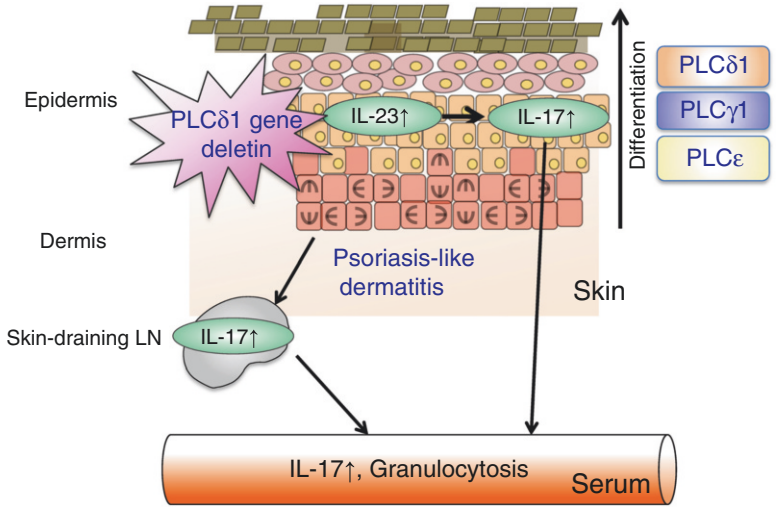

Fig. 12.2 Skin homeostasis is regulated by PLCs. PLCγ1, PLCδ1, and PLCε are abundantly expressed in keratinocytes. Loss of PLCδ1 in the epidermis resulted in epidermal hyperproliferation and overproduction of IL-23 and IL-17 in mice, which has a similar phenotype to that of psoriasis. Skin-derived IL-17 leads to systemic inflammation characterized by granulocytosis

keratinocytes, deletion of PLCδ1 gene resulted in the loss of PLC activity by 90 % in the epidermis, suggesting that PLCδ1 is probably the dominant PLC isozyme in the epidermis [21]. PLCγ1 and PLCδ1 are up-regulated during differentiation of keratinocytes and seem to regulate calcium mobilization [22, 23].

Deregulation of calcium- and skin-homeostasis by PLC gene deficiency was implicated in various skin diseases. Although the in vivo function of PLCγ1 in the epidermis has not been studied yet, in vitro studies indicate that the antisense-PLCγ1 prevents the extracellular calcium-induced differentiation of keratinocytes and inhibits the expression of involucrin and transglutaminase [24]. Similarly, the PLCδ1KO mice have an abnormal differentiation and hyperproliferation of the epidermal cells [23]. In addition, keratinocyte-specific ablation of PLCδ1 gene leads to inflammation and overproduction of the inflammatory cytokines, such as IL-23 and IL-17, a pivotal cytokine implicated in the pathogenesis of psoriasis. Interestingly, PLCδ1 protein is decreased in the epidermis of human patients with psoriasis [21]. These observations suggest that insufficient expression and activity of PLCδ1 in keratinocytes contributes to the pathogenesis of human psoriasis. Furthermore, keratinocyte-specific ablation of PLCδ1 resulted in not only skin inflammation but also in systemic inflammation. Skin-derived IL-17 caused the elevation of serum IL-17 levels, granulocytosis, and increased body temperature [21] (Fig. 12.2).

PLCδ1KO mice also showed marked hair loss [23]. Analysis of the similarity with nude mice, which is caused by a mutation of transcription factor Foxn1 gene, indicated that the Foxn1 functions as an upstream regulator of PLCδ1 expression in hair shaft formation [25]. Recently, PLCδ1 was reported as the gene responsible for

hair defects in mice with the recessive spontaneous mutation oligotriche (olt) [26]. These studies suggest that PLCδ1 has essential roles in the formation of normal hair shafts. The role of PLCε in skin inflammation and tumorigenesis is discussed later.

12.7 Requirement of PLC Isozymes in Embryonic Development

Embryonic development is a well-organized process where proliferation, differentiation, and apoptosis are precisely regulated. Analysis of KO mice models suggests that PLCγ1 and PLCδ1/PLCδ3 have been reported to be involved in these processes.

Homologous disruption of the PLCγ1 gene in mice resulted in lethality approximately at embryonic day 9 (E9) [27]. Erythropoiesis and vasculogenesis are significantly impaired in PLCγ1KO embryos at the same stage, suggesting that defects of proper differentiation may be responsible for the embryonic lethality of PLCγ1KO mice.

In addition, PLCδ1/PLCδ3 double KO (DKO) mice also resulted in embryonic lethality at E11.5–E13.5 caused by differential defects of placental development [28]. The placenta of PLCδ1/PLCδ3 DKO mice had decreased number of vessels in the labyrinth layer and showed an increased apoptosis. Furthermore, PLCδ1/PLCδ3DKO embryos supplied with a normal placenta by the tetraploid aggregation method survived beyond E14.5, indicating that the embryonic lethality is caused by a defect in the placenta. These results indicate that PLCδ1 and δ3 are essential for placental development and mediate the regulation of cellular apoptosis and survival.

12.8 Diseases Caused by the Disruption of Cellular Homeostasis

As described previously, PLCs have an important role in maintaining cellular homeostasis. Even subtle variations in the balance between cell growth, differentiation, and cell death, and/or calcium homeostasis leads to serious diseases such as tumorigenesis.

12.9 Promotive and Suppressive Roles of PLC Isozymes in Tumorigenesis

Recent studies have proposed diverse roles of PLC isozymes in tumorigenesis; some facilitate tumor formation, while others function as anti-oncogenes. PLCγ1 is well known to play a pivotal role in mitogenic signaling [27, 29, 30]. PLCγ1 binds to EGFR and PDGFR by an interaction between a phosphotyrosine residue of their receptors and the SH2 domain of PLCγ1 [31]. SH3 domain of PLCγ1 also binds to the

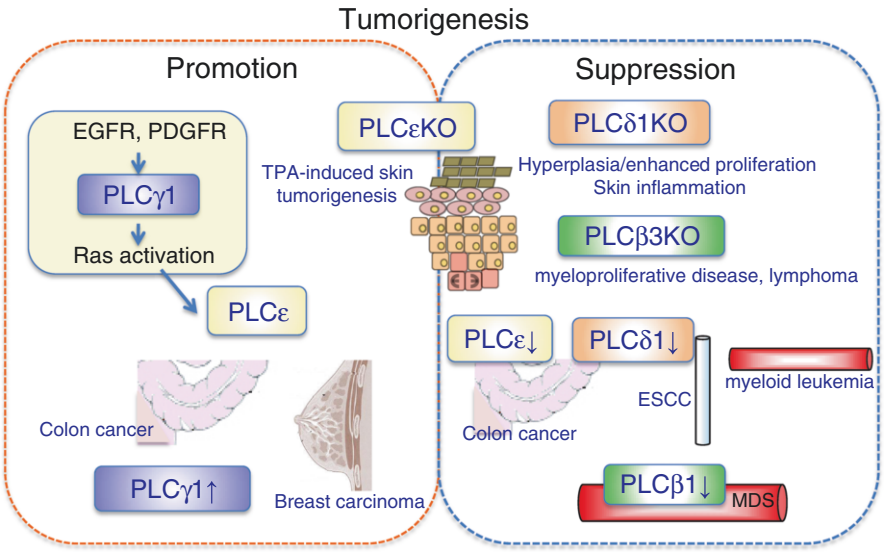

Fig. 12.3 Promotive and suppressive roles of PLC isozymes in tumorigenesis. PLCγ1 binds to EGFR and PDGFR, and also binds to the Ras exchange factor, SOS1, leading to enhancement of Ras activation. The expression of PLCγ1 is enhanced in human breast carcinoma, colorectal cancer, and so on. PLCε is identified as an effector of Ras. PLCεKO mice exhibit resistance to tumor formation and TPA-induced skin chemical carcinogenesis. PLCδ1 was identified as an anti-oncogene protein in human. The skin of PLCδ1KO mice show hyperplasia and enhanced proliferation. PLCδ1 gene is deleted frequently in colon cancer, esophageal squamous cell carcinoma (ESCC), or decreased in myeloid leukemia. Deletion of PLCβ1 gene was also detected in myelodysplastic syndromes (MDS) that progress to acute myeloid leukemia

Ras exchange factor, SOS1, leading to the enhancement of Ras activation. This implies that PLCγ1 may be correlated with tumorigenesis (Fig. 12.3). In fact, the expression of PLCγ1 was enhanced in many human cancers such as human breast carcinoma, familial adenomatous polyposis, and colorectal cancer [29] (Fig. 12.3).

On the other hand, the implications of PLCε on skin inflammation and tumorigenesis were analyzed by Kataoka's group using PLCεKO mice. They identified PLCε as an effector of Ras, and showed that PLCεKO mice exhibit resistance to tumor formation in two-stage skin chemical carcinogenesis [32] (Fig. 12.3). In this model, PLCε facilitates tumor promotion by stimulating 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation such as edema, granulocyte infiltration, and expression of a proinflammatory cytokine, interleukin-1α (IL-1 α) [33]. Recently PLCε was also reported to link neuroinflammation [34]. Meanwhile, ultraviolet (UV) β-induced skin tumor development was enhanced in PLCεKO mice. In this case, cell death in the skin was markedly suppressed in PLCεKO mice, suggesting a novel function of PLCε in regulating UVβ-induced cell death [35].

In contrast, PLCδ1 was identified as an anti-oncogene protein in human (Fig. 12.3). Since 3p22 is frequently deleted in esophageal squamous cell carcinoma (ESCC), Li et al. screened genes that are located in this region and found that PLCδ1 is a strong candidate for a tumor-suppressor gene [36]. The expression of PLCδ1 was not

detected in the primary ESCCs and ESCC cell lines, and the down-regulation of PLCδ1 protein was significantly correlated with ESCC metastasis. In addition, the decrease in the expression of PLCδ1 correlated with the clinical outcome of acute or chronic myeloid leukemia patients [37]. Furthermore, transcriptome analysis indicated that repression of PLCδ1 gene was found associated with KRAS mutation in colorectal cancer [38]. Taken together with the epidermal hyperplasia and enhanced cellular proliferation in PLCδ1KO mice [21, 23], the studies collectively indicate that PLCδ1 may play an important suppressive role in the development and progression of some cancers. On the other hand, Yuan et al. isolated a new gene, DLC-1, which is frequently deleted in liver cancer. Human DLC-1 shares high homology with rat p122 RhoGAP [39], a PLCδ1-binding protein. Since DLC-1 inhibited human cancer cell growth and the in vivo tumorigenicity in nude mice, it is possible that p122 functions as anti-oncogene by synergic interaction of PLCδ1 and by the modulation of the Rho-mediated actin cytoskeleton.

Similarly, PLCβ-type is being considered as an anti-oncogene. The involvement of PLCβ1 in hematopoietic differentiation implicated the role of PLCβ1 in hematological malignancies such as myelodysplastic syndromes (MDS) that progress to acute myeloid leukemia [40]. Fluorescence in situ hybridization (FISH) analysis demonstrated that patients bearing a mono-allelic deletion of the PLCβ1 gene had a worse clinical outcome as compared with patients having both alleles. These results suggest that PLCβ1 regulates cell survival and proliferation of MDS cells. In addition, PLCβ3KO mice develop myeloproliferative disease, lymphoma, and other tumors, suggesting that PLCβ3 is a potential tumor suppressor [41].

12.10 Conclusions

This review was focused on the role of PLC isozymes in the maintenance of cellular homeostasis and associated diseases due to the disruption of this homeostasis. Though many of the PLC isozymes may have different roles in specific cells or tissues, it would be difficult to categorize their functional role only based on the differences in their distribution in tissues, different activation mechanisms, and their regulation of calcium increase. By further analyzing the associated signaling mechanism, we could understand the pathophysiology of the diseases induced by disorders of individual PLCs, and thereby contribute to the prevention and treatment of these diseases.

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Chapter 13
Phospholipase C Isoform Functions in Immune Cells

Charlotte M. Vines

Abstract Phospholipase C (PLC) family members play critical roles in regulating immune cell functions during inflammatory responses. This chapter discusses how different family members can be activated by G-protein coupled receptors, T-cell receptors, B-cell receptors, and other tyrosine kinase receptors, in addition to many of the pathways that contribute to propagation of signaling through the intracellular signaling events that are mediated by different family members. By understanding these signaling events and immune mechanisms we will be able to better define targets for pharmacological intervention for inflammation and autoimmune diseases.

Keywords Immune cells • Phospholipase C • Signaling • Receptors

13.1 Introduction

Phospholipase C (PLC) family members are a group of enzymes that are expressed in a number of cell types, including immune cells, whose function is to hydrolyze lipids, which generates signaling intermediates. Accordingly, PLC is activated in response to receptor clustering, which generates multi-protein complexes to promote activation of intracellular signal transduction pathways. The association of proteins within these complexes is maintained via binding and affinity domains. Within these signaling complexes are src homology 2 (SH2) domains that bind to phosphorylated tyrosines, while src homology 3 (SH3) domains interact with proline-rich, and arginine/lysine-rich motifs [1–4]. Cooperative binding between these sites facilitates clustering and thereby promotes signaling [5].

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_13,

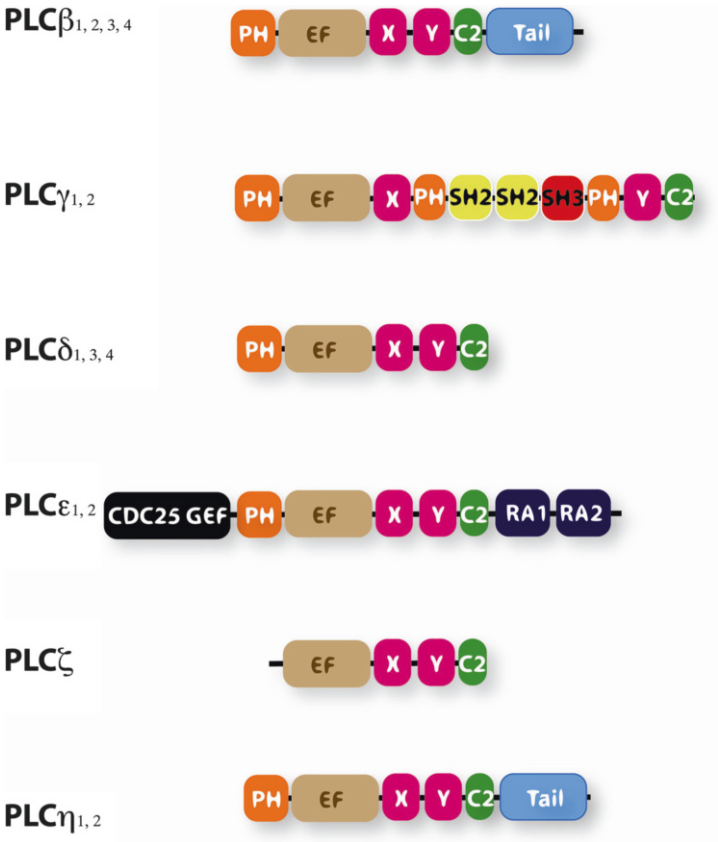

Fig. 13.1 Phospholipase C isoforms. The PH plextrin homology, EF hand, X, Y catalytic domains, and the CDC25 homology guanine nucleotide exchange factor domain

Six different PLCs have been identified (β, γ, δ, ε, ζ, and η), which consist of 13 family members (Fig. 13.1). There are four isoforms of PLCβ (PLCβ1-4), two of PLCγ (PLCγ1-2), three of PLCδ (PLCδ1, -3, -4), two of PLCη (PLCη-1 and -2), and one each of PLCε and ζ (as reviewed [6]). PLCβ1, PLCβ3, PLCγ1, and PLCγ2 are expressed in a wide range of tissues, while the expression of PLCβ2 is restricted to hematopoietic cells and PLCβ4 to neuronal cells. PLCζ and η, which are not expressed in hematopoietic cells, are not discussed any further in this chapter; however, more information can be found regarding each isoform [7, 8].

In the PLCγ enzymes the X/Y linker found within the catalytic domain promotes auto-inhibition [9]. Similar to PLCγ1 it is thought that the auto-inhibition of PLCγ2 is released by phosphorylation of the tandem SH2 domains by RTKs [10]. The Ser 707 site within PLCγ2 is found within 100 amino acids of three well-described phosphorylation sites: Tyr733, Tyr753, and Tyr 759. Therefore, the S707Y mutation observed in antibody deficiency and immune disregulation [114] Tyr site likely

affects auto-inhibition of this protein. PLC family members can promote signaling via multiple mechanisms including serving as adapter proteins and as hydrolyzing enzymes. During hydrolysis, the function of these enzymes is to cleave phosphatidyl inositol-4, 5-biphosphate (PIP2) into the second messengers inositol-1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG) (as reviewed by [6]). IP3 binds to receptors in the endoplasmic reticulum, the soluble IP3 promotes release of calcium stores, which activates protein kinase C (PKC) and activates Orai1 channels in the plasma membrane. The sustained Ca^{2+} mediated signaling leads to gene transcription [11]. In addition, DAG potentiates signaling by activating PKC family members. In response to these signaling events, cells migrate, proliferate, mediate sensory input, and differentiate.

13.2 Functions of PLC Family Members in Immune Cell Types

13.2.1 PLCβ

PLCβ is the best-characterized PLC family member expressed in immune cells [12]. Under specific conditions PLCβ can either promote or inhibit signaling to control cellular responses to immune environments. Isoforms of PLCβ promote cellular differentiation, proliferation, and migration to control innate and adaptive immune responses. Dendritic cells (DCs) and macrophages are professional antigen presenting cells, whose primary function is to orchestrate the activation of T cells during onset of the adaptive immune response. These cells are widely distributed within a host, where they serve as sentinels that detect, ingest, and present antigens. While probing their extracellular environments for antigens, immature dendritic cells continuously ruffle their membranes. This ruffling is due to the presence of high concentrations of phosphatidic acid (PA) at their plasma membranes [13]. It was observed that PA is produced by phosphorylation of DAG, downstream from stimulated PLCβ1 and PLCβ3 [13]. This was unexpected since phospholipase D had been thought to regulate PA levels in non-phagocytic cells [14, 15], and therefore marked a novel role for PLCβ in phagocytes.

In monocyte-derived dendritic cells, PLCβ1 also propagates signaling through toll-like receptors and prostaglandin E2 receptors to regulate the extent of the inflammatory response [16]. In addition, signaling through PLCβ1contributes to the maturation of dendritic cells through Ca^{2+} signaling as well. In these studies a number of diverse agonists including lipopolysaccharide (LPS), cholera toxin, dibutyryl-cyclic AMP, prostaglandin E2, and the calcium ionophore A23187, which all promote the release of Ca^{2+} from intracellular stores, led to production of IP2, and dendritic cell maturation through PLC. Maturation of dendritic cells could be blocked by using the PLC inhibitor D609 at 100 μM [17]. The authors proposed that the PLC family member involved is PLCβ, since this maturation could be

mimicked by stimulation of immature dendritic cells in the presence of *Pasteurella multocida* toxin, which promotes the activation of PLCβ1, maturation of the cells. This maturation was marked by upregulation of activation markers CD80, CD83, CD86, and HLA-DR. While maturation of dendritic cells can be induced by other stimuli such as LPS, these observations contribute to our knowledge of mechanisms used by immune cells to promote maturation.

PLCβ family members also play a significant role in regulating the signaling through G-protein coupled receptors (GPCRs), in neutrophils. Following ligand binding, GPCRs induce an exchange of GDP for GTP causing the GPCR to release its associated Gα and Gβγ subunits to induce signaling—distinct Gα subunits associate with different GPCRs. While all PLC-β isoforms can be activated by the Gq class of Gα subunits, only PLC-β2 and PLC-β3 can be activated by both the Gαi/o and Gβγ subunits [12]. In addition, these two PLC-β isoforms can be activated by the small GTP-binding proteins Rac and Cdc42 [12]. PLCβ2 and PLCβ3 play important roles in mediating signaling in neutrophils as well. Neutrophils are short-lived cells derived from the myeloid lineage, and are the most abundant leukocytes in the circulation. Neutrophils express a broad range of GPCRs including the formyl peptide receptors, leukotriene receptors, platelet-activating factor receptors, C5a receptors, and certain chemokine receptors (CXCR1, CXCR2, CCR1, CCR2, CCR5, and CCR7), which control host immunity and trigger inflammation [18–32]. Following ligand binding, GPCRs catalyze the exchange of GDP for GTP on the Gα subunits of the heterotrimeric G proteins that associate with the cytoplasmic face of the GPCR. Signaling to PLC from these GPCRs, which had been thought to be activated only in response to the release of Gαq subunits of GPCRs, was subsequently found to be activated by the Gβ/γ subunits as well [33–37]. This signaling in neutrophils is marked by a biphasic Ca^{2+} signaling [38]. The initial phase is thought to be mediated by PLCβ enzyme promoting the release of Ca^{2+} from the endoplasmic reticulum, since homozygous deletion of PLCβ2 and PLCβ3 completely blocked IP3 production, Ca^{2+} release, and the concomitant degranulation of superoxide [39]. Since Ca^{2+} mediates cell migration it was unexpected that PLCβ2$^{-/-}$ and PLCβ3$^{-/-}$ mutants migrated normally in response to stimulation of the formyl peptide receptor or CCR1. These observations likely reflect redundancy in signaling pathways, as PLCγ has been shown to regulate immune cell migration in response to chemokine receptors that are GPCRs (see below).

Mast cells mediate allergic reactions via the activation of high affinity IgE receptors (FcεRI) [40]. Using PLCβ3$^{-/-}$ mice it was shown that PLCβ3 is required for late phase cytokine production of interleukin 6 (IL-6), tumor necrosis factor α (TNFα) and IL-13. In addition, migration of sensitized mast cells was reduced in the absence of PLCβ3. Loss of PLCβ did not affect the early phase response, as Ca^{2+} mobilization, and Fyn activation and degranulation of histamine was unaltered when compared to wild-type mice. Instead, this study demonstrated a role for PLCβ3 in regulating SHIP-1 in regulation of Lyn activation, which was mediated by the adaptor function of this PLC family member since loss of the catalytic activity did not affect signaling through FcεRI.

13.2.2 PLCγ

PLCγ has two forms (PLCγ1 and PLCγ2), which have been linked to signaling in hematopoietic cells. To date, both isoforms have been observed in dendritic cells, neutrophils, mast cells, natural killer cells, and B cells [41–46]. We will discuss roles for PLCγ isoforms in each of these cell types. Homozygous deletion mutant have been studied for each PLCγ isoform. Effects of homozygous deletion of PLCγ1 on hematopoiesis are difficult to study, since the mice die at embryonic day 8.5. In contrast, mice homozygously deleted in the PLCγ2 locus have a marked reduction in the numbers of mature B cells, and lack IgM receptor-induced Ca^{2+} mobilization, which appeared to be due to loss of signaling through Bruton's tyrosine kinase (BTK) and B-cell linker protein (BLNK) [47]. T-cell differentiation in these mice, however, was unaffected. These mice are able to express IgM, IgG2a, and IgG3 at reduced levels. In addition, although platelet counts are normal, these mice have defects in collagen-induced platelet aggregation that leads to gastrointestinal hemorrhages [47]. From these studies, it was determined that although PLCγ isoforms have similar structures, these lipases are not functionally redundant.

Neutrophils which express selectins and integrin adhesion receptors on their surfaces that control localization and targeting of these cells during an innate immune response, signal through PLCγ [48]. Both selectins, which are single chain transmembrane glycoproteins and integrins, heterodimeric adhesion proteins, promote transient attachment of leukocytes, such as neutrophils to the inflamed vascular endothelium [49–52]. Specific adhesion proteins, including the P-selectin glycoprotein ligand-1 (PSGL-1) and the β1 and β2 integrins on the cell surface, play significant roles in regulating neutrophil adhesion. Signaling through both PSGL-1 and β2 integrin adhesion proteins is mediated by PLCγ2 [48].

Macrophages and dendritic cells express pattern recognition receptors, such as the Toll-like receptors (TLRs), which allow these cells to respond to microbial products and activate an immune response [53], and the resultant release of pro-inflammatory cytokines such as TNFα and IL-6. In response to CpG stimulation of these phagocytes, TNFα secretion is mediated through spleen tyrosine kinase (Syk) [53]. Downstream PLCγ2, a substrate of Syk is phosphorylated to promote TNFα secretion. In the absence of the Syk/PLCγ2 signaling, secretion of IL-6, is unaffected. This type of tailored signaling through Syk to PLCγ2 allows cells to generate responses that are specific to each targeted pathogen.

In basophils and in mast cells signaling through the high affinity, IgE receptor (FcεRI) is mediated through PLCγ1 and through PLCγ2 [47, 54–57]. FcεRI consists of an α-subunit, which has two extracellular IgE-binding domains, a transmembrane β-subunit along with two γ-subunits [58]. Immune tyrosine-based activation motifs (ITAMs) are found within the β- and the γ-subunits, which promote signal amplification and propagation [59–61]. Initially, FcεRI clustering mediates recruitment of the src family kinase, Lyn [62–64]. Lyn phosphorylates the ITAMS on the β- and γ subunits of the FcεRI. These phosphorylated ITAMs recruit

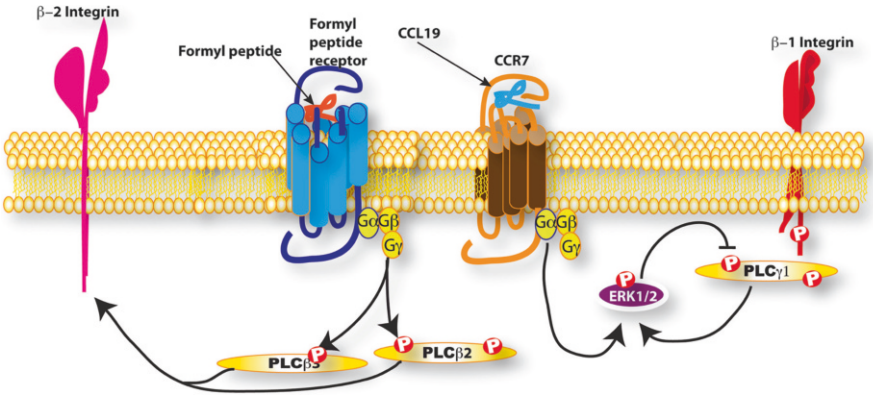

Fig. 13.2 Phospholipase Cγ1 promotes signaling following activation of the T-cell receptor. The T-cell receptor (TCR), found on the surface of T-lymphocytes is composed of ligand-binding subunits (TCR-α/TCR-β) or (TCR-γ/TCRδ), along with CD3γ, CD3δ, CD3ε, and CD3ζ [116–118]. The CD3 subunits promote signaling through the TCR following ligand binding, via phosphorylation of immune-receptor tyrosine-based activation motifs (ITAMs) found on the cytoplasmic tails of these subunits [119]

Syk [65], which is phosphorylated by Lyn to mediate the recruitment of Linker of activation of T cells (LAT). Syk phosphorylation of LAT mediates recruitment and activation of PLCγ1 and PLCγ2 [66, 67]. Following cleavage of PIP2 into IP3 and DAG, the released calcium promotes degranulation. This phase of the FcεRI signaling is part of the early response.

PLCγ1 plays a key role in the adaptive immune response in T cells as well. T cells traffic to lymph nodes in response to signaling through the C–C chemokine receptor 7 (CCR7) [68]. We have shown that migration of T cells via β1 integrins is regulated by PLCγ1 [69]. In these studies shRNA depletion of PLCγ1 in primary T cells was used to show that activation of PLCγ1 mediates T-cell migration in response to CCR7/CCL21. Although CCR7 has two ligands, migration via the second ligand appears to be mediated by a different signaling pathway, demonstrating a role for PLC family members in differential regulation of signaling through a single chemokine receptor. Since migration of immune cells via the CCR7 receptor to CCL21 is mediated by PLCγ1 [69], it is possible that other PLC family members are activated in neutrophils to promote chemotactic migration. Indeed, in support of our observations, stimulation of migration of T cells via the CXCR4-stromal derived factor 1-α (SDF1α) receptor is also regulated via PLCγ1 and not PLCβ3 [70] (Fig. 13.2).

T cells are activated through association with antigen-presenting cells through a structure termed the immunological synapse (IS) [71, 72]. T-cell activation is a complex process, during which a number of signaling proteins co-localize to promote downstream signaling events. Within the IS, microclusters form in which the ζ chain of the TCR is phosphorylated by the Src family kinase member, Lck [73–77] (Fig. 13.3). In turn the ζ-chain associated protein of 70 kDa (ZAP-70) is activated through its association with the phosphorylated ζ-chains. ZAP-70 phosphorylates

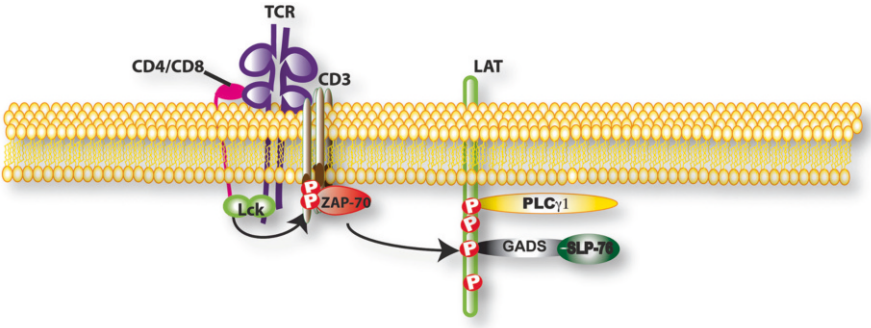

Fig. 13.3 Signaling through PLCγ1 promotes migration following activation of T cells via CCL21

LAT, a 36–38 kDa adapter protein [78] and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) [79–81]. PLCγ1 is recruited via its N-terminal SH2 domain to tyrosine 132 of LAT [61, 82–84], where it is phosphorylated and propagates signaling via cleavage of phosphatidyl inositol 1,4,5-triphosphate [78, 85]. PLC-γ then binds with SLP-76 (Fig. 13.3). Following the release of Ca^{2+} from the ER, Orai1 channels in the plasma membrane open to sustain Ca^{2+} signaling and specific gene transcription is initiated [11].

During signaling through the IS, F-actin turnover promotes phosphorylation of PLCγ1 that sustains signaling during T-cell activation, since in the presence of jasplakinolide, an F-actin stabilizing agent that perturbs actin turnover [86], phosphorylation of PLCγ1 tyrosine 783 is lost [87]. End-binding 1 microtubule-binding protein, which promotes turnover of the actin, binds directly to the TCR complex to promote vesicle recruitment to the IS. These vesicles traffic the PLCγ1/LAT complex to the IS which promotes T-cell signaling.

In natural killer (NK) cells, signaling through the γδ T-cell receptor (TCRγδ) promotes Vav1-mediated PLCγ1 signaling pathway, and lytic granules are released to promote killing [88]. Natural killer group 2 member D (NKG2D), homodimeric transmembrane C-type lectins are normally expressed by NK cells and CD8+ αβT cells, and CD4+ γδ T cells [89–91]. Ligands for NKG2D consist of MHC class-I related molecules and the MHC class I-related proteins A and B (MICA/B) and 6 UL-binding proteins. In combination with the γδ T-cell receptor (TCRγδ), signaling in response to activation of NKG2D is propagated through src family kinases to activate the Vav1-PLCγ1 and the PLCγ2 signaling pathways [92–94]. While both isoforms of PLCγ couple to activating receptors in NK cells, their functions are not redundant, as certain forms of NKG2D preferentially bind PLCγ2 [94, 95]. These pathways are the upstream of activation of jun kinase 1 (JNK1) which is required to polarize the microtubule organizing center and the cytolytic granules at the synapse with target cells [96]. In response to signaling through PLCγ, IFNγ is produced [97, 98]. As an additional regulatory mechanism, PLC promotes shedding of the glycosylphosphatidylinositol-linked isoform of NKG2D from the surface of the cells through hydrolysis of the GPI-anchor [88].

13.2.3 PLCδ

Although there is very little evidence linking PLCδ1, 2, or 3 to hematopoietic cells, Chinese hamster ovary cells transfected with PLCδ1 can be stimulated by thrombin, to produce inositol phosphates, implicating a potential role for PLCδ1 in a GPCR-signaling pathway [99]. Inflammatory mediators such as bradykinin, lysophosphatidic acid, and a calcium ionophore added to serum can stimulate expression from the PLCδ4 promoter, as well, and may implicate this PLC family member in regulation of cellular events in response to inflammation [100]. Other functions of PLCδ family members, however, do not appear to be linked to immune cell functions (as reviewed by [101]).

13.2.4 PLCε

PLCε is an isoform that is expressed at a low level, but has a significant impact on the immune states in the cells where it is expressed, even though other PLC family members are found at much higher levels. This isoform, which is expressed within the thymus, appears to contribute to inflammatory processes. Using the APCMin$^{/+}$ mouse model of colon cancer, in which inflammation mediates the development of intestinal tumors, the number of spontaneous tumors was significantly reduced in mice crossed onto a PLCε$^{-/-}$ background when compared to mice on a PLCε$^{+/+}$ background [102]. In this study it was found that levels of neutrophil and macrophage chemoattractants, CXCL1 and CXCL2, were suppressed in PLCε$^{-/-}$; ApcMin$^{/+}$ mice when compared to the PLCε$^{+/+}$; ApcMin$^{/+}$ mice. In addition, levels of cyclooxygenase 2 (COX-2) were suppressed, when compared to levels in the PLCε$^{+/+}$; ApcMin$^{/+}$ mice, which are upregulated. These reductions in myeloid cell chemokines and pro-inflammatory mediators resulted in a significant attenuation in the number and grade of tumors in the PLCε$^{-/-}$; APCMin$^{/+}$ mice. Importantly, these results demonstrate that signaling that leads to production of inflammatory cytokines can be regulated through PLCε. PLCε has also been linked to contact dermatitis. In these studies, transgenic mice that overexpress PLCε spontaneously developed lesions with adherent silvery scales, overgrowth of keratinocytes, and aberrant infiltration of IL-22 producing T cells. The dermatitis was suppressed by injection of anti-IL-23 antibodies which is produced by T cells in response to IL-22 or FK506, which inhibits the activation of calcineurin, a signaling intermediate that is activated by calcium release downstream of PLCε, via forming complexes with the immunophilin FK506-binding protein (FKBP) 12 [103, 104]. Similar to PLCδ, PLCε can be activated through GPCR binding of lysophosphatidic acid, thrombin, sphingosine 1 phosphate, isopreternol, and exendin [105]. Like the PLC-γ isoforms, PLCε can also be activated following stimulation of member receptor tyrosine kinases, such as the epidermal growth factor receptor, and the platelet-derived growth factor. Collectively, these studies highlight roles for PLCε in regulating the state of immune cell mediators.

13.3 Immune Disease-Linked Mutations

13.3.1 PLCβ3

In mice, homozygous deletion of PLC-β3 led to death of 50 % of the mice within 16 months, in contrast to 100 % survival of the wild-type animals [106]. These animals had enlarged spleens, which contained foci of myeloid and erythroid cells that appeared to be the sites of extramedullary hematopoiesis. Specifically, the spleens contained high numbers of c-Kit$^+$Sca-1$^+$Lineage-cells, granulocyte-macrophage progenitors and megakaryocyte–erythroid progenitors, when compared with age-matched mice. These defects appeared to be due to the increased Stat5 activation, as measured by phospho-Stat5 levels in the mutant mice. Markedly, the bone marrow of these mice contained unusually high numbers of CD11b$^+$Gr-1$^+$ mature granulocytes. Since these animals did not have bacterial infections and antibiotic treatment did not affect the number of granulocytes, it was concluded that the animals had myeloproliferative disease. Therefore it was not unexpected, when a second group of mice progressed into a blast crisis, similar to human chronic myeloid leukemia [107]. Examination of 128 mice revealed the presence of lymphomas with T-cell markers, skin carcinomas, and lung carcinomas [106]. By overexpressing PLCβ3 in mouse Ba/F3 cells in vitro, and in KSL cells in vivo, the authors found that PLCβ3 reduced the levels of Stat5 phosphorylation, and that this suppressor activity was localized to the C-terminus of PLC protein. The authors point out that these deletion mutants demonstrate the non-overlapping functions of PLCβ2 and PLCβ3, since PLCβ2$^{-/-}$ do not develop tumors or die prematurely. Furthermore, PLCβ3 is implicated as a tumor suppressor in myeloid cells.

13.3.2 PLCγ2

Genetic deletion of PLCγ2 leads to a loss in osteoclast differentiation. In addition, there is a loss of bone resorption both in vitro and in vivo. PLCγ2 plays an important role in regulating the innate immune response during inflammatory arthritis. Bone loss observed in patients with prosthetic implants, periodontal disease, osteoarthritis, and rheumatoid arthritis is due to an increased number and/or function of bone-resorbing osteoclasts. Normally the catalytic and adaptor domains of PLCγ2 promote osteoclast differentiation and function. To determine the role of SH2 domains in PLCγ2, a mutant was expressed that contained two tandem SH2 domains (PLCγ2 (SH2(N+C))). Bone marrow-derived macrophages expressing this mutant failed to form mature osteoclasts that could resorb bone in vitro. This appeared to be due to a loss of activation of the receptor activator of NF-kB (RANK) signaling, a key regulator of osteoclast development, since in vitro, osteoclasts differentiate from bone marrow precursors in the presence of RANK ligand (RANKL), and macrophage colony stimulating factor (M-CSF) [108] via activation of p38 and IK-Bα.

In the bone marrow, precursors expressing PLCγ2 (SH2(N + C)) downstream signaling through p38, IK-Bα was lost as well, confirming a role for the PLCγ2 catalytic and adaptor functions in promoting downstream signaling leading to osteoclastogenesis.

In mice and in retrospective studies of human patients suffering from cold urticaria, links between the gain of function mutations within PLCγ2 have been reported [109–111]. The PLCγ2 mutations and the corresponding mutations within PLCγ1 likely affect the auto inhibition region of these proteins [112]. Based on predicted structures of the PLCγ interaction domains [113], it is thought that PLCγ is misfolded in these patients. Therefore, it is reasonable to hypothesize that a point mutation found within the C-terminal SH2 domain of PLCγ2 (S707Y) led to misfolding of this protein and the resultant cold urticaria observed in all subjects.

In one family, mutation of serine 707 to tyrosine in PLCγ2 was linked to autoinflammatory disease with immunodeficiency. In this case the serine normally found in the auto-inhibitory SH2 domain that is critical for PLCγ2 regulation was mutated to tyrosine. Overexpression of PLCγ2 containing a mutation, which converts Ser707 to Tyr (pSer707Tyr) led to enhanced PLCγ2 activity. This mutation is found within a highly conserved region of PLCγ2 that appears to be crucial for the activation of PLCγ2 since it represses the activity and is found within the primary auto-inhibitory region [10]. When this domain was compared between 16 different species this site was found to be highly conserved. In one patient small corneal blisters, which eventually progressed to corneal erosions, ulcerations and cataracts appeared [114]. In addition, these patients had very few class-switched memory B cells ($CD20^{+}CD27IgM^{-}IgA^{+}$ or IgG^{+}), which likely explained their histories of developing bacterial infections. Although these cells did produce significant increases in extracellular sign-related kinase (ERK)—the numbers of naïve and memory T cells, and NK cells were normal. Overexpression of the pSer707Tyr mutant in human embryonic kidney (HEK) 293T cells or COS7 cells leads to cells that exhibit high levels of intracellular IP3, which led to increased release of Ca^{2+} from intracellular stores following stimulation with EGF. It is clear that this region plays a pivotal role in regulating the extent of immune cell activation.

13.3.3 PLCε

PLCε contributes to contact hypersensitivity, a T-cell-mediated immune inflammatory response that develops following chronic exposure to a chemical hapten [115]. In these studies using $PLC\varepsilon^{+/+}$ and $PLC\varepsilon^{-/-}$ mice, it was found that PLCε significantly contributed to inflammation induced by sensitization and challenge with 2,4-dinitrofluorobenzene. Specifically, $PLC\varepsilon^{-/-}$ mice displayed substantially reduced levels of immune inflammatory responses when compared to the $PLCe^{+/+}$ mice as measured by a reduction in the extent of ear swelling, which correlated with reduced neutrophil infiltration, and proinflammatory cytokine production such as interleukin-4 (Il-4), IL-17, interferon γ (IFN-γ), and TNF-α. By adoptively transferring

CD4+ T cells from either PLCε$^{+/+}$ and PLCε$^{-/-}$ cells to PLCε$^{+/+}$ and PLCε$^{-/-}$ backgrounds it was found that PLCε functions in a non-T-cell-dependent manner to induce contact hypersensitivity. Moreover, it appeared that the dermal fibroblasts and epidermal keratinocytes, played a role in up-regulating the levels of cytokines produced that controlled the extent of the immune response.

13.4 Conclusions

Studies defining the roles of PLC family members have revealed specific targets that may be useful in treating human illnesses, caused by misregulation of immune cell functions. A more detailed understanding of the function of the PLC family members in the future will be useful in defining disease-specific inhibitors that can be used to provide relief from disease. Since GPCRs can be activated by very high affinity ligands, which are internalized, these types of receptors could potentially be used to target PLC inhibitors to specific cell types that are involved in the development and progression of different diseases.

Acknowledgments This work was funded by a startup award through the Texas STARS program to CMV.

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Chapter 14
Phosphoinositide-Specific Phospholipase C Enzymes and Cognitive Development and Decline

Vincenza Rita Lo Vasco

Abstract The development of the mammalian nervous system is a tightly regulated and complex process, which involves a number of signal transduction pathways, which controls the cascade of events, both spatially and temporally. Complex modifications of the structural and functional bases of the activities of the nervous system also occur in the cognitive decline often observed during aging. The phosphoinositide (PI) signal transduction pathway, which contributes to regulate the calcium levels by means of converting enzymes, such as the phosphoinositide-specific phospholipase C (PLC) family, interacts at a different hierarchy of control with a number of different molecules and/or pathways involved in neural development, neurogenesis and maintenance of the synaptic plasticity. The PI pathway was suggested to be involved in the complex mechanism of memory, crucially and strictly correlated to learning abilities. Specific roles were also suggested for PLC isoforms, on the basis of numerous evidences indicating the involvement in diseases which affect the nervous system, with special regard to cognitive impairment. The nature, meaning, and developmental period of PLC involvement in cognitive development and decline are still largely unclear and will require further studies.

Keywords Phosphoinositide • PLC • Cognitive development • Aging • Mental retardation • Neurodegenerative disease • Mood disorders

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_14,

14.1 Introduction

The development of the mammalian nervous system is a complex process. During both prenatal and postnatal life, it involves a number of signal transduction pathways, which tightly regulate the process, both spatially and temporally. Complex modifications of the structural and functional bases of the neural activities also occur in the cognitive decline often observed during aging, as well as in a number of diseases [1].

Calcium is a ubiquitous second messenger involved in a variety of cell activities in all tissues, including cell proliferation, survival, differentiation, adhesion, and cytoskeleton dynamics [2]. In the nervous system, calcium acts in further important events, such as dendrite morphogenesis, axon guidance [3], and neurite morphogenesis [4]. Moreover, dendrite spine dynamics depend on actin cytoskeleton and related regulatory proteins, some of which are sensitive to changes in the calcium concentration [5–8]. The calcium influx probably drives the neural cell movements, regulating the cytoskeleton dynamics, and the positioning of vesicles in synaptic areas [9]. The phosphoinositide (PI) signal transduction pathway contributes to regulate the calcium levels in a number of cells [10] by means of various converting enzymes, such as the phosphoinositide-specific phospholipase C family of enzymes (PLC) [5]. The role of the PI system is well described in nervous cells. As an example, the PI signal transduction pathway seems to be involved in the spontaneous calcium transients exhibited by neural crest cells (NCC) [11]. NCC displaying calcium transients may generate neurons, and the blockade of the calcium-transient activity prevented that generation. The spontaneous calcium-transient activity seems to be regulated by PLC enzymes [12].

The development of the nervous system is based on the balance between self-renewal and differentiation of the neural stem cells (NSC), which are characterized by the ability to differentiate into multiple neural cell types. In the rodent, NSC are present in developing and adult brain, as they were detected from early embryonic stages until senescence in specific regions, such as sub-ventricular zone (SVZ), hippocampus, and olfactory bulb [13]. The Wnt signal transduction pathway, involved in multiple developmental processes including neurogenesis [14], diversifies into at least four branches, one of which, the Wnt/Ca^{2+} pathway, involves the activation of PLC and protein kinase C (PKC) [15]. Moreover, in NSC, the fibroblast growth factor (FGF) induces prominent extracellular signal-regulated protein kinases 1/2 (Erk1/2) and PLC γ1 activation, which mediates both the proliferation and the anti-neuronal differentiation effects of FGF.

14.2 Signal Transduction Pathways Related to PLC Activity in the Nervous System

Recent evidences indicate that the PI signal transduction pathway, via PLC enzymes, interacts at a different hierarchy of control with a number of different molecules and/or pathways involved in neural development, neurogenesis, and maintenance of

the synaptic plasticity. The interacting elements include cell adhesion molecules [16, 17], Neurin-1, an axonal growth-related molecule [18], Tenascin, an extracellular matrix glycoprotein exerting both stimulatory and inhibitory effects on axon outgrowth [19–21] and the cAMP-response element-binding protein/mitogen-activated protein kinase (CREB/MAPK) system [22, 23]. The calcium-permeable channels TRPC, supposed to act as cellular sensors, also interact with PLC enzymes. TRPC play important roles in neural development, contributing to proliferation, cerebellar granule cell survival, axon path finding, neuronal morphogenesis, and synaptogenesis. Calcium influx through TRPC3 and 6 activates calcium/calmodulin-dependent protein kinase (CaMK) and MAPK to phosphorylate CREB [7], thus leading to neuronal survival. CREB is actually considered the converging point for different signal transduction systems, including the PI pathway [24].

Further signalling molecules networking the PLC system are Neurotrimin (Ntm), which probably plays a role in the development of thalamo-cortical and ponto-cerebellar projections [25, 26], the Pituitary adenylate cyclase-activating polypeptide (PACAP), a pleiotropic neuropeptide which contributes to neurogenesis and gliogenesis [27, 28], Papaverine, an inhibitor of phosphodiesterase (PDE) 10A, playing neuroprotective/neurotrophic actions [29], and Homocysteine, which induces neurotoxicity through different cell signalling mechanisms, including those related to *N*-Methyl-D-aspartate (NMDA) receptors and voltage-dependent channels [30].

Great interest also deserved the interaction of PLC enzymes with further signal transduction systems widely represented in the nervous system, such as the pathways of serotonin, involved in craniofacial morphogenesis [31–36], of growth factors [37], with special regard to PLC γ isoform activity, and of thyroid hormones [38, 39]. Moreover, the brain-derived neurotrophic factor (BDNF), via dopamine D1–D2 receptor heteromer, activates a signal transduction pathway connecting dopamine signalling and neuronal growth through a cascade of events, primarily involving the mobilization of intracellular calcium via Gq, PLC, and inositol trisphosphate (IP3), a downstream product of the PLC activity [40–43]. PLC enzymes were also described to interact with muscarinic receptors in the human fetal brain [44], and with metabotropic receptors (mGluRs) [45–48], which mediate the interaction with glutamate, the main excitatory neurotransmitter in the central nervous system [49–51].

14.3 The Role of PLC in Memory and Learning

Recently, the PI pathway was suggested to be involved in the complex mechanism of memory, crucially and strictly related to learning abilities. The activity of PLC enzymes is directly modulated by opioids. The PLC β3 isoform is probably simultaneously implicated in both the analgesic and the amnesic effects induced by administration of morphine to mice [52]. That suggests common molecular mechanisms underlying drug-induced analgesia and memory impairment [52].

PKC is involved in synaptic remodelling, induction of protein synthesis, and many other processes. PKC activation is closely tied to the activity of the PI system, as it depends on diacylglycerol (DAG), another downstream product of the PLC activity [7]. Activation of neuronal PKC is probably related to all phases of learning, including acquisition, consolidation, and reconsolidation [53]. PKC interacts with pathways networking insulin, which can activate the PKC pathway via PLC γ, Erk 1/2, MAPK, and Src stimulation. Probably, the interaction of PKC/insulin in the differentiated nervous system induces synaptogenesis, enhances memory, reduces the risk for Alzheimer's disease (AD), and stimulates the repair [53]. Authors suggest that the PKC/insulin interaction might oppose the mechanisms inducing AD [53]. ERK regulate a variety of cellular functions, including proliferation, differentiation, and plasticity. ERK2 and PLC subfamilies β and γ interact in the rat hippocampus, playing critical roles in learning and memory, as well as a variety of other neuronal functions [54].

BDNF, already cited as an important regulator of synaptic transmission, is also involved in the long-term potentiation (LTP) in the hippocampus and in other brain regions. BDNF plays a role in the formation of selected forms of memory. The effects of BDNF are mediated by tropomyosin-related kinase B (TrkB) receptors, coupled to the activation of Ras/ERK, phosphatidylinositol 3-kinase/Akt and PLC γ [55]. BDNF regulates the transport of mRNAs to the synapse along dendrites, by modulating the initiation and elongation phases of protein synthesis, and by acting on specific miRNAs [55]. Furthermore, the effect of BDNF on the regulation of transcription may further contribute to long-term changes in the synaptic proteome. Therefore, BDNF might influence learning and memory formation via the PI/PLC pathway [55].

14.4 Abnormality of PLC Genes in Clinical Cases Presenting Nervous Symptoms

Mental retardation (MR) affects about 2–3 % of the population, but its causes remain unexplained in 40 % of cases. Subtle telomeric rearrangements are responsible for about 1 % of MR [56]. A number of genetic abnormalities have been associated to isolated as well as syndromic MR. Specific roles were recently suggested also for PLC isoforms, on the basis of their tissue-specific expression and of evidences witnessing their involvement in diseases affecting the nervous system.

PLC β1, an isoform highly expressed in the cerebral cortex and hippocampus [57], is activated by G-protein-coupled receptors (GPCR) that signal through Gq/11. PLC β1 mediates the activity-dependent cortical development and the synaptic plasticity [47, 58]. PLC β1-knockout mice develop epilepsy, minor abnormalities in the hippocampus [59], and behavioral deficits in location recognition, probably due to excess in neurogenesis and aberrant migration of adult-born neurons [60, 61]. The activity-dependent regulation of synapse and dendrite spine morphology in the developing barrel cortex requires the presence of PLC β1 [58].

A recent report described a male child affected with epileptic encephalopathy associated with loss-of-function mutation in the gene which codifies for PLC β1 (PLCB1, OMIM *607120) [62]. A 0.5-Mb region was identified between rs6118078 (8,048,714 bp) and rs6086520 (8,507,651 bp) located within an extended region of homozygosity on chromosome 20. Subsequent analyses detected homozygous deletion on chromosome 20 involving exclusively PLCβ1; the deletion involved exons 1, 2, and 3, which seemed to be completely lost [62]. The patient was the first male child of consanguineous healthy parents. During the third trimester of pregnancy, mild intrauterine growth retardation was referred [62]. Focal seizures, begun at 10 weeks, were successfully treated pharmacologically. Clinical examination showed mild axial hypotonia and microcephaly (0.4th centile). Neurological examination was normal and neurodevelopmental assessment was age-appropriate. No further seizures presented until 6 months of age, when recurrence was successfully managed for 2 months. Then, the patient developed the clinical and EEG features of West syndrome [62]. At 10 months, and over the next 2 years, the patient had recurrent tonic and generalized tonic-clonic seizures. By 13 months, the EEG showed encephalopathy process characterized by generalized slowing. Seizure control was not achieved despite the use of multiple anti-epileptic agents. Progressive developmental regression was recorded. MRI brain scans (at ages of 5 and 13 months) were normal. At 2.9 years of age, the patient developed lethal respiratory infection [62].

In another patient, affected with malignant migrating partial seizures (MMPEI), abnormalities of PLCB1 were also identified [63]. MMPEI is a rare form of epilepsy characterized by early onset of multiple seizures types, pharmaco-resistant seizures, and overall poor prognosis [64]. Chromosomal microarray analysis of the patient identified three copy number variations (CNV): homozygous 476 kb deletion of chromosome 20p12.3 and heterozygous in chromosome 7p21.3 and in chromosome 12q24.12. Both parents, first cousins, were heterozygous for the 20p12.3 deletion. The 20p12.3 deletion covered exons 1, 2, and 3 of PLCB1 [63]. The patient, a male child, was born at a term after uncomplicated pregnancy; the postnatal development was delayed but progressing. Seizures began at 6 months [63]. Neurologic examination showed marked truncal and appendicular hypotonia. EEG showed multifocal interictal spikes and abundant seizures arising from the right and left temporal lobes independently, at times with migration from one hemisphere to the other within a seizure. Inefficient treatment was attempted with multiple antiepileptic pharmaceuticals [63]. MRI at 6, 7, 8, and 9 months revealed mildly prominent cerebrospinal fluid spaces. MR spectroscopy performed at 9 months was normal. Laboratory investigations for inborn errors of metabolism, neurotransmitter disorders, and mutations in other genes were unrevealing [63].

PLCB1 abnormalities were also described in autism affected patients. A recent study investigated the CNV implicated in autism and intellectual disability. These variants are large and affect many genes but lack clear specificity toward autism as opposed to developmental-delay phenotypes [65]. The analysis identified recurrent gene-disruptive events in selected genes, including PLCB1 [65].

Recently, PLCH2 (OMIM *612836), the gene which codifies for PLC η2, was suggested to be involved both in syndromic and isolated mental retardation [66].

Deletions in the distal region of the short arm of chromosome 1 (1p36) are widely diffused, both as somatic abnormalities in tumors [67] and as a congenital syndrome. Constitutional deletion of 1p36 results in a recognizable syndromic pattern (1p36 Deletion Syndrome, OMIM #607872) considered due to deletion of contiguous genes. The syndrome is characterized by a number of features, including MR of variable degree [68, 69]. The frequency of monosomy 1p36 is 1 in 5–10,000 births, no gender and ethnicity differences have been registered [69, 70]. The deletion of 1p36 region may result from both interstitial and terminal deletions of variable size and different breakpoints [71]. Deletions vary from size 1.5 to 10 Mb, with common break points located from 1p36.13 to 1p36.33 [72], although the severity of the phenotype is only partially related to the extent of the deletion [71, 72]. The great number of genes mapping in this region complicated the identification of candidates. The human 1p36 chromosomal region contains a number of genes, including the proto-oncogene V-Ski Avian Sarcoma Viral Oncogene Homolog (SKI; OMIM *164780) [73], matrix metalloprotease 23 A (MMP23A; OMIM *603320), matrix metalloprotease 23 B (MMP23B; OMIM *603321) [73], potassium channel voltage-gated shaker-related subfamily beta member 2 gene (KCNAB2; OMIM *601142) [74], and human gamma-aminobutyric acid A receptor delta-subunit gene (GABRD; OMIM *137163) [75]. The finding of a patient bearing a complex rearrangement including 1p36.32 deletion in which the GABRD locus was not involved suggested that the neurological features might be correlated to anomalous expression of other genes [76]. The patient, a 9-year-old female, presented with dysmorphic features, learning disability, ear problems, and hypermetropia. Prenatal scan detected nuchal oedema and ventriculomegaly. Developmental retardation such as delayed ability to sit, speech delay, and late walking was reported. Molecular cytogenetic analyses in the proband detected 1p36.32 deletion 1.4–2 Mb long not involving the GABRD locus [76]. Further analyses detected the deletion of PLCH2, which maps on 1p36.32 [76].

PLC η2 enzyme, expressed in the brain after birth, is a key player in calcium mobilization and in signal transduction systems acting in neurons [77]. In the nervous system, PLC η2 is abundantly expressed in hippocampus pyramidal cells and olfactory bulb [78], organs which contribute to memory circuits. PLC η2 is also expressed in the cerebral cortex, a region involved in memory, thinking and understanding language processes [78]. Therefore, PLC η2 might be involved in these functions. PLC η2 was also found abundant in mouse habenula and retina, which both contribute to regulate the circadian rhythm [79]. As PLC η2 is involved in the formation and maintenance of neuronal networks and memory circuits, its absence might imply impairment of these functions and subsequent abnormal neuronal and intellectual development.

Moreover, disarrangement of the PI pattern was described and/or supposed in a number of conditions presenting MR, such as Costello syndrome (OMIM #218040), Lowe syndrome (OMIM 309000), CHIME syndrome (OMIM #280000), Zellweger syndrome (OMIM #214100), and hyperphosphatasia mental retardation syndrome (OMIM #614207). Although the complete panel of MR presenting syndromes which may involve PLC enzymes is probably incomplete, further investigations about the PI/PLC signal transduction system in the selected group of patients might help to better understand the etiopathogenesis of MR in complex syndromes.

14.5 PLC and Aging of the Nervous System

Many evidences witness the existence of age-related deficits of the PI signal transduction in the hippocampus mediated by the mGluRs, although independently upon the receptor expression [80]. The absence of a decline in neurotransmitter receptor expression is consistent with the evidence that neuron loss does not occur in the hippocampus, even in experimental animals presenting with cognitive deficits [81, 82]. Deficits in the PI signal transduction pathway are mainly represented by the decrease of PLC β1 activity. Accordingly to findings of functional abnormalities in the hippocampus during aging [83–89], the age-related changes in the PI/PLC signal transduction system might substantially contribute to cognitive decline [80]. In the hippocampus of young and aged Long-Evans rats behaviorally characterized for spatial learning in the Morris water maze, the maximal PI turnover mediated by the type-1 mGluR resulted blunt in the aged rat population [80]. The decrease of the PI turnover was significantly correlated with the age-related spatial memory decline. A significant decrease in the immunoreactivity of PLC β1, however, was observed in the hippocampus of aged rats. PLC β1 levels significantly correlated with spatial learning only unifying the young and aged rat groups. The decrease of mGluR-mediated signal transduction in the hippocampus, related to cognitive impairment in aging, might follow the reduction of PLC β1 expression. Therefore, an age-related alteration in PI signal transduction system may provide the functional basis for cognitive decline independently from the neuron loss [80].

Moreover, during brain aging or progression of AD, the levels of amyloid beta (Abeta) and proinflammatory cytokines accumulate in the tissue prior to major degenerative changes. This event might affect related signal transduction pathways critical for neuronal health. Neurotrophin signal transduction system is critically involved in synaptic plasticity, learning, memory, and neuronal health. Exposure to low levels of Abeta impairs BDNF/TrkB signal transduction, suppressing the Ras/ERK, and the PI3-K/Akt pathways, and not the PLC γ pathway [90]. Then, the downstream regulation of gene expression and neuronal viability are impaired. Evidences suggest that accumulation of amyloid beta (Abeta) and proinflammatory cytokines in the brain during aging generates a sort of "neurotrophin resistance" which might induce the brain susceptibility for cognitive decline and dementia [90].

14.6 PLC Enzymes and Cognitive Impairment in Neurodegenerative Diseases

The PI signal transduction pathway is described to be involved in the most common neurodegenerative diseases. Interestingly, in neurodegenerative illnesses presenting with cognitive impairment, a role for PLC pathway was also described.

PLC β3 is described in the nervous tissue [7], and a specific isoform to human cone photoreceptor neurons was identified [91]. The human gene which codifies for PLC β3 (PLCB3; OMIM *600230) maps to a genomic region associated to

neurodegenerative diseases, such as Bardet–Biedl syndrome, a complex disease also presenting with MR (OMIM #209900), and Best's vitelliform dystrophy (OMIM #153700). Furthermore, many evidences indicate that selected PLC enzymes play a role in the initiation/progression of neurodegenerative disease, especially in those forms presenting with cognitive impairment.

Evidences indicate consistent disturbances in the G protein-associated signal transduction processes in cerebral cortex and cerebellum of adults affected with Down syndrome (DS; OMIM #190685) or Alzheimer's disease (AD; OMIM #104300). In fact, premature aging and neuropathological features of AD are commonly observed in DS. The PLC pathway was found severely disturbed in the aged brains of patients affected with DS or AD. However, the alterations observed in DS are usually more severe, and differed to some extent from those observed in AD [92]. DS is the most common genetic form of MR, occurring in 1/700–1,000 live births, and results from cytogenetic abnormalities of the chromosome 21 [93]. A characteristic feature of DS is premature aging. Many affected individuals develop Alzheimer-like neuropathology by the fourth decade of life. These neurodegenerative changes are characterized by progressive accumulation of senile plaques and neurofibrillary tangles, and occur with a regional distribution comparable to that observed in AD [94]. In the brains of patients affected with AD, the neurotransmission is compromised, due to disrupted postreceptor signal transduction, with special regard to the process mediated by G-protein regulated adenylyl cyclase (AC) and PI hydrolysis, linked to PLC pathways [95–98]. Studies performed in the DS experimental model Ts65Dn mouse demonstrated reduction of the PLC activity, with special regard to low expression of the PLC β4 isoform [99]. There was also a significant reduction in the responses of the cerebral cortex to stimulation by GTPγS, serotonergic, and cholinergic agonists, as well as in the response to carbachol in the cerebellum, compared to controls.

On the other hand, in AD significant reduction in the response to carbachol was evident. The results obtained in AD brains confirm previous findings on agonist-stimulated PI hydrolysis [92, 97]. The lower hydrolysis of (3H)-phosphatidyl inositol (4,5) bisphosphate (PIP2) in response to carbachol in the brains of both DS and AD affected patients reduces the DAG production and the subsequent PKC activation. That event probably disturbs a secretase-mediated cleavage of amyloid precursor protein (APP), favoring the Abeta production and subsequent apoptosis, which is a consistent brain feature in DS and AD. Disturbances in the PLC system were suggested to affect the apoptosis phenomenon both increasing Abeta-related apoptosis and reducing neuroprotection [92]. The concurrent reduction of responsiveness to cholinergic and serotonergic stimulation observed in DS brains reflects the impairment in the activity of the PLC pathway. Furthermore, the abnormalities of the pre- and post-synaptic abilities to convey cholinergic and serotonergic information may explain some of the cognitive and behavioral features associated with aging in DS, such as the decline in speech, in memory and the behavioral depression. The dysregulation of G-protein-associated signal transduction in cerebral cortex and cerebellum of adults with DS or AD may play a crucial role in the impairment of cognitive functions. As PLC enzymes are supposed to contribute to the neuronal

information storage, derangement of the PI system might contribute to the cognitive deficits in aged patients affected with DS or AD, as suggested by the findings obtained using carbachol upon PIP2 hydrolysis.

Dementia with Lewy bodies (DLB) is a primary neurodegenerative disease in the elders, which shares selected features with Parkinson's disease (PD) and with AD. DLB presents with fluctuating disturbances of consciousness, recurrent visual hallucinations, sleep disorders, and cognitive decline to dementia [100–107]. Clinical features of DLB include the presence of the so-called Lewy bodies, neural inclusions composed of abnormally phosphorylated neurofilament proteins aggregated with ubiquitin, and α-synuclein. Lewy bodies are also the hallmark of PD. Changes characteristic of AD were also described in the DLB, including senile plaques and neurofibrillary tangles [108–110]. Interestingly, abnormal α-synuclein/PLC β1 interactions associated with impaired mGluR function were described in the cerebral cortex of patients presenting with DLB [111, 112].

PD is a multisystemic neurodegenerative disease that affects selected nuclei of medulla oblongata, pons, olfactory bulb and tract, intestinal ganglionic plexus, substantia nigra (pars compacta), amygdala, nucleus basalis of Meynert, and cerebral cortex [113]. The main neuropathological hallmark is the presence of Lewy bodies and aberrant neurites filled with abnormal protein aggregates, of which the most important component is α-synuclein. The α-synuclein, abnormally phosphorylated, nitrated, and oxidized, shows altered solubility, aggregation, and facility to fibril formation [114]. Classic PD presents as a complex motor disorder resulting from the reduced dopaminergic input of the substantia nigra to the striatum and from the altered basal ganglia modulation of motor control [115]. Cognitive impairment occurs in most cases with advanced PD. More important for diagnosis, altered cortical function can be detected in some individuals before the appearance of motor symptoms, and cortical dysfunction is common [116]. Clinical observations suggested that the altered cortical function is not related to the presence of Lewy bodies and aberrant neurites in the cerebral cortex [117]. Therefore, further factors which might contribute to the abnormal cortical function were investigated [118], such as impaired function of selected metabolic pathways, including a mild alteration in the PI system [118–123].

Huntington's disease (HD; OMIM #143100) is a genetic neurodegenerative disease presenting in the adult age, characterized by progressive and lethal degeneration of cognitive functions. The disease is due to abnormal expansion of a CAG codon in exon 1 of the huntingtin gene (HTT; OMIM *613004) [124], resulting in cognitive, psychological, and motor disturbances [125]. The primary sites of neurodegeneration are striatum and cerebral cortex [126], although other structures involved in cognition, including the hippocampus, are also affected in early stages of the disease [127]. Cognitive impairment can appear first and motor symptoms follow later [128, 129]. Symptoms usually precede the neuronal loss [126], suggesting that the neurological symptoms may be due to the underlying neuronal dysfunction rather than due to the neuronal death. Following the observation that decrease of BDNF expression is related to learning impairment during the progression of HD, interacting signal pathways were investigated [130–132]. Observations indicate that

learning deficits may be attributed to the decrease in BDNF levels observed in selected experimental genotypes [130–135]. A targeted mutation in PLC γ docking sites to TrkB is sufficient to impair LTP in the hippocampus [134, 135]. The overexpression of the same receptor induces increased PLC γ activity together with improved learning [136]. BDNF modulates the cognitive function in different learning and memory tasks. Impaired learning was described in patients and in experimental HD models [131–133]. Mutant huntingtin alters the activity and function of BDNF. Actually, the decrease of BDNF, which consequently affects the integrity of BDNF-TrkB-PLC γ signal transduction pathway, is thought to be involved in the learning impairment observed in HD [130].

14.7 PLC in Psychiatric Disorders

Mood disorders are common mental health problems, afflicting 154 million people around the world [137]. They exist in many forms, including unipolar depression, bipolar depression, schizophrenia/schizoaffective disorder spectrum, mania, mixed syndromes, and subsyndromes. These conditions can co-occur with other psychiatric and physical disorders [138]. Mood disorders are a public health problem associated with considerable burden of disease, suicides, physical comorbidities, high economic costs, and poor quality of life. As an example, major depression is currently the third leading cause of disability worldwide [139, 140]. Approximately 30–40 % of patients with major depression obtain only a partial response to the available pharmacological and psychotherapeutic interventions. Therefore, due to the incidence, the clinical outcome and the problematic therapeutic approach, mood disorders are actually considered a major medical need [137]. Abnormalities in signal transduction are supposed to play a role in the pathogenesis of mood disorders. The cAMP, PI, MAPK, and glycogen synthase kinase cascades were mainly indicated as pivotal actors in the pathogenesis [141–143]. The possible role of PLC enzymes in the ethiopathogenesis and/or the progression of mood disorders was suggested. Recently, PLC expression in bipolar disorder [144], major depression [145], and schizophrenia [146, 147] was investigated.

Schizophrenia is a deteriorating psychiatric disorder, affecting higher human cognitive functions, such as attention, motivation, execution, and emotion. In spite of decades of research efforts, its exact pathological mechanisms remain elusive [148]. Neuropathological data on postmortem brains indicated that neuronal reduction occurred in several brain regions includes prefrontal cortex, hippocampus, and thalamus of schizophrenia affected patients [149–151]. Functional neuroimaging studies showed progressive gray matter loss and enlargement of the brain ventricles, an early feature in the disorder [152–156]. The apoptotic process is characterized by layer-specific neuronal reduction, dendrite deficits and brain volume loss [157]. Schizophrenia is considered a neurological disorder of developmental or genetic origin due to its high genetic susceptibility. However, the presence of a large number of sporadic schizophrenia patients might suggest that abnormalities in the

neurotransmitter system function also play important roles in disease etiology [158, 159]. A number of research efforts were addressed to identify selected signal transduction pathways which may play a critical role in the pathogenesis of schizophrenia, including the PI system, focussing on PLC enzymes. Recently, D1 and D2 receptor heterooligomer activated by the specific agonist SKF83959 was shown to stimulate PLC-related intracellular calcium release in the brain [157]. Moreover, the overstimulation of this calcium-related pathway by high concentration of dopamine and SKF83959 induced cortical neuronal apoptosis through calcium disturbance. Prolonged stimulation of dopamine and SKF83959 in cortical neurons can reduce the dendrite extension at early stage [157]. Moreover, it can also induce neuronal apoptosis later on through PLC–calcium-related pathways, which might provide important apoptotic mechanisms in order to highlight the schizophrenia pathogenesis [157, 160–162].

Recently, deletion of PLCB1 gene was identified in paraffin-embedded samples of orbito-frontal cortex in 4 out of 15 patients affected with schizophrenia [146], in 1 out of 15 patients affected with bipolar disorder [144]. No cytogenetic detectable deletion both in 15 major depression-affected patients and in 15 normal controls was found [145]. Further studies demonstrated that the mRNAs of PLC β1 splicing variants a and b were decreased (−33 % and −50 %, respectively) in short-duration schizophrenia [147]. By contrast, only variant a mRNA was decreased in long-duration schizophrenia [147]. As authors did not find the changes in levels of mRNA to translate into a change at the level of protein, they suggested that protein expression might be regulated independently of mRNA [147]. It remains to be determined whether there is a functional consequence of this change in mRNA related to schizophrenia. Moreover, several structural and cellular changes, including marked glial anomalies, have been observed in mood disorders, with special regard to astroglia [163]. Studies have further identified specific cytoarchitectural abnormalities, especially reductions of cell number and density [164]. Astrocytes are the most abundant form of glial cells and are commonly further divided into protoplasmic and fibrous subtypes on the basis of their presence in the gray or white brain matter respectively. Recently, it has become clear that there is a much greater level of heterogeneity included in this general class of astrocytes [165]. Neuroimaging studies showed that the volume of the subgenual part of Brodmann area 24 is reduced in familial forms of major depression and bipolar disorder. The numbers of glia cells were reduced in both major depression and bipolar disorder [166–168]. A number of studies employing animal models of depression support the hypothesis that astrocyte pathology is associated with mood disorders [169–171]. A relationship between the astrocyte pathology observed in mood disorders and the emerging evidence of abnormalities within the glutamatergic neurotransmitter systems of mood disorder subjects has been postulated http://neuroscience.sciencedirect.com/article/S0006322313003909—bib48, considering the central role of astrocytes in mediating amino acid neurotransmitter clearance and metabolism [163, 172, 173]. Interestingly, previous studies conducted in rats indicated that PLC isoforms are differently expressed in activated astrocytes with respect to the corresponding quiescent counterpart [174–177]. That represents an interesting trigger point in order to develop novel therapeutic strategies targeting astrocytes.

14.8 Conclusions

Considerable research efforts were performed in order to delineate the metabolic pathways acting in the central nervous system. However, the complex interplay of the signalling molecules in the neuronal metabolism requires further studies in order to be elucidated, with respect to the embryonic and developmental period, as well as during aging, and in the alteration of the cognitive process in a number of pathologic conditions. Many evidences suggested that the PI signal transduction pathway is involved in the nervous development. More recent evidences indicate that PLC enzymes act in different events, influencing the activity of a number of molecules, at several levels in the control of neural development, thanks to a complex network. A number of observations reported that PLC enzymes might be involved in the alteration of neurotransmission. The nature, meaning, and developmental period of PLC involvement in cognitive development and decline are still largely unclear and will require further studies. Besides an increase in knowledge, to delineate the interplay of the signalling pathways recruited in the nervous system might allow the delineation of the pathogenesis and the clinical history of a number of nervous diseases. That will be helpful in order to define the diagnosis and prognosis, which often are difficult to define. This promising field of research might also provide useful insights in order to open the way to novel molecular therapeutic strategies.

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Chapter 15
Where Life Begins: Sperm PLCζ in Mammalian Egg Activation and Implications in Male Infertility

Michail Nomikos, Maria Theodoridou, and F. Anthony Lai

Abstract Egg activation is the earliest step of embryonic development following mammalian fertilization and is triggered by a characteristic series of cytoplasmic calcium (Ca^{2+}) transients, known as Ca^{2+} oscillations. It has been proposed that following sperm–egg fusion in mammals, it is the fertilizing sperm that causes these Ca^{2+} oscillations by introducing a sperm-specific protein factor into the egg cytoplasm. Mounting scientific and clinical evidence supports the notion that this protein is a sperm-specific phospholipase C (PLC), PLC-zeta (PLCζ). PLCζ has been singularly shown to stimulate cytoplasmic Ca^{2+} oscillations matching those at fertilization that trigger the early events of embryo development in many mammalian species. Sperm-delivered PLCζ is responsible for catalyzing phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis within the fertilized egg stimulating the inositol 1,4,5-trisphosphate (IP_3) signaling pathway, leading to Ca^{2+} oscillations. PLC zeta (PLCζ) is the smallest with the most elementary domain organization among all mammalian PLC isoforms, exhibiting a typical PLC domain structure. Importantly, the role of PLCζ in mammalian fertilization is highlighted by a number of recent clinical reports that have linked defects in human PLCζ with cases of egg activation deficiency resulting in male infertility. Herein, we describe the current paradigm of PLCζ in this fundamental biological process, summarizing recent important advances in our knowledge of the biochemical and physiological properties of this enzyme that is so crucial to successful fertilization and embryogenesis. We also describe how PLCζ is linked to cases of egg activation deficiencies and postulate the therapeutic and diagnostic roles this enzyme presents within a clinical setting.

Keywords Sperm • Phospholipase C • PLC zeta • Inositol 1,4,5 trisphosphate • Calcium oscillations • Egg activation • Development • Embryogenesis • Fertilization • Infertility

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_15,

15.1 Introduction

The term "egg activation" is used to describe the process that mammalian eggs (or oocytes) go through to prepare the oocyte for development, following fusion of the sperm and egg plasma membranes. Egg activation is the earliest step of mammalian embryonic development after fertilization and is triggered by an increase in the levels of intracellular calcium ion concentration [Ca^{2+}] [1, 2]. This striking Ca^{2+} signaling phenomenon is both necessary and sufficient for all the events of egg activation, such as cortical granule exocytosis (CGE) to prevent polyspermy, the resumption and completion of meiosis, and pronuclei formation [3]. The importance of Ca^{2+} extends beyond mammals, since egg activation is accompanied by an increase in the level of intracellular [Ca^{2+}] in nonmammalian species such as sea urchins and frogs. In these species, the Ca^{2+} increase takes the form of a single rise, whereas mammalian and ascidian eggs show persistent and repetitive Ca^{2+} spikes, known as Ca^{2+} oscillations, which occur after sperm–egg fusion and last for several hours (Fig. 15.1) [2–4]. The frequency and duration of Ca^{2+} oscillations varies between species with some eggs displaying a Ca^{2+} transient once every 2 min and other eggs once every hour [3, 5, 6].

It has been established in all species studied to date that the fertilization-associated Ca^{2+} oscillations involve the activation of the phosphoinositide (PI) signaling pathway [7]. Activation of the PI pathway in eggs results in the production of the

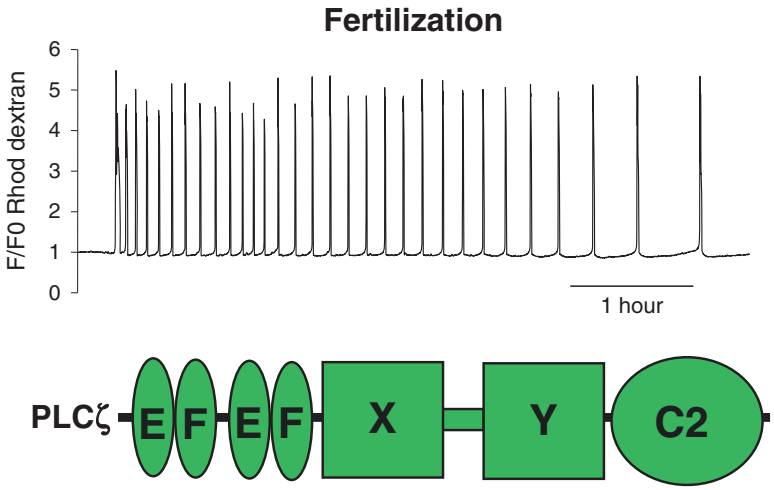

Fig. 15.1 Sperm-mediated Ca^{2+} oscillations in mouse eggs and schematic representation of PLC zeta (PLCζ) domain structure. A representative trace of mouse sperm-induced cytoplasmic Ca^{2+} oscillations recorded using the calcium indicator, Rhod dextran, following in vitro fertilization of a mouse egg (*upper panel*). The *lower panel* represents a schematic linear representation of PLCζ showing the major structural domains; the tandem putative Ca^{2+}-binding motifs (EF hands), the central catalytic domains (X and Y), and the potential lipid-binding domain (C2). All these domains are common to the other phospholipase C (PLC) isoforms (β, γ, δ, ε, and η). Note the absence of a PH domain from PLCζ sequence. Modified from [3]

Ca^{2+}-mobilizing messenger, IP_3 and diacylglycerol (DAG) via the hydrolysis of PIP_2 by a phosphoinositide-specific phospholipase C (PLC) isoform [8]. Mammalian PLC is a ubiquitous family of cytoplasmic enzymes that play a central role in activating intracellular signal transduction pathways. PLCs catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) liberating IP_3 and DAG. Both products of this PIP pathway are involved in shaping Ca^{2+} responses. IP_3 is involved directly in mediating cytosolic Ca^{2+} release by binding and gating its integral membrane protein receptor, the type I IP_3 (IP_3R1), a tetrameric ligand-gated Ca^{2+} channel located on the endoplasmic reticulum (ER) membrane. Binding of IP_3 to the IP_3R1 causes a conformational change and consequently the opening of the intrinsic IP_3R1 channel resulting in a rapid Ca^{2+} release from internal stores. Production of DAG may indirectly be involved in the Ca^{2+} influx via activation of protein kinase C (PKC) [8]. The involvement of IP_3 and the IP_3R in fertilization has been illustrated by studies showing that Ca^{2+} oscillations at fertilization can be inhibited by injection of an IP_3R function-blocking monoclonal antibody or by down-regulation of IP_3R expression [9–11]. In addition, sustained injection of IP_3, or microinjection of the IP_3 analogue adenophostin, can also lead to a series of Ca^{2+} oscillations in eggs [12, 13].

Four distinct hypotheses have been proposed to explain the nature of the Ca^{2+} oscillations in mammalian eggs at fertilization. (1) the "Ca^{2+} bomb" hypothesis, (2) the "conduit" hypothesis, (3) the "contact" hypothesis, and (4) the "sperm factor" hypothesis (for details on the proposed hypotheses explaining Ca^{2+} oscillations at fertilization, see review [3]). There are now several lines of study providing convincing evidence that the "sperm factor" hypothesis of egg activation is the most appropriate model [2–4, 14, 15]. The "sperm factor" hypothesis proposes that the spermatozoon itself contains a soluble factor that, upon sperm–oocyte fusion, diffuses into the oocyte cytosol and stimulates the IP_3 pathway and the subsequent Ca^{2+} oscillations in fertilized oocytes [4]. Evidence for this hypothesis in mammals comes from the finding that microinjecting protein-based sperm extracts into oocytes from a number of mammalian species triggers Ca^{2+} oscillations similar to those seen at fertilization [4, 16]. The microinjection of whole spermatozoa into mammalian oocytes by intracytoplasmic sperm injection (ICSI), a frequently used clinical technique for in vitro fertilization (IVF), which avoids any surface contact between the oocyte and spermatozoon, also elicits a similar set of Ca^{2+} oscillations [15, 17]. Moreover, the Ca^{2+}-releasing sperm factor is apparently a sperm-specific protein, as extracts from other tissues do not elicit Ca^{2+} increases when injected into oocytes [4, 18]. However, this soluble sperm factor mechanism is not species specific, because sperm extracts from hamsters, humans, pigs, cows, frogs, and chickens can each trigger Ca^{2+} oscillations in mouse eggs [18, 19].

15.2 Discovery of Sperm-Specific PLCζ

Early candidates for the sperm factor were believed to be small molecules such as IP_3 [20], NO [21], or NAADP [22]. Although these molecules have the ability to generate Ca^{2+} release from intracellular stores in nonmammalian species, none of

these could fully mimic the response seen at IVF in mammalian eggs [12]. In addition, various fractionation studies suggested that the sperm factor was a protein ~30–100 kDa in size [23, 24]. Different proteins have been hypothesized to be the sperm factor, including a 33 kDa protein [25], a truncated form of the *kit* receptor, tr-kit [26], and a post-acrosomal sheath WW domain-binding protein called PAWP [27]. However, none of these proteins has been demonstrated to be capable of generating the characteristic pattern of Ca^{2+} oscillations observed during fertilization in mammalian eggs [3]. Experiments employing in vitro PLC assays using mammalian sperm extracts showed that these extracts possess a PLC enzyme activity at least 100-fold greater than that present in other tissues known to express several PLC isoforms [23]. Uniquely, the PLC activity of the sperm extracts was high even at the basal cytoplasmic Ca^{2+} levels (~0.1 μM) typical of mammalian eggs at the time of fertilization. These observations supported the idea that the sperm factor may therefore be a PLC isoform itself. Several known PLC isoforms have been shown to also be expressed in mammalian sperm [28]. However, microinjection of recombinant proteins corresponding to most of the known PLC isoforms expressed in sperm failed to initiate Ca^{2+} oscillations in mouse eggs or did so only at nonphysiological concentrations [2, 3, 29]. Furthermore, chromatographic fractionation of sperm extracts revealed that none of the known PLC isoforms were present in the protein fraction displaying potent Ca^{2+} oscillation-inducing activity [24]. All these observations suggested that if the sperm factor was a PLC, then it would likely be a novel isoform.

Experimental evidence for a novel PLC isoform was first obtained after a search of a mouse-expressed sequence tag (EST) database, which revealed potentially novel PLC sequences derived from testis [30]. This led to the isolation of a novel PLC from mouse testis, termed PLC zeta (PLCζ). PLCζ is unusual in that it appears to be a gamete-specific protein that is expressed during spermatogenesis only in spermatids. PLCζ is a protein of about 70 kDa, which makes it the smallest in size compared to the other known somatic PLC isoforms [30, 31]. Numerous studies now support the notion that PLCζ is the sole physiological agent responsible for mammalian oocyte activation. Initially, microinjection of complementary RNA (cRNA) encoding the mouse, human, and cynomolgus monkey PLCζ into mouse eggs triggered the characteristic Ca^{2+} oscillations that are also specifically observed at fertilization [30, 31]. In addition, microinjection of cRNA corresponding to PLCζ triggered both Ca^{2+} oscillations and consequent egg activation in human and pig eggs [32, 33]. Immunodepletion of PLCζ from native sperm extracts by a specific anti-PLCζ antibody abolished their ability to induce Ca^{2+} oscillations in mouse eggs [30].

Consistent with the PLCζ cRNA microinjection experiments, recombinant mouse and human PLCζ protein was capable of triggering Ca^{2+} oscillations in mouse and human eggs [34, 35]. Further, these PLCζ injections also supported early embryonic development of mouse eggs to the blastocyst stage. Moreover, sperm obtained from transgenic mice with significantly reduced expression of PLCζ displayed a premature termination of Ca^{2+} oscillations following IVF [36]. Immunofluorescence localization experiments showed that PLCζ is localized to the post-acrosomal region of the sperm head, which is consistent with the proposal that

a sperm factor should be localized in a region within the sperm that would gain rapid access to the ooplasm to initiate Ca^{2+} oscillations within a few minutes after sperm–egg fusion [35].

The importance of PLCζ in mammalian fertilization has been further highlighted by the mounting clinical evidence that directly linked abnormal forms or aberrant function of PLCζ with documented cases of human male infertility [37–40]. It was shown that sperm of infertile males, which routinely fail clinical procedures such as IVF and ICSI, produced either no Ca^{2+} oscillations upon injection into mouse eggs or produced significantly diminished Ca^{2+} oscillation profiles in both frequency and amplitude relative to those observed from fertile males [37]. Moreover, immunofluorescence and immunoblot analysis revealed that infertile patients whose sperm failed ICSI and were unable to induce Ca^{2+} oscillations exhibited reduced or absent levels of PLCζ within the sperm head [37]. All these data, therefore, suggest that sperm-specific PLCζ is the sole sperm factor required for the initiation of a new life.

15.3 Distinctive Properties and Structure of PLCζ

PLCζ is a gamete-specific protein that is expressed only in spermatids. PLCζ is the smallest and the most elementary in domain organization among all the mammalian PLC isoforms identified to date [2, 30]. Sperm PLCζ enzymes are similar in size for all species so far studied, being 70–75 kDa, but they display great variation in their calculated isoelectric points (pI), from 5.29 in rat to 9.14 in human [41]. The reason why there should be such a wide species range in predicted pI is unknown. Similar to other PLC isoforms, PLCζ demonstrates a typical PLC domain structure with four tandem Ca^{2+}-binding EF hands at the N-terminus, followed by the characteristic X and Y catalytic domains, which form the active site in all PLC isoforms and a single C2 domain at the C-terminus (Fig. 15.1) [2, 30]. There is an intervening segment between the X and Y domains, giving rise to a large loop, the XY-linker sequence, which differs considerably between PLC isozymes. PLCζ is closest in domain structure and primary sequence to the PLC isoforms of the δ class. Sequence alignment analysis indicates that PLCζ has the greatest homology with PLCδ1 (47 % similarity, 33 % identity) [30]. However, the major structural difference which distinguishes PLCζ from PLCδ1 and all the other somatic PLC isoforms is that it lacks the presence of a typical pleckstrin homology (PH) from its sequence, a domain which has been found in all the other somatic PLC isoforms [2]. PH domains are well-defined structural modules of about ~120 amino acids residues that mediate the membrane binding of somatic PLC isoforms. This suggests that PLCζ may have to employ a novel mechanism to target biological membranes. Another unique feature of PLCζ compared to somatic PLC isoforms is its high Ca^{2+} sensitivity. PLCζ appears to be 100-fold more sensitive to Ca^{2+} than PLCδ1 with an EC_{50} of 80 nM [42]. This is within the range of the reported rested Ca^{2+} concentrations in mammalian eggs, suggesting that PLCζ may become enzymatically active immediately when it is introduced from the sperm cytosol into the ooplasm upon

gamete fusion at fertilization. Significantly, each of the individual PLCζ domains appears to have an essential role in the distinct biochemical characteristics and the unique mode of regulation of this gamete-specific PLC isozyme.

15.4 EF Hand Domains

PLCζ contains two pairs of EF hand domains at its N-terminus. The EF hand domains consist of four helix-loop-helix motifs divided in two pair-wise lobes. The EF hand motifs occur in pairs because one loop helps to stabilize the other. In PLCδ1, these domains form a flexible link between the XY catalytic domain and the PH domain and possess Ca^{2+} ion-binding residues, which have also been identified in various other calcium-binding proteins such as calmodulin and troponin [3, 41]. Experimental evidence suggests that the EF hand domains play an important role in the way PLCζ generates IP_3 in an egg. Deletion of one or both pairs of EF hand domains of PLCζ completely abolishes its Ca^{2+}-oscillation-inducing activity in mouse eggs [42]. PLCζ EF hand domains play an important role in the high Ca^{2+} sensitivity relative to the other PLC isoforms, and especially in comparison with PLCδ1. Interestingly, deletion of both EF hands dramatically changed the EC_{50} for Ca^{2+} of PLCζ from 80 nM to 30 μM and also decreased the Hill coefficient from ~4 to ~1 [42]. Removal of the first EF hand domain also raised the EC_{50} for Ca^{2+} by ~9-fold. This suggests that EF hand truncation would ablate the enzyme's ability to generate IP_3 in an intact cell which has a basal Ca^{2+} concentration of ~100 nM [42]. Another recent study showed that replacement of the EF hand domains of PLCζ with the corresponding EF hand domains of PLCδ1 resulted in a ~10-fold increase of the Ca^{2+} sensitivity of PLCζ, reducing its in vivo Ca^{2+} oscillation-inducing activity without affecting its in vitro affinity for the substrate, PIP_2 [43].

Interestingly, an additional unexpected role of the EF hand domains in nuclear translocation of mouse PLCζ has been proposed, following experimental evidence suggesting that point mutations within this region disrupt the nuclear translocation process [44]. This contrasts with reports that the XY-linker region in mouse PLCζ comprises the nuclear localization signal [2, 45]; however, the EF hands might contribute to the nuclear translocation ability of mouse PLCζ by participating in specifically effective folding of the molecule required for this process [44].

15.5 X and Y Catalytic Domains

The XY catalytic domain is responsible for the enzymatic activity of PLCζ and consists of two distinct domains, X and Y. There is an intervening segment between the X and Y domains, giving rise to a large loop, the XY-linker sequence, which differs considerably between PLC isozymes [2, 3]. The XY catalytic domain is the most highly conserved region of PLC compared to the other regulatory domains.

The XY sequence similarity between all PLC isoforms is ~60 %, and even higher within those of the same class. The PLCζ catalytic domain displays 64 % similarity with that of PLCδ1 [30]. By homology with PLCδ1, the PLCζ XY domain is predicted to be organized in repetitive beta sheet/alpha helix sequences, forming a distorted barrel. Five essential active site residues within the catalytic domain of PLCδ1 (His^{311}, Glu^{341}, Asp^{343}, His^{356}, and Glu^{390}) are conserved in PLCζ, suggesting a similar mechanism of catalytic activation for PLCζ and PLCδ1. A point mutation of Asp^{210} ($D^{210}R$) in the catalytic domain of PLCζ, corresponding to the essential active site Asp^{343} residue of PLCδ1, has been shown to cause the complete loss of PLCζ-dependent Ca^{2+}-oscillation-inducing activity in mouse eggs [3, 30]. Despite the high sequence similarity between the XY domains of PLCζ and PLCδ1, a recent study showed that replacement of PLCζ XY catalytic domain from the corresponding domain of PLCδ1 completely abolished the ability of PLCζ to trigger Ca^{2+} oscillations in mouse eggs, dramatically affecting its in vitro enzymatic activity and the interaction of PLCζ with PIP_2 [43].

15.6 XY-Linker Region

The other region of PLCζ that plays an important role in regulating its enzymatic activity and its substrate targeting is the segment that joins the X and Y catalytic domains, termed the XY-linker region. Notably, the XY-linker region is the only part of the PLCδ1 structure that has not been resolved by X-ray crystallography. This region of PLCζ is more extended and notably comprises more basic residues relative to its PLCδ1 counterpart [2, 3]. The XY-linker of PLCζ is also the least well-conserved region between species being shortest in length in the *Homo sapiens* PLCζ sequence due to apparent skipping of an exon that is retained in the monkey protein [31]. The significance of this XY-linker diversity is unclear but may contribute to the different rates of PIP_2 hydrolysis and relative potency that have been observed in inducing Ca^{2+} oscillations between the PLCζ of different species [3].

Structural and biochemical evidence suggests that the XY-linker region of PLCβ, γ, δ, and η mediates potent auto-inhibition of their enzymatic activity. Such data are consistent with the negatively charged XY-linker region of these PLC isoforms, which may confer electrostatic repulsion alongside steric hindrance to occlude PIP_2 from the active site [46, 47]. In contrast with somatic PLCs, recent evidence suggests that PLCζ operates via a novel enzymatic mechanism, as the absence of the XY-linker of PLCζ significantly diminishes both its in vitro PIP_2 hydrolysis and in vivo Ca^{2+} oscillation-inducing activity [48]. It has been proposed that the positively charged XY-linker region of PLCζ may be involved in the targeting of PLCζ to its membrane-bound, negatively charged PIP_2 via electrostatic interactions, assisting in anchoring PLCζ to membranes, while enhancing local PIP_2 concentrations [49, 50]. This is also consistent with a recent study, which showed that replacement of PLCζ XY-linker region with the corresponding region of PLCδ1 completely abolished the ability of PLCζ to induce Ca^{2+} release in mouse eggs, dramatically affecting its in

vitro PIP_2 hydrolysis activity and its interaction with its substrate PIP_2 [43]. Interestingly, a proteolysis study has demonstrated that the porcine PLCζ remains functionally active after cleavage has occurred in the XY-linker region, suggesting the intact enzyme is not essential for PIP_2 hydrolysis [51]. As mentioned earlier, there is also evidence that the basic residues in the XY-linker region comprise a nuclear localization sequence (NLS) in mouse PLCζ, which is discussed in more detail later.

15.7 C2 Domain

The C2 domain is a ~120 residue structural motif found in numerous proteins and it is known that most C2 domains can bind to Ca^{2+}, and this property is a critical determinant for the associated enzyme activity. However, there are some of them that do not bind Ca^{2+} ions, such as the C2 domains of the ApIII PKC and PI3K-C2β, which bind to phospholipids with relatively low affinity and specificity [3]. The C2 domain has a vital role in PLCζ function since deletion or replacement of this domain with that of PLCδ1 abolishes its Ca^{2+} oscillation-inducing activity in intact eggs, although enzyme activity was retained and its sensitivity to Ca^{2+} was unaffected [42, 43]. There is also biochemical evidence for low affinity binding of the PLCζ C2 domain to membrane phospholipids containing phosphatidylinositol 3-phosphate PI(3)P and phosphatidylinositol 5-phosphate PI(5)P [50, 52]. It has been suggested that the association of C2 with PI(3)P may play a role in PLCζ targeting or regulation of activity, since it has been shown that presence of PI(3)P reduces in vitro PIP_2 hydrolysis by PLCζ (Fig. 15.2) [52].

15.8 PLCζ Targets Intracellular PIP_2 Stores in Mammalian Eggs

The subcellular localization of PLCζ in the egg has been extensively studied using Venus- or YFP-tagged PLCζ fusion proteins. In either case, PLCζ does not localize to the plasma membrane, but instead appears to be distributed uniformly within the egg cytoplasm [53, 54]. Immunocytochemistry studies that examined the distribution of PLCζ in eggs showed that PLCζ is localized in small (<1 μM) vesicles in the cytoplasm of the eggs. By using a specific anti-PIP_2 antibody, it was shown that small intracellular vesicles contain PIP_2 stores [54]. The significance of the intracellular PIP_2 was investigated by experiments which employed targeting of an inositol phosphatase into different subcellular compartments of mouse eggs. Inositol phosphatases are enzymes which catalyze the removal of the phosphates from the PIP_2 and has been previously successfully used to deplete PIP_2 levels in cells. It was found that targeting the inositol phosphatase to the plasma membrane did not block the Ca^{2+} oscillations triggered by PLCζ; however, targeting this phosphatase to the cytosolic small vesicles by fusing it with an inactive form of PLCζ, the Ca^{2+}

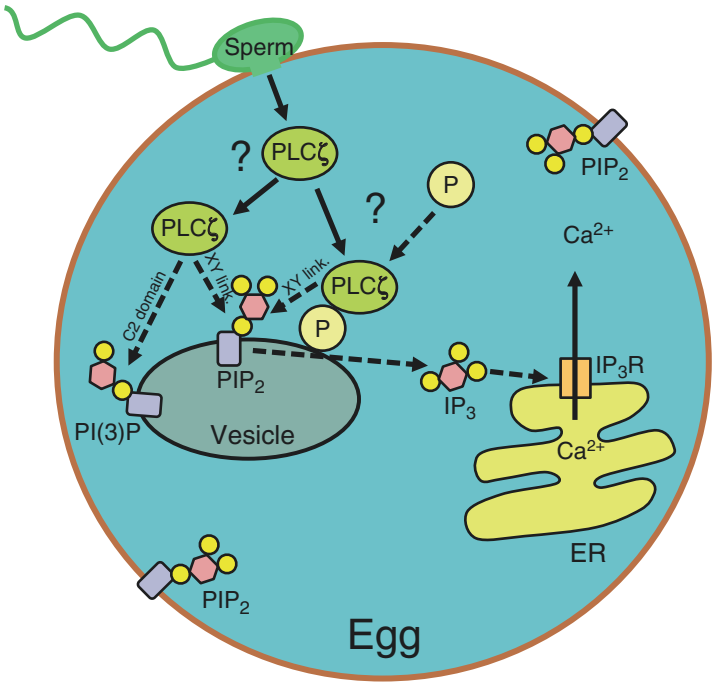

Fig. 15.2 Putative mechanism of PLCζ function after sperm-egg fusion. PLCζ diffuses from the sperm head into the egg cytosol and targets a distinct intracellular vesicular PIP_2-containing membrane. Association of PLCζ with its specific membrane target may be mediated by interaction of the C2 domain with either PI(3)P or an unidentified egg membrane-targeting protein. The positively charged amino acids in the XY linker region might further assist the anchoring of PLCζ to the membrane, by enhancing the local PIP_2 concentration adjacent to the catalytic domain via electrostatic interactions with the negatively charged PIP_2. Once PLCζ is associated with the membrane PIP_2 the catalytic X/Y barrel binds and hydrolyzes its substrate [2]. Modified from [2]

oscillations induced either by sperm or PLCζ were significantly inhibited [54]. The idea of an intracellular vesicular source of PIP_2 gains support from studies indicating that in fertilizing ascidian oocytes, Ca^{2+} oscillations may be driven by Ca^{2+}-dependent PLC activity analogous to PLCζ, with a fertilization-like profile of Ca^{2+} release only possible in the presence of a uniform cytoplasmic source of PIP_2, alongside uniform cytoplasmic PLC activity (Fig. 15.2) [55]. However, the precise mechanism underlying this specific vesicular/organelle localization of PLCζ is still unknown and requires further elucidation.

15.9 Regulation of PLCζ Activity in Mammalian Eggs

The complete mechanism of PLCζ regulation in mammalian eggs is still unclear. However, it has been shown that in mouse eggs the cessation of Ca^{2+} oscillations coincides with PLCζ translocation into the newly formed pronuclei. PLCζ contains in its

XY-linker region, a predicted nuclear localization signal (NLS) sequence, the same region that is also thought to be necessary for PLCζ membrane association through its electrostatic interaction with the negatively charged PIP_2 [2, 3]. Substitution of basic for acidic residues in the mouse PLCζ NLS resulted in the loss of mouse PLCζ nuclear translocation ability without affecting its in vivo Ca^{2+} oscillation-inducing activity, enabling Ca^{2+} oscillations to proceed beyond pronuclei formation [45]. However, it remains unclear how Ca^{2+} oscillations cease in eggs of other species, since bovine, rat, and human PLCζ do not appear to undergo nuclear localization, although they do contain a putative NLS sequence [56]. Interestingly, rat PLCζ does not translocate to the pronuclei of rat zygotes, while mouse PLCζ does, although rat and mouse PLCζ NLS sequence share 87 % sequence identity [56]. Thus, while PLCζ nuclear sequestration may play a key role in the termination of Ca^{2+} oscillations at interphase in mouse embryos, different mechanisms may be involved in other organisms.

An alternative explanation is that PLCζ may require association with a specific egg factor in order to achieve an active state and subsequent dissociation of this factor may result in inactivation of PLCζ and thus termination of Ca^{2+} oscillations. Support for this hypothesis comes from studies in CHO cells, in which PLCζ was expressed to levels 1,000 times higher than that which is active in eggs and this did not cause any significant Ca^{2+} changes even following ATP-induced Ca^{2+} release, suggesting that PLCζ is inactive [57]. Surprisingly, microinjection of such PLCζ-transfected CHO cells or extracts made from these cells into mouse eggs induced Ca^{2+} oscillations [57]. Hence, the "egg factor" hypothesis could potentially explain why PLCζ is kept inactive within sperm, where it is present at significantly higher concentrations than within eggs.

15.10 Defects in PLCζ and Human Infertility

Advances in our understanding of oocyte activation and the paradigms of the PLCζ mechanism and regulation within this essential process at fertilization stand to provide significant benefit to assisted reproductive technology (ART), clinical laboratory techniques that aim to combat human infertility, a condition affecting ~1 in 7 couples [2]. While ART has successfully led to total birth rates approaching ~7 % in some developing countries, several conditions such as severe male infertility (19–57 % of cases of infertility) remain untreatable. Intracytoplasmic sperm injection (ICSI) is a powerful modification of the IVF technique whereby a single sperm is directly injected into individual eggs. However, even up to 5 % of ICSI cycles still fail, affecting over 1,000 couples per year in the UK alone, with deficiencies in oocyte activation currently regarded as the main reason for this failure [2].

A number of clinical reports have linked defects in human PLCζ protein with cases of egg activation deficiency. One report identified a number of patients whose gametes repeatedly failed to fertilize after ICSI and this was found to be due to egg activation failure. Sperm from these patients were unable to trigger the Ca^{2+}

oscillations required for egg activation and the authors provided significant evidence that this deficiency was associated with reduced expression levels or absence of PLCζ in the sperm of these patients [37]. In addition, two clinical studies reported two novel PLCζ mutations in a heterozygous patient who was a case of failed ICSI [38, 39]. These point mutations are located on either the X or Y catalytic domains (H233L and H398P) of human PLCζ and they have been found to dramatically reduce the in vitro PIP2 hydrolysis activity of recombinant PLCζ and its ability to produce the normal pattern of repetitive Ca^{2+} release in mouse eggs [35, 40]. Interestingly, the two mutations were shown to be inherited, one from the father and the other from the mother, indicating that male infertility could be conveyed by the maternal germ line. Furthermore, the identification of these mutations indicates that PLCζ may be contributing not only towards male infertility, but also in cases of male sub-fertility [2, 58].

15.11 PLCζ as a Potential Therapeutic Agent for Male Infertility

It has been reported that in some cases of egg activation failure after ICSI, the eggs were successfully treated by applying Ca^{2+} ionophores during ICSI, even though this does not induce Ca^{2+} oscillations [2, 3]. However, it remains to be determined whether ionophore treatment represents the safest or most effective method for overcoming egg activation failure. This is because Ca^{2+} ionophore treatment may pose concerns for the future health of the embryo due to the potential for cytotoxic, mutagenic, and teratogenic effects on the eggs and embryos [2, 59]. Current ionophore protocols for treatment of egg activation failure may not be beneficial for all patients presenting with egg activation-related issues. Furthermore, the abnormal Ca^{2+} signal induced, which often manifests as a single Ca^{2+} transient, is a potential threat to ensuing development at later stages with potential repercussions on epigenetic processes [60]. Thus, a more endogenous therapeutic agent is urgently required as a replacement for current synthetic methodology and hence recombinant PLCζ may therefore be potentially more suitable as the presumptive physiological therapeutic agent to treat such cases of failed fertilization after ICSI.

The production of pure and active recombinant form of human PLCζ has been a key goal over recent years, an aim which seems to have been finally achieved following a recent key study. It has recently been reported that recombinant human PLCζ has been produced in a stable state using a bacterial expression system. Recombinant human PLCζ protein prepared in this way was able to generate Ca^{2+} oscillations in a physiological range in mouse and human eggs [35]. The same study also demonstrated that the deleterious effects of mutant versions of PLCζ may also be efficiently overcome by utilization of purified recombinant PLCζ protein leading to efficient blastocyst formation (Fig. 15.3) [35]. However, while this work is extremely encouraging, such advancements require urgent extrapolation from

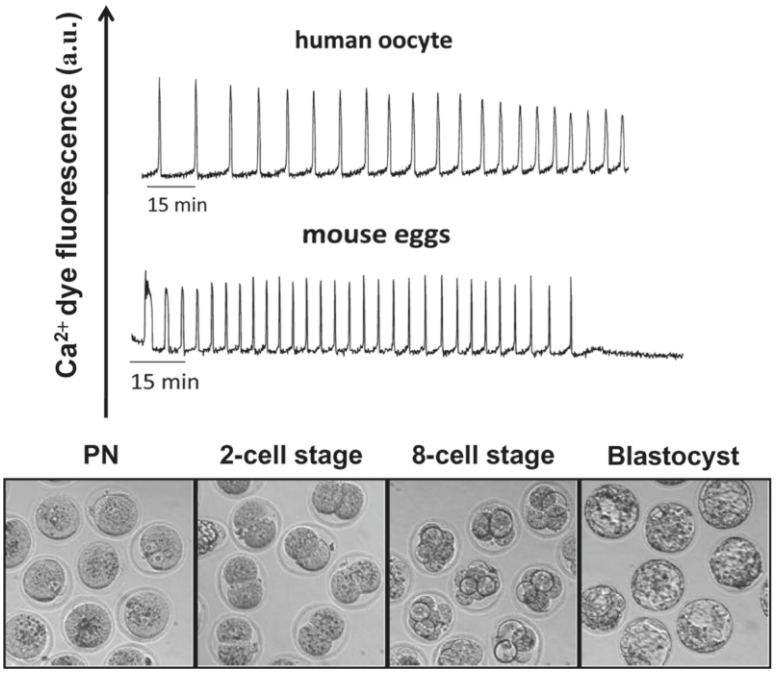

Fig. 15.3 Microinjection of recombinant human PLCζ protein induces Ca^{2+} oscillations in human and mouse eggs and initiates early embryo development. Representative fluorescence (a.u.; arbitrary units) recordings reporting the Ca^{2+} concentration changes in a human oocyte and a mouse egg following microinjection of human PLCζ recombinant protein (*upper panel*). *Lower panel* shows micrographs illustrating mouse embryos at the various early developmental stages (pronuclear formation [PN], two-cell and eight-cell stages, and blastocyst stage) achieved after egg microinjection with ~80 fg of purified human PLCζ recombinant protein. Modified from [35]

laboratory models to a clinical setting, before widespread use in fertility clinics may be advised. Moreover, recombinant human PLCζ protein could potentially be used in regenerative medicine approaches via generation of parthenogenetic embryos and blastocysts that may facilitate stem cell derivation and differentiation [35].

15.12 PLCζ as a Diagnostic Marker of Male Fertility

Considering the central importance of PLCζ to successful fertilization and embryogenesis, PLCζ may represent a powerful biomarker to examine sperm functional competency. A recent study utilizing PLCζ as a biomarker indicated that motile sperm organelle morphology evaluation (MSOME) methodology, relying on high-powered magnification analysis of human sperm before a modified version of ICSI is applied (IMSI), may enable selection of sperm with higher total levels of

PLCζ, as well as selecting a higher proportion of sperm exhibiting the presence of PLCζ [61]. Thus, a significant application of PLCζ may be its use as a prognostic indicator of sperm oocyte activation capability, and thus fertility. A diagnostic test based on human sperm microinjection into mouse oocytes (known as the mouse oocyte activation test; MOAT) has previously been developed as a heterologous model to evaluate the activation capacity of human sperm [62]. However, considering that human PLCζ is thought to be more potent in its activity compared to mouse PLCζ, application of the MOAT technique may only detect extreme cases of PLCζ deficiency, where PLCζ is completely absent from sperm, and not where a more subtle reduction is present in a clinical setting.

Thus a more attractive option would be the direct examination of sperm PLCζ. Previous immunofluorescence studies on human sperm have demonstrated a pattern of PLCζ localization in the sperm head that would be consistent with normal fertile sperm, with abnormal patterns evident in ICSI-failed sperm [38, 63]. PLCζ analysis may also identify cases of male sub-fertility when compared to routine clinical semen parameters, indicating that analysis of PLCζ status may benefit the wider male population and not just cases of ICSI-failure.

15.13 Conclusions

In 2002 the discovery of sperm PLCζ was a significant breakthrough in the fertilization field and began a shift in thinking about how fertilization works in mammals and other animals. PLCζ participates in a standard biochemical pathway (phosphoinositide signaling) that is known to be present in all types of cells in the body, but uniquely, PLCζ appears to be able to work only in egg cells. PLCζ also seems to interact with small membranous vesicles inside eggs, which is very different from the way other types of PLC proteins work by interacting with the inner leaflet of the cell surface membrane. There are also big variations in the activity of PLCζ in eggs from different animal species that has proven difficult to explain. Also, while we know that all parts of the PLCζ protein are important for it to work, we don't fully understand how all these parts work together. A step forward would be the high-resolution structure determination of full-length PLCζ. This would help to reveal all the critical ion and lipid-/protein-binding sites in the protein, providing a useful tool for understanding the complex regulatory mechanism of this enzyme. The availability of purified, active recombinant human PLCζ protein appears to represent both a highly practical and the most physiologic therapeutic agent for overcoming failed ICSI cases resulting from aberrant sperm PLCζ. This potential therapeutic approach requires extrapolation from laboratory models to a practical fertility clinic setting. Further investigation of PLCζ should help to fully elucidate the fundamental mechanism of the earliest events required for a new life to begin.

Acknowledgments We are grateful for grant support from the Wellcome Trust and helpful discussion with Professor Karl Swann.

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Chapter 16
Oocyte Activation and Phospholipase C Zeta (PLCζ): Male Infertility and Implications for Therapeutic Intervention

Junaid Kashir, Celine Jones, and Kevin Coward

Abstract Infertility is a condition that now affects an estimated one in seven couples. In approximately 40 % of cases, the primary cause of infertility rests with male-derived factors associated with a variety of anatomical, physiological, and molecular deficiencies. In a proportion of such cases, the functional ability of sperm to successfully fertilise and activate the oocyte is compromised. While assisted reproductive technology can successfully circumvent some of these issues via the application of artificial oocyte-activating agents, there is significant ongoing debate as to whether these chemical agents should be replaced with an endogenous alternative. Phospholipase C zeta (PLCζ) is the sperm-specific protein responsible for activating the quiescent oocyte following gamete fusion. Identified in a number of mammalian and non-mammalian organisms, PLCζ plays a fundamental role in the process of oocyte activation by inducing the controlled release of calcium in the ooplasm via an inositol triphosphate (IP_3)-mediated signalling cascade. A growing body of evidence shows clear association between abnormalities in PLCζ structure, expression, localisation, and function to characterised states of human male infertility. Consequently there is significant global interest in PLCζ as both an endogenous therapeutic target to rescue infertile states associated with PLCζ-linked oocyte activation deficiency, and a diagnostic marker for oocyte activation ability. Here, we discuss the present status of PLCζ research and contemplate future applications of this fundamental sperm PLC in the clinic.

Keywords Phospholipase C zeta (PLCζ) • Oocyte activation • Male infertility • Sperm • Therapy • Diagnostic • Assisted reproductive technology

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_16,

16.1 Introduction

Infertility (the inability to conceive a child naturally) is now estimated to affect approximately 10 % of couples. This worrying statistic has contributed to the phenomenal growth of assisted reproductive technology (ART) over recent years. While ART has resulted in the birth of over five million babies worldwide [1], pregnancy and live birth rates remain poor, and rarely exceed ~40 % [2]. Pregnancy and delivery via routine conventional ART procedures such as in vitro fertilisation (IVF; whereby sperm and oocytes are co-incubated in culture media) or intracytoplasmic sperm injection (ICSI; in which a selected individual sperm is microinjected directly into the ooplasm) remain as low as 22.4 % and 23.3 %, respectively [2]. Consequently, there is a very clear need to improve success rates in order to provide couples with the best chances of conception.

Significant concern surrounds a phenomenon known as recurrent ICSI-failure, which involves cases where oocytes fail to fertilise, even following ICSI [3–5]. On average, ICSI results in fertilisation rates of approximately 70 % [4, 5]. However, complete or virtually complete fertilisation failure still occurs in 1–5 % of all ICSI cycles [3, 5–7]. The underlying causes of this condition have been attributed to a variety of physiological, biochemical, or genetic deficiencies in the fertilising sperm [5], and a growing body of evidence now indicates that deficiencies in phospholipase C zeta (PLCζ), the oocyte activation factor, plays a key role.

In this chapter, we discuss our current understanding of the potential roles played by the PLC family at fertilisation and the phenomenon known as oocyte activation, focusing specifically upon PLCζ, the sperm-derived oocyte activation factor. We present recent discoveries linking PLCζ to characterised states of infertility, and discuss how recent advancements in our discipline may assist future diagnostic and treatment options in the clinic.

16.2 Oocyte Activation and Calcium Oscillations

Oocyte activation is characterised by the formation of the second polar body, the male and female pronuclei, and the subsequent initiation of embryogenesis [8]. Collectively, this fundamental process involves cortical granule exocytosis, progression of the cell cycle, maternal mRNA recruitment, and the alleviation of meiotic arrest in the fertilised oocyte [8–12]. In mammals, oocytes are arrested at the second metaphase of meiosis (MII) following the exclusion of the first polar body [13, 14]. It is now well regarded that the initiation of oocyte activation is dependent upon the release of intracellular calcium ions (Ca^{2+}) within the oocyte which can occur either in a single transient wave traversing from one side of the oocyte to the other as seen in the sea urchin or as a series of repetitive oscillations as seen in the human [15, 16]. The temporal pattern of Ca^{2+} oscillations in activating oocytes is largely species specific in terms of amplitude, duration, and frequency over time [17–20], and it is believed that these specific features play subtle roles in the molecular processes associated with activation.

Oocytes display considerable sensitivity to each wave of oscillations, with early cortical granule exocytosis requiring fewer oscillations than later events such as the alleviation of MII arrest [21, 22]. Protein expression profiles in early mouse embryos are influenced by the frequency and amplitude of Ca^{2+} oscillations [19], which can also influence embryonic development in rabbits [8, 10]. The frequency and amplitudes of Ca^{2+} oscillations are directly responsible for cell cycle progression and can induce variation in cell cycle progression rates [19, 20]. Considering that the rate of progression to the 2- and 4-cell stages of human oocytes is considered as an indicator of normal embryogenesis [23], it follows that the frequency and amplitude of Ca^{2+} oscillations at fertilisation may also be important for embryogenesis, and not just for oocyte activation as first believed.

While it is well established that Ca^{2+} oscillations are of utmost importance for oocyte activation, the relative roles played by the respective gametes during fusion have been subject to intense scrutiny with three models hypothesised to explain how the fertilising sperm initiates these oscillations in mammals: (1) the Ca^{2+} conduit model [24–27], (2) the membrane receptor model [28–31], and (3) the soluble sperm factor model [31–33].

While there was initial controversy surrounding the debate as to which model was correct, a series of studies provided overwhelming support for the sperm factor theory of oocyte activation (for review see [5, 16]). This model proposed that oocyte activation is triggered by the introduction of a soluble factor released from the sperm into the oocyte during or immediately post-gamete fusion. Indeed, the injection of sperm extracts into the eggs and oocytes from a variety of species, including marine worms and ascidians, has demonstrated successful Ca^{2+} release and oocyte activation [15, 34, 35]. Furthermore, sperm extracts from frogs, chickens, and tilapia fish also trigger Ca^{2+} oscillations when injected into mouse oocytes [36–38], suggesting the existence of a similar sperm-based mechanism throughout a wide spectra of species.

Initial data indicated that the mammalian sperm factor was likely to be a sperm-specific PLC, as it appeared to possess distinct enzymatic properties compared to other known PLCs [18, 39]. This observation correlated well with the hypothesis that oocyte activation involved Ca^{2+} oscillations generated in an inositol triphosphate (IP_3)-mediated manner, which in turn supported the notion that the soluble sperm factor was likely to be a PLC which mediated the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 [12, 17, 40]. General consensus agreed that the factor responsible for Ca^{2+} release within oocytes must be sperm specific, as extracts from other tissues did not result in Ca^{2+} induction upon oocyte injection [34, 41].

16.3 PLCs at Fertilisation and Oocyte Activation

Phosphoinositide metabolism is a vital intracellular signalling system and implicated in multiple cellular functions such as hormone secretion, neurotransmitter transduction, growth factor signalling, membrane trafficking, and cytoskeletal

regulation, and has also been linked to fertilisation and embryogenesis [42–46]. PLC and IP_3 signalling mechanisms have also been shown to be involved in sperm thermotaxis, where elevated levels of Ca^{2+} may modify flagellar bending and sperm motion paths [47–50].

Thirteen mammalian PLC isozymes have now been categorised on the basis of their structure and regulatory activation mechanisms. These are PLCdelta (PLCδ1, 3, and 4), PLCbeta (PLCβ1–4), PLCgamma (PLCγ1 and 2), PLCepsilon (PLCε), PLCzeta (PLCζ), and PLCeta (PLCη1 and 2) [46, 51–54]. PLC isozymes generally contain catalytic X and Y domains, and regulatory domains, such as the C2 domain, the EF-hand motif, and the pleckstrin homology (PH) domain. These domains exist in various conformations, depending on the particular isozyme. Some isozymes may also exhibit subtype-specific domains, which are thought to confer specific regulatory properties, for example, the Src homology (SH) domain observed in PLCγ [51] and the Ras-associating and Ras-GTPase exchange factor-like domains found in PLCε [55, 56].

Investigation of sperm from PLCδ4 knockout (KO) mice showed that these sperm induced activation in fewer oocytes following IVF, and failed to elicit Ca^{2+} oscillations, which suggests an essential role for sperm-borne PLCδ4 in oocyte activation [57]. Additionally, studies have shown that solubilised mouse zona pellucida (ZP) was able to induce an acrosome reaction (an essential step in fertilisation) in sperm from normal mice, but was unable to do so in PLCδ4 KO sperm. Here, the elevation of Ca^{2+} levels was thought to play a significant role [58, 59]. Sperm from normal mice treated with ZP exhibited continuous elevations in Ca^{2+} while incubation of ZP with PLCδ4 KO sperm induced only small increases in Ca^{2+}, suggesting that PLCδ4 played a significant role in the ZP-induced acrosome reaction [45, 46, 60].

Data suggests that when modulated by tyrosine phosphorylation, PLCγ1 may be activated in mouse spermatozoa [61–63]. Immunostaining studies have indicated that PLCγ1 is found in close proximity to the sperm head, and that capacitation induces change in this pattern of localisation [61]. ZP treatment leads to increased PLCγ1 activity, although this elevation was prevented by suppressing ZP-induced acrosomal exocytosis [63, 64]. While no direct evidence supports a role for PLCβ in sperm, both PLCβ1 and β3 have been identified in acrosomal regions of mouse sperm, along with Gαq/11 [57, 65]. PLCβ is activated by pertussis toxin-insensitive GTP-binding proteins Gq and G11 in somatic cells, which, along with the observation that progesterone-stimulated DAG formation was not blocked by pertussin toxin, suggests a role for PLCβ in acrosomal exocytosis [63, 66]. Indeed, sperm from PLCβ1 KO mice exhibited lower acrosome reaction rates than their normal counterparts [67]. However, further, in-depth, studies are required to identify the exact roles of these PLC isozymes during fertilisation [63].

There may also be a key role for endogenous oocyte PLCs during oocyte activation [40], and to date, this area has received only scant attention. Oocytes contain significant levels of PLC isoforms, including β, γ, and δ, which may be regulated by the Ca^{2+} oscillations that occur at fertilisation [68]. Reduced levels of oocyte PLCβ1 reduce the amplitude of Ca^{2+} oscillations at oocyte activation, but do not mediate their duration or frequency. Over-expression of PLCβ1 in oocytes prior to

fertilisation did not result in spontaneous Ca^{2+} oscillations, but instead, altered the Ca^{2+} oscillation profile following fertilisation, thus indicating a role for oocyte-derived PLCs in sperm-induced oocyte activation in mammals [68]. PLCβ1 has also been implicated in nuclear translocation following meiotic resumption in mouse oocytes, apparently to perichromatin and interchromatin granules, followed by a subsequent shift to the nucleoplasm [69, 70].

In starfish eggs, a Ca^{2+} rise associated with activation requires the presence of an egg Src family kinase (SFK) which activates PLCγ via an SH2 domain-mediated mechanism involving the endoplasmic reticulum (ER) [71, 72], although the role and concentration of PLCδ, ε, or ζ isoforms in echinoderm eggs are not yet known. It has been shown that PLCβ may be activated by heterotrimeric G protein-coupled receptors, while PLCγ may be activated by receptor and non-receptor protein tyrosine kinases (PTKs), or via translocation to the plasma membrane [72, 73]. However, considerable debate surrounds whether G protein-PLCβ or PTK-PLCγ plays a role during egg activation in other invertebrates, or whether these pathways function synergistically [73].

Coward et al. [74] identified a new PLCδ isoform in sea urchin gametes, termed PLCδsu, although the precise role of this PLC during fertilisation and early embryogenesis currently remains unknown. While a green fluorescent protein-tagged PLCδsu PH domain was observed to localise to the plasma membrane of eggs with increasing concentration at fertilisation, recombinant PLCδsu protein failed to elicit Ca^{2+} signals that are characteristic of fertilisation when injected into mouse oocytes and sea urchin eggs. These observations suggested that PLCδsu may not be directly involved in egg activation, but may, instead, play a role in other downstream extracellular signalling processes. Interestingly, the in vivo expression of PLCδsu cRNA did not result in Ca^{2+} transients in either mouse oocytes or sea urchin eggs. This observation is consistent with the behaviour of recombinant PLCβ1, PLCγ1, PLCγ2, PLCδ1, PLCδ3, and PLCδ4 protein and cRNA, none of which cause Ca^{2+} release in mouse oocytes.

The specific PLC isozyme responsible for oocyte activation in mammals remained undiscovered until Saunders et al. [40], using mouse expressed sequence tag (EST) databases, identified a novel and testis-specific PLC, termed PLCzeta (PLCζ), a ~74 kDa protein which was proven to play a critical role in oocyte activation. Subsequent studies have identified further mammalian orthologues of PLCζ in human, hamster, monkey, and horse sperm [40, 75–80].

16.4 PLCζ, the Mammalian Oocyte Activation Factor

PLCζ exhibits a typical PLC domain structure [40] with characteristic X and Y catalytic domains [81–83], a single C2 domain, and four tandem EF-hand domains. Although other PLCs demonstrate pleckstrin homology (PH) and Src homology (SH) domains, these are absent in PLCζ, making it the smallest known mammalian PLC with a molecular mass of ~70 kDa in humans and ~74 kDa in mice [40].

PLCζ is also highly sensitive to Ca^{2+} [84] with conserved catalytic X and Y domains, and demonstrates a total loss of Ca^{2+}-oscillatory ability following mutagenesis of active site residues [32, 85–89].

The X–Y linker region is poorly conserved amongst PLCζ isoforms, except in the presence of positively charged amino acids, which has prompted speculation that differing motifs in this region may describe species-specific patterns of Ca^{2+} oscillations amongst mammals [32, 33, 90]. Nomikos et al. [86, 87] suggested that charged amino acids may play an important role in the interaction of PLCζ with PIP_2 [86, 91, 92]. Yu et al. [93] further demonstrated that while PLCδ targets PIP_2 at the oolemma, PLCζ appeared to target intracellular membranous PIP_2 on distinct vesicular structures within the mouse oocyte cortex. These studies suggest that specific factors within the ooplasm may be required for PLCζ-mediated Ca^{2+} release, as well as providing evidence for the Ca^{2+} releasing target of PLCζ [16, 94]. The identity of such factors, however, remains a mystery.

Several studies have attempted to determine when PLCζ is first expressed during spermatogenesis. Saunders et al. [40] first detected PLCζ mRNA in mouse spermatids, while more systematic studies during porcine spermatogenesis were able to identify PLCζ mRNA translation in elongating spermatids [76]. Northern blot analyses of testes from post-natal hamsters showed that PLCζ mRNA was present as early as day 17 [78]. It has not yet been possible to investigate the expression levels of PLCζ mRNA within the human testis, largely owing to problems associated with ethics and supply. However, available data clearly shows PLCζ to be a sperm-specific protein and provides convincing evidence to support PLCζ as the oocyte activation factor. For example, recombinant PLCζ RNA can initiate Ca^{2+} oscillations and embryonic development to the blastocyst stage in mice [40, 95]. Immunodepletion of PLCζ suppresses the release of Ca^{2+} when treated sperm extracts are injected into mouse oocytes [40]. Sperm fractionation studies clearly correlated the presence of PLCζ in sperm to their ability to induce Ca^{2+} oscillations [96, 97]. Furthermore, transgenic mice exhibiting disrupted PLCζ expression in the testis have been produced through RNA interference (RNAi) experiments. Sperm from these mice induced Ca^{2+} oscillations that ended prematurely, with a clear reduction in litter size [98]. Further data strongly suggest that PLCζ may be a universal feature of vertebrate oocyte activation. For example, sperm extracts and PLCζ cRNA from one species are readily able to elicit Ca^{2+} release upon microinjection into oocytes from another species [75, 99]. Moreover, non-mammalian testis-specific PLCζ homologues have been identified in the chicken [38], medaka fish [100], and quail [101].

Growing evidence implicates a significant role for PLCζ in some forms of male-factor infertility. Sperm from infertile men which consistently fail IVF and ICSI also fail to induce Ca^{2+} oscillations upon injection into mouse oocytes, or cause abnormal patterns of Ca^{2+} release when compared with those of fertile males [85, 102]. Sperm from such patients also exhibited abnormalities in the level or localisation pattern of PLCζ [85, 102]. The first genetic link between PLCζ and male infertility was reported by Heytens et al. [85] who identified a substitution mutation in an infertile male diagnosed with oocyte activation deficiency (OAD). This case involved a substitution of a histidine with proline within the Y domain of

the catalytic site of the protein, at position 398 of the PLCζ open reading frame ($PLC\zeta^{H398P}$). Microinjection of sperm possessing this mutation, as well as $PLC\zeta^{H398P}$ cRNA, into mouse oocytes failed to induce Ca^{2+} oscillations, or resulted in very atypical patterns of Ca^{2+} release [85]. An equivalent mutation in mouse PLCζ ($PLC\zeta^{H435P}$) also resulted in major structural changes to the PLCζ protein, resulting in functional inactivation [87].

More recently, Kashir et al. [88] identified a second novel point mutation from the same patient in which the H398P mutation was first identified. This second mutation involved a substitution of a histidine with leucine in the catalytic X domain at position 233 of the PLCζ open reading frame ($PLC\zeta^{H233L}$). While microinjection of $PLC\zeta^{H233L}$ cRNA resulted in an abnormal Ca^{2+} release profile and a failure to activate oocytes, this mutation was not as detrimental to calcium release as the H398P mutation. A further intriguing observation was that both the $PLC\zeta^{H398P}$ and $PLC\zeta^{H233L}$ mutations, which are heterozygous in nature, originated from different parental origins: $PLC\zeta^{H398P}$ was paternal in origin, while $PLC\zeta^{H233L}$ was maternal. These findings represented the first description of an autosomal point mutation resulting in male fertility via the maternal lineage [88]. It is possible that mutations in PLCζ may be recessive in nature, requiring mutation on both parental alleles for full infertility to occur [88, 89]. One could also reason that heterozygous mutations in PLCζ may result in cases of sub-fertility. Indeed, Kashir et al. [103] reported that HEK293T cells over-expressing fluorescently tagged $PLC\zeta^{H398P}$ exhibited a lower level of fluorescence compared to HEK293T cells over-expressing fluorescent-PLCζWT, perhaps indicating the manner in which H233L and H398P affected levels of PLCζ in the afflicted patient's sperm.

16.5 Clinical Prospects for Oocyte Activation and PLCζ

ART is responsible for 1.5 % and ~7 % of all births in the UK [16, 104] and developed countries [105], respectively. However, while conventional IVF methodology provides effective treatment for many infertile couples, several conditions such as severe male infertility (accounting for 19–57 % of cases) remain untreatable [106]. In such cases, the implementation of ICSI has proven to be a highly effective approach [5, 16, 107]. However, an estimated 1–5 % of ICSI cycles still fail [7, 105], affecting ~1,000 couples per year in the UK alone [16].

A deficiency in the mechanism of oocyte activation is currently regarded as the principal cause of fertilisation failure following ICSI, accounting for an estimated 40 % of failed cases [3, 5, 108, 109]. A range of post-gamete fusion events are crucial for successful fertilisation [110], and may be attributable to factors determining inherent oocyte quality [16]. Thus, furthering our understanding of sperm and oocyte mechanisms relating to the efficacy of oocyte activation may allow the discovery and establishment of novel therapeutics and diagnostics for clinical use to further improve ART success rates, and provide hope for patients diagnosed with idiopathic (unknown) infertility.

Considering that absent/reduced levels of PLCζ in sperm have been implicated in forms of male infertility where such sperm is repeatedly unable to activate oocytes [85, 102, 111], it is plausible that cases in which PLCζ is absent or severely reduced may be due to destabilising effects caused by mutation in highly conserved regions of PLCζ. However, it is important that future studies examine the effect of mutant PLCζ in testicular germ cells to investigate whether such a trend is reproducible, and to determine whether such loss-of-activity mutations are potentially disruptive to the overall folding of the PLCζ protein, in a manner similar to the somatic cell models used previously.

Cases of oocyte activation failure are currently treatable by exposing fertilising oocytes to chemicals that artificially induce Ca^{2+} release, via methodology referred to as assisted oocyte activation (AOA). Currently, Ca^{2+} ionophores or strontium chloride are the most popular artificial agents [7, 111–113]. Taylor et al. [111] demonstrated high rates of fertilisation, and a successful pregnancy, in PLCζ-deficient patients using a Ca^{2+} ionophore to artificially activate oocytes following ICSI. However, there is significant concern as to how such chemicals may be detrimental to embryo viability and future health due to potential cytotoxic, mutagenic, and teratogenic effects on oocytes and embryos [105]. Thus, a more endogenous therapeutic agent is urgently required as a replacement for current synthetic methodology [5, 16, 107]. Indeed, recent evidence suggests that current AOA protocols may not be beneficial for all patients presenting with oocyte activation-related issues within the infertility clinic, since they enhance fertilisation rates of cases with complete fertilisation, but not cases with low rates of successful fertilisation [114].

Rogers et al. [115] first showed that it was possible to generate blastocysts parthenogenetically following the injection of PLCζ cRNA into human oocytes. In a subsequent study, Yoon et al. [102] demonstrated that the failure of sperm exhibiting abnormal PLCζ localisation/levels to activate an oocyte could be rescued upon co-injection with mouse PLCζ mRNA. Together, these two studies provided significant support, and proof-of-principle for the clinical use of PLCζ as a therapeutic. However, the therapeutic utilisation of PLCζ cRNA is unlikely to be viable, due to issues with uncontrollable transcription, and the potential for reverse transcription within the oocyte [19, 115–117]. Consequently, the synthesis of a pure and active recombinant form of PLCζ has been a key goal over recent years, an aim which seems to have been finally achieved following the publication of three recent studies [107, 118, 119]. However, while encouraging (Fig. 16.1), such findings urgently need to be extrapolated from laboratory models to a clinical setting, before widespread use in fertility clinics can be initiated.

A further significant clinical application of PLCζ may be as a novel prognostic indicator of sperm–oocyte activation capability, and thus fertility [5, 120]. Indeed, previous work utilising immunofluorescent assays has demonstrated a pattern of PLCζ localisation in the sperm head that is consistent with fertile sperm [77, 85], and an abnormal pattern evident in ICSI-failed sperm [85, 102, 103], implicating a correlation between an abnormal localisation pattern of PLCζ and aberrant function/infertility (Fig. 16.2).

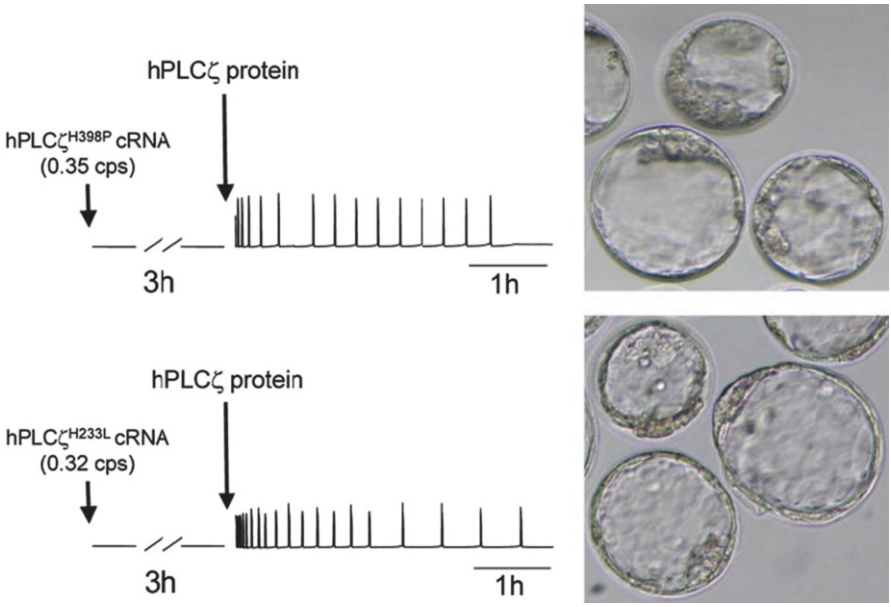

Fig. 16.1 Use of wild type recombinant PLCζ to rescue oocyte activation in mouse oocytes injected with H398P and H223L PLCζ mutants. Traces on the *left* represent levels of Ca^{2+} in unfertilised mouse oocytes following injection of mutant cRNA at time zero followed 3 h later by wild type recombinant PLCζ. Panels on the *right* depict representative blastocysts observed 96 h after injection of the wild type recombinant protein. Reproduced from Nomikos and colleagues [93] with permission

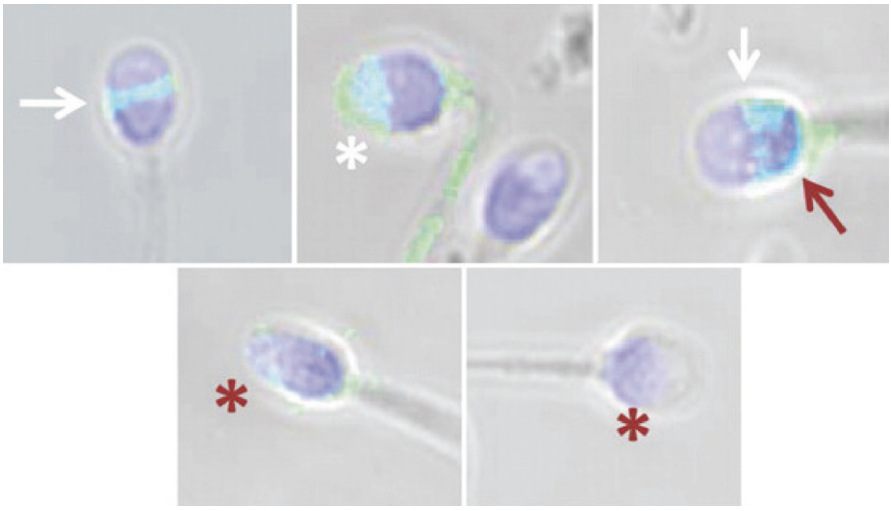

Fig. 16.2 Representative confocal images of PLCζ immunofluorescence in human sperm, showing characteristic localisation patterns. Images represent overlays of nuclei (*blue*), PLCζ (*green*), and DIC images. *White arrow* indicates equatorial localisation; *white asterisk* (*) indicates acrosomal localisation; *red arrows* indicate post-acrosomal localisation; and *red asterisk* indicates reduced/absent PLCζ levels and abnormal localisation in the sperm head. Reproduced and adapted from Kashir et al. [103] with permission

However, there seems to be significant species-specific differences in the concentration and activity of PLCζ delivered into oocytes during fertilisation [79]. PLCζ has been detected in sperm from many species, and is localised to distinct regions in the sperm head, with suggestions of differential functional roles for each population [5, 16, 77, 78, 103, 107]. Three distinct populations of PLCζ have been identified in the human sperm head; acrosomal, equatorial, and post-acrosomal [77, 85, 102, 103, 120, 121], whereas in mouse and bovine sperm, two populations have been identified—acrosomal and post-acrosomal [78, 96, 122]. In equine sperm, PLCζ was reported to be localised to the acrosome, equatorial segment, and head mid-piece, as well as principle piece of the flagellum [80]. In the pig, PLCζ was identified in the post-acrosomal region and the tail [123]. It remains to be ascertained as to whether these different populations have functional ramifications.

However, a particular conundrum was presented by Kashir et al. [121], who did not observe a consistent motif with regard to localisation pattern and total levels of PLCζ in sperm from either fertile males or ICSI-failed males. While it is not yet clear whether a particular pattern of localisation is required, or whether a combination of different populations is required for functional ability, the equatorial and post-acrosomal populations would indeed permit rapid access to the ooplasm following sperm–oocyte fusion [5, 16, 124–126]. However, further evidence is urgently required before any validation of suggestions that there are multiple isoforms of PLCζ in sperm, and whether these perform functions other than oocyte activation [16, 107]. However, the findings of Kashir et al. [121] indicated that sperm from fertile males consistently exhibited higher proportions of sperm exhibiting PLCζ immunofluorescence compared to infertile sperm, suggesting that proportional analysis of PLCζ is likely to serve as a more useful diagnostic test rather than simply comparing mean fluorescence (Fig. 16.3).

Furthermore, preliminary data suggest that proportional PLCζ analysis could also indicate cases of male sub-fertility when compared to conventional clinical semen parameters used in routine clinical practice, perhaps indicating that such analysis could benefit the wider male population and not just cases of ICSI-failure [121]. While further detailed investigation is still required, it is plainly evident that PLCζ may represent a powerful marker with which to investigate sperm health within the clinic. Furthermore, given the apparent species-specific differences between PLCζ (in both gametes and via expression in somatic cell models), this enzyme may also yet represent an invaluable target with which to study the convergent biochemical evolution of enzymes between different species, and how they may adapt to their specific roles.

Given the compelling body of evidence to support the key role played by PLCζ during oocyte activation, it follows that PLCζ may represent a powerful biomarker

Fig. 16.3 (continued) (percentages) exhibiting PLCζ immunofluorescence (**b**) exhibited by sperm from individual control and oocyte activation-deficient (OAD) patients. Fluorescence intensity was quantified in arbitrary units using ImageJ software. *Asterisks* (*, **, ***) denote statistically significant differences ($P \leq 0.05$), whereas *hash marks* (#, ##, ###, #′, ##′) denote statistically insignificant differences. Combinations of marks indicate comparisons between different groups (e.g. # indicates comparison with one group and ## indicates comparison with another). Data are shown as mean ± SEM. Figure reproduced and adapted from Kashir et al. [121] with permission

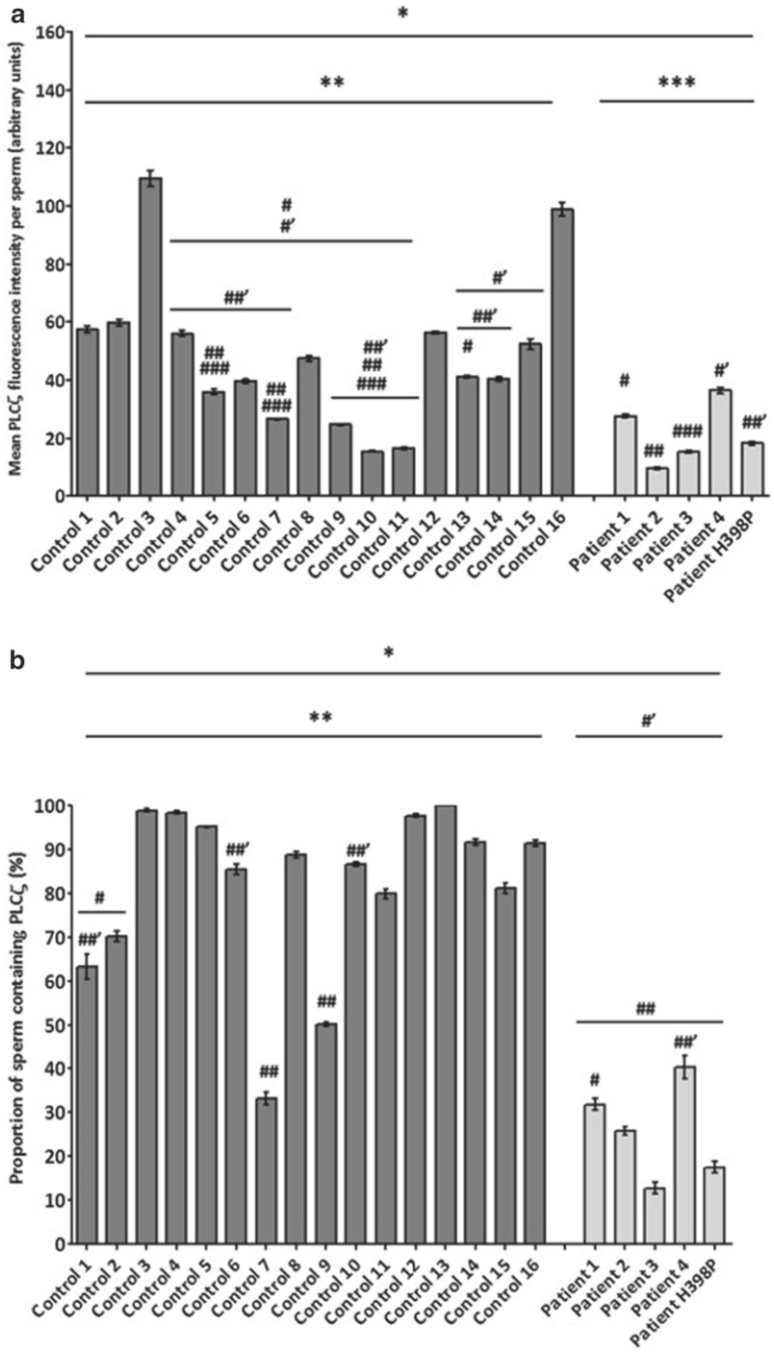

Fig. 16.3 Histograms illustrating complexity in utilising quantitative immunofluorescent analysis as a prognostic measure following extensive clinical analysis. Histograms illustrate mean relative total phospholipase C zeta (PLCζ) fluorescence levels (**a**) and mean proportions of sperm

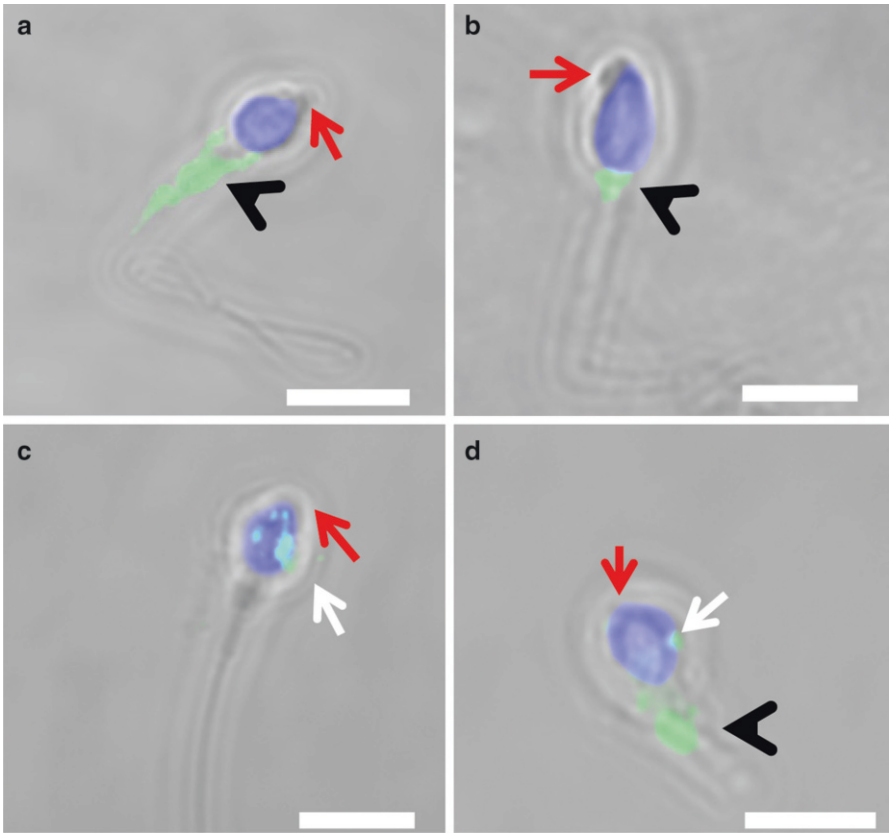

Fig. 16.4 Representative confocal images of PLCζ immunofluorescence in motile sperm organelle morphology evaluation (MSOME)-selected globozoospermic sperm exhibiting an acrosomal bud (*red arrow*). PLCζ (*green*) was localised to the mid-piece (*black arrowheads*, **a**, **b**), or as a punctate pattern in the sperm head (*white arrows*, **c**), or in combination (**d**). Images were captured at ×63 optical magnification, and represent overlays of PLCζ (*green*), nuclei (*blue*), and DIC images. *White scale bar* represents 5 μm. The MSOME procedure selected a higher proportion of globozoospermic sperm exhibiting PLCζ immunofluorescence, as well as selected sperm with higher total levels of PLCζ. Image reproduced and adapted from Kashir et al. [128] with permission

with which to examine the functional competency of sperm [127]. Indeed, Kashir et al. [120] utilised PLCζ as a biomarker to examine the effects that routine clinical cryopreservation may exert upon sperm from fertile males. These studies indicated that cryopreservation led to a reduction in the total level of PLCζ. In another study, motile sperm organelle morphology evaluation (MSOME), a new sperm selection technique that relies on the analysis of human sperm in high magnification prior to a modified version of ICSI (IMSI; intracytoplasmic morphologically selected sperm injection), was shown to select sperm from globozoospermic patients with higher total levels of PLCζ, as well as selecting a higher proportion of sperm exhibiting the presence of PLCζ [128] (Fig. 16.4). Such experiments indicate the potential applications of PLCζ in assisting the further enhancement of current ART methodology to improve current clinical treatment protocols.

Indeed, Kashir et al. [120] indicated that cryopreservation, a common technique utilised for the preservation of fertility in patients undergoing fertility treatment as well as radio/chemotherapy or surgery [129], had a significant detrimental effect upon the levels of PLCζ compared with fresh sperm. A further finding of this particular study was that density gradient washing (DGW; a centrifugation method used to isolate the best quality sperm based on motility parameters) led to a significant increase in the proportion of sperm exhibiting PLCζ immunofluorescence in fertile male donors [120], thus increasing the likelihood of successful activation. Furthermore, Nakai et al. [123] showed that the pretreatment of pig sperm reduced oocyte-activating ability via significant reductions in the levels of PLCζ compared with untreated sperm. Given that low PLCζ concentrations in sperm are linked with infertility, these studies further support the notion that PLCζ represents a highly beneficial biomarker for ART [16, 107].

16.6 Conclusions

Collectively, it is clear that PLCζ imparts significant effect upon the fertility status of mammals. While there is still controversy surrounding the identity of the sperm factor (see [5, 16, 107] for detailed reviews), it is clear that PLCζ plays a key role in determining not only male fertility but also potentially the efficacy of embryogenesis by influencing the precise nature of Ca^{2+} oscillations during oocyte activation and beyond. It thus represents an extremely powerful therapeutic and prognostic tool to utilise in the infertility clinic. Given the fundamental role played by PLCζ in oocyte activation, it also follows that this protein could represent a potential male contraceptive target. While non-hormonal in its mechanism of action, an anti-PLCζ contraceptive would represent a non-barrier method of contraception which could limit its appeal to both pharmaceutical companies and the end user. Future elucidation of the three-dimensional structure of PLCζ would permit the selection of potential inhibitors, but of course care must be taken to ensure that such inhibitors act only upon PLCζ and do not cause deleterious side effects by interfering with cellular signalling processes involved with other metabolic processes. However, despite many exciting advances over the last few years, both from molecular and clinical perspectives, there is still a significant body of work to be undertaken before the clinical application of this fundamental protein can be realised.

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Chapter 17
Phospholipase C Signaling in Heart Disease

Elizabeth A. Woodcock

Abstract Phospholipase C (PLC) expression and activity have repeatedly been reported to be elevated in cardiomyocytes under pathological conditions, including ischemia/reperfusion, hypertrophy, and chamber dilatation. In recent studies the subtypes of PLC involved have been identified, paving the way for studies of the mechanisms by which PLC may be activated under pathological conditions and how this may contribute to disease progression. PLC subtypes are localized by subtype- and tissue-specific binding to scaffolding proteins providing the possibility of developing cardiac-specific therapies based on inhibition of the localization of particular PLC subtypes in cardiomyocytes.

Keywords Ischemia/reperfusion • Hypertrophy • Dilatation • Scaffolding protein

17.1 Introduction

Phosphatidylinositol-specific phospholipases C (PLCs) are enzymes that cleave the plasma membrane phospholipid, phosphatidylinositol(4,5)*bis*phosphate (PIP_2), to generate inositol(1,4,5)*tris*phosphate ($Ins(1,4,5)P_3$), a Ca^{2+} releasing intracellular messenger, and *sn*-1,2-diacylglyerol (DAG), an activator of conventional subtypes of protein kinase C. The substrate lipid and the two products all have critical roles in regulating cellular responses and therefore PLCs are of central importance in the functioning of all cell types. Furthermore, perturbations in PLC activity may contribute substantially to disease phenotypes in a range of different tissues. As expected from a family of enzymes with such a central role in signaling, PLCs can be

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_17,

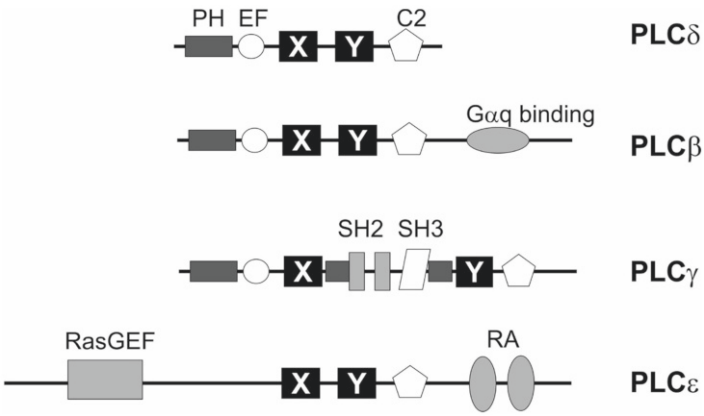

Fig. 17.1 Diagram showing the relationship between the different classes of the PLC family of proteins, emphasizing the structural motifs present

regulated in many different ways. PLCs are classified into six major classes (β, γ, δ, ε, ν, ζ), each of which includes multiple subtypes and splice variants (Fig. 17.1) [1]. PLCβ family members (PLCβ1-4) respond to G protein subunits activated downstream of seven transmembrane spanning receptors (also called G protein-coupled receptors, GPCR) [2]. PLCβ1 and PLCβ3 are expressed in cardiomyocytes, but PLCβ2 is not. PLCβ1 exists as two splice variants that differ only in their extreme C-terminal sequences, PLCβ1a (MW 150kD) and PLCβ1b (MW 140kDa, Fig. 17.2). Whilst both splice variants are expressed in neonatal rat cardiomyocytes [3], only PLCβ1b is expressed in adult human, rat, and mouse heart [4]. PLCγ members (PLCγ1 and PLCγ2) translocate to the plasma membrane subsequent to the activation of receptor tyrosine kinases, following stimulation with the appropriate growth factor [5]. Hearts express primarily PLCγ1 [6]. PLCδ subtypes are more sensitive to activation by Ca^{2+} than other subtypes, and hearts express PLCδ1, but the physiological importance of this has not been firmly established [7, 8]. PLCε regulation is complex involving a variety of activators including monomeric G proteins of the Ras family, as well as heterotrimeric G proteins of the $G_{12/13}$ family and Gβγ [9]. Thus receptor activation can lead to PLCε activation by a variety of signaling mechanisms, often well downstream of receptor activation. There is only a single PLCε gene product, but this is expressed as two N-terminal splice variants [10]. Other PLC subtypes are not expressed in heart and will not be considered further.

17.2 The Regulation of PLC Activity in Heart

Early studies showed that activation of α_1-adrenergic receptors [11], M2 muscarinc cholinergic receptors [12] or endothelin receptors [13] resulted in generation of Ins(1,4,5)P_3 and its metabolites. Subsequently, activation via purinergic receptors

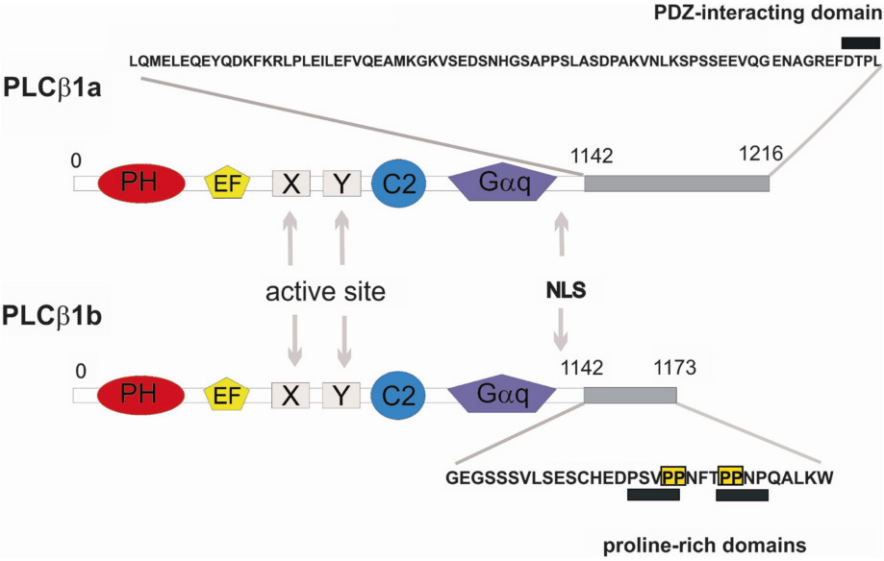

Fig. 17.2 The splice variants of PLCβ1. Diagram showing the structures of PLCβ1a and PLCβ1b outlining the sequence differences in the C-terminal regions of the proteins. Proline-rich domains and PDZ-interacting domains are indicated. NLS is nuclear localization sequence

was reported [3]. All of these factors bind receptors coupled to Gq and would therefore be expected to activate PLCβ family members [14]. There have also been reports of activation via growth factor receptors that would be expected to activate PLCγ subtypes [15]. More recently the novel PLCε subtype has been identified in cardiomyocytes [16] and shown to be activated via thrombin (protease activated receptor 1, PAR1) and sphingosine1-phosphate (S1P) receptors [17]. In addition to activation by hormones and neurotransmitters, PLC in intact hearts and in cardiomyocytes in culture responds to acute stretch [18–21].

17.3 Localization of PLC Subtypes in Heart

To be active PLCs must be located close to their substrate PIP_2, localized primarily or exclusively at the sarcolemma. It is now well recognized that PLC subtypes are specifically localized to particular membrane regions by binding scaffolding proteins. These scaffolds are selective for particular PLC subtypes and, in some cases, are also tissue specific.

In the case of the PLCβ family, such scaffolding interactions generally involve a C-terminal PDZ-interacting domain, present in all PLCβ1 subtypes except PLCβ1b. These PDZ-interacting domains associate with particular PDZ (postsynaptic density protein, Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein domain) proteins. PLCβ3, for instance, binds to cell polarity proteins,

Par3 and Par6, in renal tubular epithelial cells, SH3 domain and ankyrin repeat protein 2 (Shank2) at glutamatergic synapses in neuronal tissues [22], and the sodium hydrogen exchange regulatory protein 2 (NHERF2) in Cos7 cells [23]. All of these interactions require PDZ domain interactions via the C-terminal sequence, NTQL. PLCβ3 is not localized to the sarcolemma, at least in neonatal rat cardiomyocytes [24], suggesting that suitable scaffolding proteins are not expressed, or are not associated with the sarcolemma. The C-terminal PDZ-interacting domain of PLCβ1a (DTPL) binds selectively to the first PDZ domain (nearest the N-terminal) of the scaffolding protein, sodium hydrogen exchange regulatory factor 1 (NHERF1), but not NHERF2, in HEK293 cells [25]. The first PDZ domain of NHERF1 also binds PLCβ2 via the sequence ESRL [26, 27]. NHERF1 is not expressed in heart providing an explanation for the cytoplasmic localization of PLCβ1a when expressed in cardiomyocytes [24]. As noted above, PLCβ1b does not have a C-terminal PDZ-interacting domain and so must target to membranes by a different mechanism from that used by other PLCβ subtypes. The presence of two proline-rich domains at the C-terminal end points to targeting by an SH3 domain- [28] or a WW domain-containing protein [29]. In cardiomyocytes the scaffolding protein for PLCβ1b was identified as Shank3. Shank3 is a high MW protein with multiple protein interaction motifs. Importantly, Shank3 has a type 1 SH3 domain suitable for binding the PPNP (1165–1168 in the human PLCβ1b sequence) proline-rich sequence in the extreme C-terminal region of PLCβ1b [30]. In addition to its SH3 domain, Shank3 has an N-terminal ankyrin-rich repeat sequence that binds α-fodrin, a PDZ domain, a long proline-rich sequence that binds the Homer family of proteins and cortactin, and finally a C-terminal sterile alpha motif (SAM) that facilitates dimerization. Association with fodrin likely localizes Shank3 close to the sarcolemma. Thus, association with Shank3 makes PLCβ1b part of a multi-protein system that may be critical for downstream signaling and cellular responses (Fig. 17.3). Importantly, Shank3 is expressed in only a limited number of tissues, primarily heart and glutamatergic neurons [30], and thus the binding of PLCβ1b (also with limited tissue distribution) to Shank3 provides a possible heart-specific drug target.

PLCδ1 is expressed in heart [4], although no function has unequivocally been ascribed. PLCδ subtypes have a high affinity PH domain that shows high selectivity for PIP_2 and this is sufficient to localize these to the sarcolemma [31].

PLCγ family members are activated following phosphorylation by receptor tyrosine kinases and this facilitates binding to SH2 domains present in growth factor receptors localizing these PLCs close to the plasma membrane and their substrate PIP_2 [5]. As with PLCβ subtypes, localization and activation of PLCγ members may also involve binding to other signaling proteins. PLCγ subtypes have been reported to bind to sodium-hydrogen exchanger 3 (NHE3), a plasma membrane-localized ion exchanger, and regulate its activity [32]. Interestingly, PLCγ1 has been shown to interact directly with canonical transient receptor 3 (TrpC3) to control its cell surface expression [33]. TrpC3 is implicated as contributing to pathological cardiomyocyte hypertrophy [34]; however, PLCγ1 has not been implicated in this response.

As outlined earlier, PLCε is structurally more complex that other PLCs and, as a consequence of this, its regulation also is multifactorial. Like other PLC subtypes,

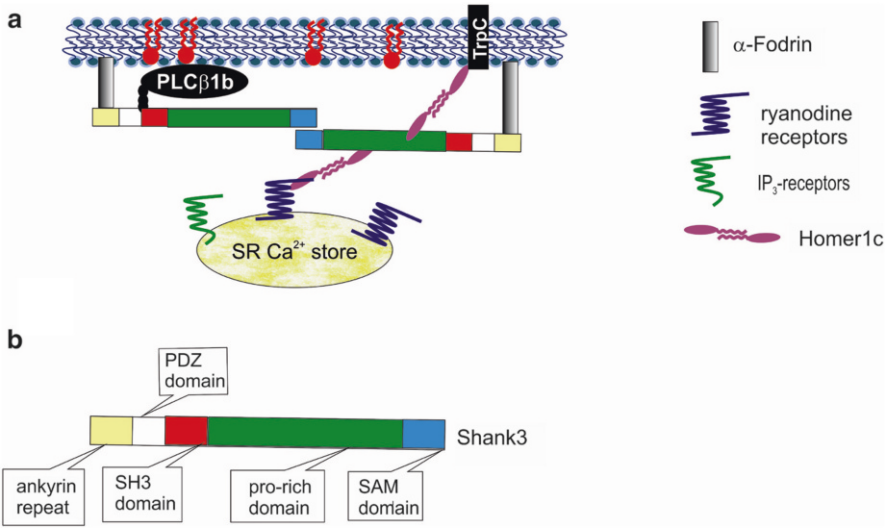

Fig. 17.3 (**a**) PLCβ1b binding to a Shank3 complex localized below the sarcolemmal membrane. Shank3 forms homodimers via its C-terminal SAM domains and is bound to α-fodrin via ank repeats in the N-terminal sequence. Dimeric Homer proteins cross-link Shank3 to TrpC channels and to intracellular Ca^{2+} channels. (**b**) Diagram showing domain structure of Shank3

PLCε binds to a scaffolding protein via sequences in its C-terminal region, in this case its (Ras association 1) RA1 domain. The RA1 domain of PLCε binds to the first spectrin repeat domain of muscle A-kinase-anchoring protein β (mAKAPβ) localizing this PLC subtype principally to the nuclear envelope in cardiomyocytes [35]. mAKAPβ, like Shank and NHERF proteins, is a multidomain scaffold and thus PLCε probably functions as part of a large protein complex.

17.4 Pathological Responses in the Heart

The primary function of the heart is to supply blood to all tissues of the body at sufficient level to optimize their function. The pump function of the heart can be compromised by a loss of contractile function of the muscle that reduces cardiac output resulting in failure to adequately supply blood to the body, a condition known as heart failure. Ineffective pumping can also be caused by a loss in organization of the contraction of the individual muscle cells, a condition known as arrhythmia. Heart failure and arrhythmia often occur together, each worsens the other and both can result from chronic hypertrophic growth of the myocardium. Because of this, there is an interest in developing therapies targeted at reducing pathological hypertrophic cardiomyocyte growth, improving contractile function (inotropic agents), or reducing arrhythmia (anti-arrhythmic agents). Currently used pharmaceuticals commonly

target cell surface receptors or ion channels, their ligands, or the downstream signaling pathways, including drugs that reduce the generation or the receptor binding of angiotensin II, blockers of β-adrenergic receptors, Ca^{2+} channel blockers, and agents that reduce the metabolism of cAMP [36]. There is clearly a need for the development of better tolerated therapies, particularly if they can be made relatively cardiac-specific.

17.5 How Might PLC Activation Contribute to Pathology?

PLC enzymes hydrolyze the sarcolemmal phospholipid, PIP_2, to generate Ins(1,4,5)P_3 that can release Ca^{2+} from intracellular stores [37] and *sn*-1,2-diacylglycerol (DAG), an activator of conventional PKC subtypes [38], PKD [39] and some TrpC channels [40]. Each of these factors, individually and in concert, can have critical effects on cellular responses.

17.5.1 Ins(1,4,5)P_3

Ins(1,4,5)P_3 binds and activates IP_3-R localized on intracellular Ca^{2+} stores [41]. The expression level of IP_3-R in cardiomyocytes is low compared with that in most other tissues and compared with the highly expressed ryanodine receptors [42] that are primarily responsible for the intracellular Ca^{2+} cycling that regulates the heart beat. Furthermore, IP_3-R in ventricular myocytes are localized around the nuclear membrane [43], seemingly distal from the site of generation of Ins(1,4,5)P_3 following activation of cell surface receptors. These nuclear membrane-localized IP_3-R(2) may supply the localized Ca^{2+} signals required to activate calmodulin-activated protein kinases (CaMKII) involved in transcriptional regulation [44]. Ins(1,4,5)P_3 has been suggested to be involved in arrhythmogenesis [45–47] and in hypertrophy [48], although direct evidence for either of these is lacking.

17.5.2 DAG

The other product generated by PLC, DAG, has a complex spectrum of activities, all of which could contribute to pathology. DAG was initially discovered as an activator of PKC [38], particularly the “conventional” PKC subtypes (PKC α, β, γ, δ ε, η, θ) [49]. DAG also activates some TrpC channels [50] and protein kinase D directly [39], in addition to actions dependent on PKC. In contrast to the controversy surrounding the contribution of Ins(1,4,5)P_3 and IP_3-R to cardiac physiology/pathophysiology, DAG and the PKC family are well accepted as a contributor to cardiac regulation. The contribution of PKC to regulation in the heart is complex,

varying with the PKC subtype, the stage of development, and the mechanism of activation. PKCα activation serves to suppress contractility [51], but can have profound pathological consequences when the regulatory domain that limits catalytic activity is removed by calpain cleavage under conditions of ischemia and reperfusion [52]. PKCβ subtypes have been shown to be involved in diabetic cardiomyopathy [53]. PKCδ has been considered an important contributor to cardiac pathology and cardiac remodeling, apparently related to activation of mitochondrial apoptotic responses [54]. PKCε primarily has a protective role in heart and is a component of preconditioning mechanism that reduces subsequent ischemic damage, discussed in more detail subsequently [55]. A recent review provides detailed information about PKC contribution to cardiac signaling under physiological and pathological conditions [56].

17.5.3 PIP_2

The process of PLC activation depletes PIP_2 as it generates $Ins(1,4,5)P_3$ and DAG. Reductions in PIP_2 are often localized and transient with the PIP_2 being replaced immediately, presumably by phosphorylation of PIP [57, 58]. However, PLC-induced localized changes in PIP_2 regulate ion channels and exchangers that are critical in maintaining heart rhythm [59], for a review see [40]. PIP_2 is also critical for maintaining the cytoskeleton via its association with actin-binding proteins [60] and PIP_2 is essential for localizing proteins to the plasma membrane [61].

17.6 PLC Involvement in Ischemia and Post-ischemia Reperfusion

Cardiac ischemia occurs when there is an interruption in the blood supply to the heart, depriving it of oxygen and nutrients, a condition associated with arrhythmia and cardiomyocyte death. The reintroduction of flow, reperfusion, also is associated with arrhythmia, cell death and contractile dysfunction. A number of studies have reported increased activity of PLC in animal models of acute cardiac ischemia [62–64]. Substantially increased PLC activation has been reported in early post-ischemic reperfusion following a brief period of ischemia [65–67], and inhibition of PLC under these conditions successfully prevents reperfusion arrhythmias [45, 46, 68] in addition to improving functional recovery [69]. However, the subtypes of PLC activated by ischemia/reperfusion are unknown as are the mechanisms leading to the heightened PLC response.

Increased expression of PLCβ, as well as of activating G proteins, has been reported in border zone and remote myocardium following myocardial infarction in humans, suggesting the likelihood of enhanced PLC activation [70] and pointing to a possible involvement in the heart's responses to chronic ischemia.

Other studies reported that protection from chronic ischemic damage by ethanol is mediated by elevation of PLC activity, but the subtype of PLC was not identified [71].

Defining contributions of PLC, its substrate and products, to ischemic or reperfusion responses is confounded by the likelihood that one or other of these might contribute to preconditioning, a phenomenon that can provide protection from arrhythmia and infarction following an ischemic insult [72]. Preconditioning involves subjecting hearts to brief periods of ischemia and reperfusion prior to the main ischemia/reperfusion procedure. This pretreatment procedure is sufficient to limit PLC activation in early post-ischemic reperfusion [73]. Preconditioning protection can be mimicked by activation of some of the PKC subtypes that are activated downstream of PLC, and to further complicate the situation, different PKC subtypes can have opposing effects on preconditioning [74]. Overexpression of either subtype of α_1-adrenergic receptors (α_{1A}- or α_{1B}-) results in heightened PLC responses to endogenous or exogenous norepinephrine. However, whilst PLC activity in these overexpressing transgenic strains was heightened in normoxia, the exaggerated response during early reperfusion was eliminated, along with the reperfusion arrhythmias [75, 76]. Presumably, this apparent contradiction is related to activation of preconditioning pathways possibly initiated by PKC activation. Taken together, these studies imply that factors downstream of PLC, most likely PKC-initiated responses, effectively precondition the myocardium, and that preconditioning reduces PLC activation.

17.7 PLC in Acute and Chronic Dilatation of the Myocardium

The myocardium responds to acute stretch by increasing cardiac output in order to manage the increase in blood volume. Thus, acute stretch results in increased rate and force of contraction. Acute stretch of the right atrium causes substantial release of atrial natriuretic peptide, possibly to facilitate a lowering of blood volume [77]. As noted earlier, in addition to activation by ligand receptor binding, PLC in heart can be activated acutely by stretch [18–21]. In perfused rat heart preparations, right atrial stretch caused PLC activation that correlated with release of atrial natriuretic peptide [78]. Stretch activation of PLC requires Gq and may involve angiotensin II receptors (AT1) acting in a ligand-independent manner [21]. The involvement of Gq and AT1 receptors implicates PLCβ subtypes as major contributors to the response to acute stretch.

Chronically increased wall tension results in chamber dilatation and wall thinning that eventually limit contractile performance and these are the hallmarks of dilated cardiomyopathies. Dilatation of the atria is observed in patients with valve diseases and is also seen in association with ventricular failure. Interestingly, substantially heightened PLC activity was observed in the dilated atria of patients suffering from valvular heart disease, as well as in atria from a mouse model of dilated cardiomyopathy that has severe atrial enlargement together with conduction

block and a sensitivity to atrial fibrillation [4, 79]. Furthermore, in both humans and mice, PLC activity correlated with atrial volume, suggesting that PLC activation was either a cause or a consequence of dilatation. Dilated atrial tissue from both humans and mice showed increased expression of only one PLC subtype, PLCβ1b, providing suggestive evidence that PLCβ1b is selectively involved in the response to chronic dilatation. There were no changes in expression of PLCβ3, PLCδ1, or PLCγ1 associated with atrial dilatation [4]. PLCε was not measured in these studies and a role for this subtype, therefore, cannot be discounted. PLCβ1a, although expressed in neonatal rat cardiomyocytes, was not expressed at measurable levels in adult human myocardium. The two splice variants of PLCβ1, PLCβ1a and PLCβ1b, differ only in their extreme C-terminal sequences as shown in Fig. 17.2. Whilst the catalytic domains and the Gαq-binding regions are identical, the differences in the C-terminal sequences would be expected to result in different localization, and consequently different activities.

Overexpression of a constitutively active Gαq is sufficient to cause severe chamber enlargement together with heightened PLC activity [80], but there are conflicting opinions about the role of PLC in promoting atrial dilatation in these Gαq-overexpressing models. Overexpression of a Gαq mutant with reduced ability to activate PLCβ, unlike the wild-type, did not result in chamber dilatation [81], providing powerful evidence for a requirement for PLC activity for the pathological responses initiated by Gq. Other studies showed that atrial remodeling in Gαq-overexpressing mice was reversed by co-expression of diacylglycerol (DAG) kinase ζ, an enzyme that depletes DAG, one of the immediate products of PLC activation [82], supporting a critical role for PLC and its immediate product, DAG, in atrial dilatation. However, in contrast to these findings, studies comparing two different Gαq-expressing transgenic lines reported that the degree of dilatation did not correlate with the extent of PLC activation [83]. These apparent discrepancies might be accounted for if there was a maximal level of PLC activation, above which further increases produced no greater effect on chamber dilatation.

At the cellular level, chamber dilatation and wall thinning are thought to involve loss of functional myocytes by apoptotic and non-apoptotic mechanisms. The ability of activated mutants of Gαq to induce apoptosis in cardiomyocytes is well documented [84], and more recently overexpression of wild-type PLCβ1b has also been shown to cause cardiomyocyte apoptosis [85]. Thus, heightened PLCβ1b activity could contribute to a dilated phenotype by promoting apoptotic death of cardiomyocytes. In summary, there is evidence for an involvement of PLC, and in particular PLCβ1b, in responses to acute and chronic dilatation of the myocardium, but the mechanisms involved remain to be established.

17.8 PLC Involvement in Cardiac Hypertrophy

Early studies using isolated cardiomyocytes or genetically modified mice pointed to a role for Gq family members in pathological growth and remodeling of the heart. Overexpression of Gαq, either the wild-type [86] or a constitutively active mutant [80],

was sufficient to cause cardiomyocyte hypertrophy, and when expressed in vivo, Gαq promoted hypertrophy and heart failure [84]. More importantly, Gq inhibitors expressed in the heart were found to substantially reduce hypertrophic growth in response to the clinically relevant challenges of pressure or volume overload [87–89]. The apparent central role of Gq in these pathological responses suggests mediation by PLCβ subtypes, as these are the best understood effectors of Gq [90]. However, members of the Rho family of monomeric G proteins are activated downstream of Gq [91] and these may also contribute to hypertrophic responses [92].

Of the PLCβ family, only PLCβ1b causes hypertrophy when overexpressed in cardiomyocytes, and this selectivity depends on its sarcolemmal localization facilitated by selective association of the splice variant-specific C-terminal sequence with the scaffolding protein Shank3 [24, 85]. Furthermore, inhibition of PLCβ1b binding to Shank3 prevented hypertrophy in response to Gq activation [85], suggesting that the sarcolemmal targeting of PLCβ1b might provide a novel target to limit hypertrophy and chamber dilatation. Both PLCβ1b and Shank3 have a limited tissue distribution opening up the possibility of cardiac-specific therapy. In addition to cardiomyocytes, Shank3 is expressed primarily in postsynaptic density fractions from central glutamatergic neurons [30], where PLCβ1b is not expressed. In neurons, Shank3 acts as a scaffold facilitating interactions between receptors and early signaling proteins [93]. In heart, Shank3 appears to function similarly, binding fodrin [94] and Homer1c [95] in addition to its association with the C-terminal sequence of PLCβ1b. Homer1c forms homodimers that can cross-link Shank3 to form large molecular scaffolds [96]. Homers promote crosstalk between intracellular Ca^{2+} channels, IP_3-R and ryanodine receptors, and cell surface canonical transient receptor potential channels (TrpC) and thus are regulators of local Ca^{2+} responses [97]. Expression of PLCβ1b in cardiomyocytes results in increased expression of Homer1c as well as its translocation to the Shank3/PLCβ1b complex [95]. The mechanisms involved in these responses are unknown, but they appear to be critical for the hypertrophic response.

The possibility that PLCε was involved in cardiac pathology was first suggested when elevated expression was reported in failed human left ventricle [16]. This idea was supported by studies showing that PLCε−/− mice exhibited exacerbated hypertrophic responses leading to the idea that PLCε, in contrast to PLCβ1b, was protective to the myocardium by inhibiting hypertrophic signaling. However, subsequent studies in isolated cardiomyocytes have questioned this conclusion. These studies found that treatment with si-RNA to knockdown PLCε inhibited hypertrophy in response to endothelin or α_1-adrenergic agonists [35], implying an involvement in Gq-initiated hypertrophy that other studies have shown involves PLCβ1b [85]. Importantly, PLC activity was absolutely required for this contribution of PLCε to hypertrophy, an important finding given the multiple functions of this complex PLC subtype. In cardiomyocytes, PLCε is localized onto the nuclear membrane by association with muscle A-kinase-activating protein (mAKAPβ, AKAP5) [35]. Such localization is suggestive of a role downstream of early signaling responses, such as initiated by PLCβ1b. In agreement with this, knockdown of PLCε inhibited hypertrophy in response to multiple stimuli, including both Gq hypertrophy that models

pathological hypertrophy and hypertrophy caused by IGF treatment, considered a model of physiological hypertrophy that is independent of Gq [35]. This contrasts to PLCβ1b, where inhibition selectively prevented Gq-mediated hypertrophy [85]. There is clearly substantial evidence for an involvement of PLC in hypertrophy of the myocardium, with current data supporting roles for PLCβ1b and PLCε, most likely at different stages in the signaling response.

17.9 Conclusions

Under physiological conditions the functioning of the heart is regulated primarily by pathways that are independent of PLC activation. However, PLC expression and activity have been shown to increase under a range of pathological conditions, including ischemia/reperfusion, hypertrophy, and dilatation and it is likely that PLC contribute to the progression of these diseases.

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Chapter 18
Activation of Phospholipase C in Cardiac Hypertrophy

Paramjit S. Tappia and Naranjan S. Dhalla

Abstract Norepinephrine is considered to mediate the cardiomyocyte hypertrophic response through α_1-adrenoceptor activation of phospholipase C (PLC). However, the regulation of specific PLC isozyme gene and protein expression as well as activities in normal and hypertrophied myocardium is not completely defined. In this chapter, we provide an overview of the role of PLC-mediated signal transduction pathways in cardiac hypertrophy. We also identify some of the mechanisms that might be involved in the regulation of PLC isozyme gene expression, protein abundance, and activities. While PLC has a key role in cardiomyocyte hypertrophy, the evidence provided here suggests that PLC activities regulate their own gene expression that perpetuates the hypertrophic signal to produce a rapid progression of cardiac hypertrophy and ultimate transition to heart failure.

Keywords Phospholipase C • Adult cardiomyocytes • PLC-mediated signal transduction • Regulation of PLC gene expression • Cardiac hypertrophy • Norepinephrine • α_1-Adrenoceptors, angiotensin II, and endothelin-1

18.1 Introduction

Myocardial hypertrophy is an adaptive response to hormonal and mechanical stimuli that increase cardiac work [1]. Initially, elevated cardiac mass confers ventricular hyperactivity to compensate for the increased stress on the myocardium;

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_18,

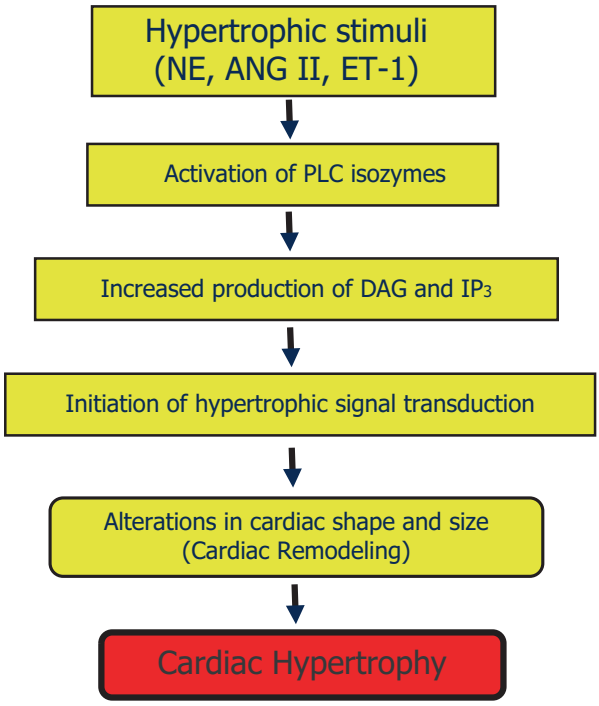

Fig. 18.1 Activation of phospholipase C isozymes in response to different stimuli leading to cardiac hypertrophy. *NE* norepinephrine, *Ang II* angiotensin II, *ET-1* endothelin-1, *PLC* phospholipase C, *DAG* diacylglycerol, IP_3 inositol trisphosphate

however, prolonged stress eventually results in congestive heart failure (CHF). Cardiac hypertrophy is characterized by an increase in cell size in the absence of cell division, an increase in protein content per individual cell [2], and a re-expression of the so-called fetal genes, i.e., α-skeletal actin, α-smooth muscle actin, β-myosin heavy chain, and atrial natriuretic factor (ANF). However, exercise-induced cardiac hypertrophy and thyroid hormone-induced cardiac hypertrophy are not accompanied by re-expression of the fetal gene program. A number of different stimuli [3–9], including norepinephrine (NE), angiotensin II (Ang II), and endothelin-1 (ET-1) [10–15], trigger cardiac hypertrophy through the activation of phospholipase C (PLC) (Fig. 18.1). Furthermore, each of these different stimuli induces a distinct phenotype as characterized by gene expression pattern and cellular morphology. This chapter focuses on the role of PLC in cardiac hypertrophy and identifies some of the mechanisms involved in the regulation of PLC isozyme gene expression.

18.2 Myocardial Phospholipase C Isozymes

PLC isozymes play a central role in activating different intracellular signal transduction pathways [16–18], particularly during early key events in the regulation of various cell functions [19]. A number of different agonists, including NE, Ang II,

and ET-1, bind to their respective G-protein (Gq subfamily)-coupled receptors and activate PLC [19–29]. The PLC isozyme family consists of six subfamilies: PLC β, γ, δ, ε, ζ, and η [19, 30–34]; however, PLC β, δ, γ, and ε isozymes appear to be the predominant forms expressed in the heart [35–37]. Although these isozymes display differences in structure and activating mechanisms, Ca^{2+} is required for their activity, but their Ca^{2+}-sensitivity varies and thus it has been argued that the activation of PLC isozymes is both Ca^{2+}-dependent and Ca^{2+}-independent [19, 30, 38]. However the distinct role of each of the PLC isozymes in the cardiomyocyte hypertrophic response and the extent of their overlap have yet to be completely defined. PLC isozymes are invariably present in the cytosolic compartment of the cardiomyocyte and they migrate to the membrane where their lipid substrates reside [25]. The activation of PLC results in the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) to produce diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG acts in conjunction with phosphatidylserine and in some cases Ca^{2+} to activate protein kinase C (PKC) isoforms containing a cysteine-rich C-1 domain [39]. PKC family members that are activated by DAG modulate contractile properties and promote cell growth and survival [40–44].

On the other hand, the functional significance of IP_3 in the heart has been debated [45, 46]. The IP_3 receptors (IP_3Rs) are intimately associated with intracellular Ca^{2+}-release channels [47]. However, relative to ryanodine receptor (RyR), which is the main source of Ca^{2+} in the excitation–contraction coupling (ECC), low levels of IP_3R are present in the cardiomyocyte [48]. It has been suggested that IP_3R may contribute to altered ECC and arrhythmogenesis in the atria [49, 50]. The type 2 IP_3R, which is the predominant subtype in cardiomyocytes, is located mainly in the nuclear envelope in ventricular cardiomyocytes, but its role in the heart is poorly understood. In this regard, ET-1 has been reported to elicit local nuclear envelope Ca^{2+}-release via type 2 IP_3R [47]. Furthermore, local Ca^{2+}-release results in the activation of transcription, that implicates a role of PLC-derived IP_3 in the regulation of gene expression in hypertrophy; the so-called excitation–transcription coupling [47]. The exchange protein directly activated by cAMP (Epac) is emerging as a new regulator of cardiac pathophysiology [51]. Epac can induce SR Ca^{2+}-release in a PLC- and Ca^{2+}/calmodulin kinase II (CaMKII)-dependent manner [52]. Furthermore, Epac has been suggested to have a role in activating the excitation–transcription coupling and induce cardiomyocyte hypertrophy [51, 52] through the activation of a signal transduction pathway involving PLC.

18.3 Role of Phospholipase C in Cardiac Hypertrophy

We have previously reported that the NE induced increases in ANF gene expression and protein synthesis that can be attenuated by U73122, a PLC inhibitor, as well as by prazosin, an α_1-adrenoceptor (α_1-AR) blocker, in the isolated adult left ventricular (LV) cardiomyocytes [20]. Interestingly, overexpression of the myocardial-specific α_{1A}-AR results in a small increase in the NE-stimulated PLC activity

without affecting the basal PLC activity. However, no morphological, histological, or echocardiographic evidence of LV hypertrophy was observed [53] in this study. In addition, apart from an increase in ANF mRNA, expression of other hypertrophy-associated genes was unchanged. On the other hand, cardiac-specific expression of α_{1B}-AR in mice results in the activation of PLC as evidenced by an increase in myocardial DAG content [54]. Furthermore, a phenotype consistent with cardiac hypertrophy developed in the adult transgenic mice with an increase in heart/body weight ratio, cardiomyocyte cross-sectional areas, and ventricular ANF mRNA levels [54]. Thus, it would appear that the α_{1B}-AR is primarily implicated in hypertrophy.

It is pointed out that the NE-induced IP_3 generation in neonatal rat cardiomyocytes has been reported to be primarily due to α_1-AR-mediated activation of PLC β_1 [55]. PLC β_1 exists as two splice variants, PLC β_{1a} and PLC β_{1b}, which differ only in their C-terminal sequences of 64 and 31 amino acids, respectively. While PLC β_{1a} is localized in the cytoplasm, PLC β_{1b} targets to the SL and is enriched in caveolae, where α_1-AR signaling is also localized [56]. Furthermore, in cardiomyocytes, responses initiated by α_1-AR activation involve only PLC β_{1b}; thus, the selective targeting of this splice variant to the SL membrane provides a potential target to reduce hypertrophy [56].

The role of PLC in the development of different types of cardiac hypertrophy has been well documented. For example, the development of cardiac hypertrophy in stroke-prone spontaneously hypertensive rats has been reported to involve PLC [57, 58]. The development of cardiac hypertrophy in cardiomyopathic hamster (BIO 14.6) is also reported to be associated with an increase in PLC activity [59]. We have previously reported an increase in PLC isozyme gene and protein expression as well as activities in the hypertrophied rat heart; due to volume overload induced by an arteriovenous shunt [60, 61]. Specifically, increased PLC β_1 and γ_1 were associated with the hypertrophic stage in this volume overload model [33]. In contrast, PLC β_1 and Gαq protein levels have been reported to be unchanged during hypertrophy due to pressure overload induced by ligation of the descending thoracic aorta in the guinea pig [62]. However, translocation of PKC isozymes from cytosol to membranous fractions was elevated. These investigators suggested that PKC translocation occurred without changes in Gαq and PLC β protein abundance and that it might be due to increases in Gαq and PLC β_1 activity rather than upregulation of expression [62], but PLC β_1 activity was not determined in this study.

An upregulation of PLC β_3 protein expression as well as activity has been reported in the development of cardiac hypertrophy in 2K1C hypertensive rats [63]. Furthermore these investigators observed an increase in the protein levels of PLC β_3 in neonatal rat cardiomyocytes in response to Ang II that could be inhibited with losartan, an AT_1 receptor blocker [63]. Interestingly, we have previously demonstrated that treatment with losartan immediately after the induction of volume overload hypertrophy (due to arteriovenous shunt) resulted in an attenuation of PLC isozyme gene expression (and possibly the PLC activities), which was found to be correlated to regression of cardiac hypertrophy [61]. Other studies have also shown that antagonism of the Ang II type 1 receptor as well as α_1-AR and ET-1 type A receptor types results in mitigation of cardiac hypertrophy and its progression to heart failure [64–72].

Mechanical stress induced by cell stretching in neonatal cardiomyocytes has also been reported to increase PLC activity [73]. However, in these studies [73, 74] no attempt was made to identify the PLC isozymes responsible for such responses. Stimulation of signaling pathways via Gαq and rac1 provokes cardiac hypertrophy in cultured cardiomyocytes and transgenic mouse models [75–78]. Furthermore, Ang II type 1 receptor (a Gαq-coupled receptor) overexpression has been reported to induce cardiac hypertrophy [79]. The first transgenic murine cardiac hypertrophy model to support a Gαq mechanism of hypertrophy was overexpression of the wild-type Gαq in the heart using the α-MHC promoter [75]. Indeed, a fourfold overexpression of Gαq resulted in increased heart weight and cardiomyocyte size along with marked increases in ANF, α-skeletal actin, and β-myosin heavy chain expression. Since PLC β is an essential downstream effector for Gαq [19], these observations would appear to implicate the activation of PLC β isozymes in cardiac hypertrophy. Gαq expression in vivo constitutively elevates cardiac PLC β activity [80, 81]. The transgenic mouse line (αq*52) in which cardiac-specific expression of hemagglutinin (HA) epitope-tagged constitutively active mutant of the Gαq subunit (HAαq*) leads to activation of PLC β, the immediate downstream target of HAαq*, with subsequent development of cardiac hypertrophy and dilation. However, in a second, independent line in the same genetic background (αq*44h) with lower expression of HAαq* protein that ultimately results in the same phenotype of dilated cardiomyopathy, no correlation with PLC activity was seen [82].

G proteins are regulated by RGS (regulators of G protein signaling) proteins, which shorten the duration of the cellular response to external signals and generally cause a reduction in hormone sensitivity [83]. Although the primary mode of action of RGS proteins is to accelerate termination of the signal by decreasing the lifetime of active, GTP-bound Gα subunits, some RGS proteins can also inhibit signal generation by antagonizing Gα-mediated effector activation [84]. In this regard, endogenous ventricular RGS2 expression has been reported to be selectively reduced in two different models of cardiac hypertrophy (transgenic Gαq expression and pressure overload), which was linked to elevated PLC β activity [85]. It was suggested that endogenous RGS2 exerts a functionally important inhibitory restraint on Gq/11-mediated PLC β activation and hypertrophy and concluded that loss of cardiac fine-tuning of PLC β signaling by RGS2 down-regulation could potentially play a pathophysiological role in the development of Gq/11-mediated cardiac hypertrophy. However, despite evidence for a role of RGS2 in negatively regulating Gq/11 signaling and hypertrophy, cardiomyocyte-specific RGS2 overexpression in transgenic mice, in vivo, did not attenuate ventricular Gq/11-mediated signaling and hypertrophy in response to pressure overload [86].

While the activation of PLC isozymes is an important signaling event in hypertrophy of the adult heart [20, 21, 60, 87], a loss of PLC ε signaling in PLC ε knockout mice has been reported to sensitize the heart to development of hypertrophy in response to chronic isoproterenol treatment [37]. On the other hand, PLC ε depletion, using siRNA, reduces the hypertrophic response to NE, ET-1, and insulin-like growth factor-1 (IGF-1) in neonatal rat cardiomyocytes [88]. These authors also observed that PLC ε activity was required for hypertrophic development; yet PLC ε

depletion did not reduce inositol phosphate production suggesting a requirement for localized PLC activity. Since PLC ε is scaffolded to a muscle-specific A kinase anchoring protein (mAKAP β) that is localized to the nuclear envelope in neonatal rat cardiomyocytes, it was suggested that PLC ε may be involved in the integration of upstream signal transduction to generate nuclear signals that regulate cardiac hypertrophy [88]. Indeed, in a follow-up study by the same group, it was reported that perinuclear PLC ε generates DAG in the Golgi apparatus, in close proximity to the nuclear envelope, to regulate activation of nuclear protein kinase D and hypertrophic signaling pathways [89]. Although, cardiac hypertrophy independent of PLC activation has also been reported [82, 90], it is evident from the aforementioned discussion that specific PLC isozymes might play a contributory role in the signal transduction pathways activated in cardiac hypertrophy.

18.4 Regulation of Phospholipase C Isozyme Gene Expression

In neonatal rat cardiomyocytes, NE has been shown to increase the expression of PLC β_1 [8]. On the other hand, in the same study, growth hormone and IGF-1 both induced a substantial increase in PLC β_3 mRNA expression. Furthermore, it was shown that the upregulation of PLC β_3 by IGF-1 was abolished by preincubation of cardiomyocytes with IGF-1 analog, an IGF-1 receptor antagonist, genistein, a tyrosine kinase inhibitor PD98059, an extracellular signal-regulated kinase (ERK) inhibitor, wortmannin, a phosphatidylinositol-3 (PI-3)-kinase inhibitor and rapamycin, a p70 S6 kinase inhibitor [8]. It is interesting to note that the induction of the immediate early genes c-myc, c-fos, and c-jun by IGF-1 was abolished by preincubation with antisense oligos against PLC β_3. This study demonstrated differential regulation of PLC β isozyme gene expression by different hypertrophic stimuli [8]. Moreover, the upregulation of PLC β_3 by IGF-1 was found to be mediated through tyrosine kinase, ERK, PI-3 kinase, and p70 S6 kinase. Importantly, PLC β_3 expression appeared to be required for the induction of immediate early genes by IGF-1.

Overexpression of IP_3 5-phosphatase has been shown to result in reduced IP_3 responses to α_1-AR agonists acutely, but with longer stimulation, an overall increase in PLC activity was observed, which was associated with a selective increase in expression of PLC β_1, that served to normalize IP_3 content in neonatal rat cardiomyocytes [46]. These investigators suggested that the level of IP_3 selectively regulates the expression of PLC β_1. Furthermore, it was also demonstrated that hearts from type 2 IP_3R knock-out mice had higher expression levels of PLC β_1. Accordingly, it was concluded that IP_3 and type 2 IP_3R regulate PLC β_1 and thus maintain levels of IP_3 [46], providing further functional significance for IP_3 in the heart. Previously, we have reported that NE-mediated cardiac hypertrophy may occur due to stimulation of α_1-AR and PLC activity [20]. We have also examined the signal transduction mechanisms involved in the regulation of PLC isozyme gene expression in adult cardiomyocytes in response to NE [24]. In this study, the

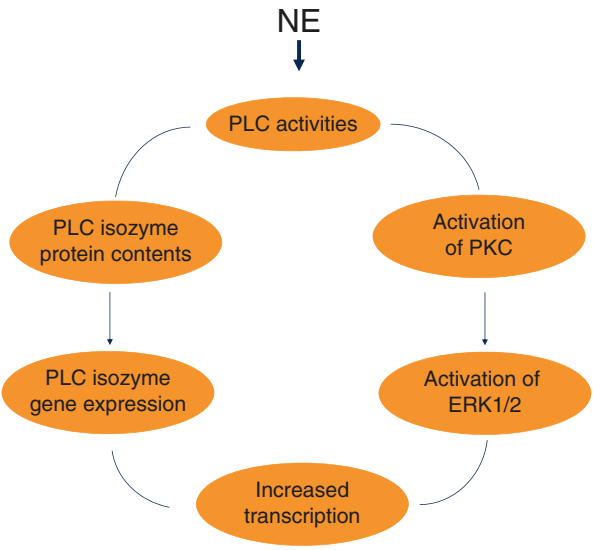

Fig. 18.2 Mechanisms of self-phospholipase C isozyme gene expression in response to norepinephrine. *NE* norepinephrine, *PLC* phospholipase C, *PKC* protein kinase C, *ERK1/2* extracellular-regulated kinase 1 and 2

NE-induced increases in PLC β_1, β_3, γ_1, and δ_1 isozyme mRNA and protein levels were attenuated in cardiomyocytes pretreated with either prazosin or U73122. The effects of prazosin and U73122 were associated with inhibition of PLC activity. We also observed inhibition of NE-stimulated PLC protein and gene expression by bisindolylmaleimide-1, a PKC inhibitor, and PD98059, an ERK1/2 inhibitor, indicating that PKC-MAPK signaling may be involved in this signal transduction pathway. Conversely, increases in PLC isozyme gene and protein levels were observed in cardiomyocytes in response to treatment with phorbol 12-myristate 13-acetate, a PKC activator. Taken together, it was suggested that PLC isozymes may regulate their own gene expression through a PKC- and ERK1/2-dependent pathway (Fig. 18.2).

Elevated levels of *c-fos* have been observed in rat heart following administration of NE [91, 92]. Stretching of isolated neonatal cardiomyocytes or exposure to NE also elevates *c-fos* mRNA levels and produces cellular hypertrophy [93–95]. Although the pathway that mediates the NE-induction of *c-fos* in other cell types has been shown to involve PKC, the identity of the specific PLC isozymes that may be part of this signaling pathway is not known. In addition, since ERK1/2 is considered to play a major role in the upregulation of the mRNA and protein levels of *c-jun* [95], it is possible that this transcription factor may play a role in the regulation of PLC isozyme mRNA levels in response to α_1-AR stimulation in adult cardiomyocytes. Although it is well known that both *c-fos* and *c-jun* regulate the expression of a number of genes in the heart [2, 96–98], we have reported that these transcription factors are also involved in regulating specific PLC isozyme gene expression [99]. In this study, transfection of cardiomyocytes with PLC isozyme-specific siRNA was found to prevent the NE-mediated increases in the corresponding PLC isozyme gene expression, protein content, and activity. Unlike PLC γ_1 gene, silencing of

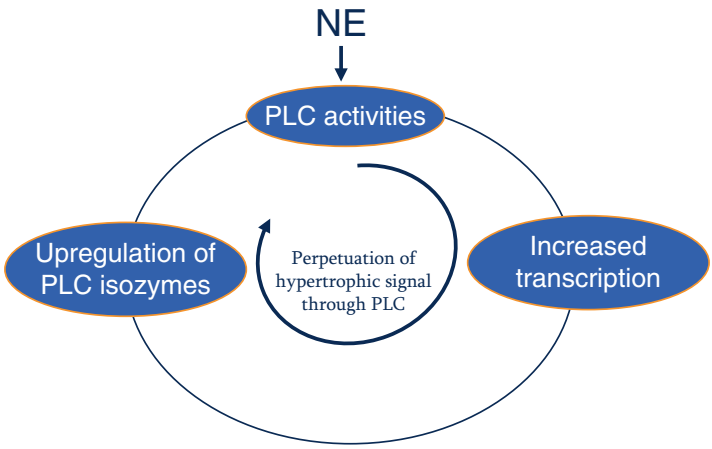

Fig. 18.3 Perpetuation of hypertrophic response due to upregulation of phospholipase C. *NE* norepinephrine, *PLC* phospholipase C

PLC β_1, β_3, and δ_1 genes with siRNA prevented the increases in *c-fos* and *c-jun* gene expression in response to NE. On the other hand, transfection with *c-jun* siRNA suppressed the NE-induced increase in *c-jun* as well as PLC β_1, β_3, and δ_1 gene expression, but had no effect on PLC γ_1 gene expression. Transfection of cardiomyocytes with *c-fos* siRNA prevented NE-induced expression of *c-fos*, PLC β_1, and PLC β_3 genes, however; it did not affect the increases in PLC δ_1 and PLC γ_1 gene expression. Silencing of either *c-fos* or *c-jun* also depressed the NE-mediated increases in PLC β_1, β_3, and γ_1 protein content and activity in an isozyme-specific manner. Furthermore, silencing of all PLC isozymes as well as of *c-fos* and *c-jun* resulted in prevention of the NE-mediated increase in ANF gene expression. These findings raise the intriguing possibility of a reciprocal regulation of PLC isozyme and *c-fos* and *c-jun* gene expression in adult cardiomyocytes that may be involved in the perpetuation of PLC-mediated cardiomyocyte hypertrophy (Fig. 18.3).

We have also reported that *c-fos* and *c-jun* mRNA levels, unlike other transcriptional factors examined, are increased by both NE and phenylephrine, a specific α_1-AR agonist [100]. The increases in *c-fos* and *c-jun* gene expression due to NE were attenuated by both prazosin and U73122. Activation of PKC with phorbol myristate acetate increased *c-fos* and *c-jun* mRNA, whereas inhibition of PKC with bisindolylmaleimide as well as inhibition of ERK1/2 with PD98059 abolished the NE-induced increase in *c-fos* and *c-jun* gene expression. Reduction of *c-jun* phosphorylation by SP600125, an inhibitor of JNK activity, was associated with an attenuation of the NE-induced increases in PLC gene expression. These findings suggested that *c-fos* and *c-jun* gene expression is regulated by PLC in adult cardiomyocytes through a PKC- and ERK1/2-dependent pathway, providing further evidence for a role of PLC in the cycle of events that result in the progression of cardiac hypertrophy.

18.5 Conclusions

This review has presented the possible involvement of PLC in cardiac hypertrophy (summarized in Fig. 18.4) as well as identified some of the signal transduction mechanisms involved in the regulation of PLC isozyme gene expression and protein levels in the heart. Although the role of PLC β_1 and PLC β_3 has been extensively studied in myocardial hypertrophy, PLCβ_4, which is also expressed in human, rat, and murine heart as well as in HL-1 cardiomyocytes, has been reported to be upregulated in HL-1 cardiomyocytes in response to Ang II [101] and thus may also be of relevance in the development of cardiac hypertrophy. The contribution of PLC with respect to other myocardial signaling systems, involved in the hypertrophic response needs to be defined. Although the molecular mechanisms responsible for the regulation of PLC isozyme gene expression need to be fully understood, the precise mechanisms of regulation of PLC activities in the heart also remain to be completely addressed, particularly since it is now understood that mRNA expression correlates to a very limited extent to enzyme expression and/or activity.

While some studies have shown prazosin in mitigating the progression of cardiac hypertrophy to heart failure [64, 65, 102–104] and losartan to attenuate cardiac hypertrophy, a direct inhibition of PLC (isozyme gene expression, protein contents, and activities) and regression of cardiac hypertrophy need to be demonstrated in vivo. To verify the involvement of specific PLC isozymes in cardiac hypertrophy it would be worthwhile to determine if overexpression of specific PLC isozymes in vitro and in vivo results in the cardiomyocyte hypertrophic response. While this review is not meant to de-emphasize the activation of other signal transduction pathways and their role in cardiac hypertrophy, it can be suggested from the evidence provided that specific PLC isozymes might constitute additional therapeutic targets for drug discovery for the treatment of cardiac hypertrophy and its progression to heart failure.

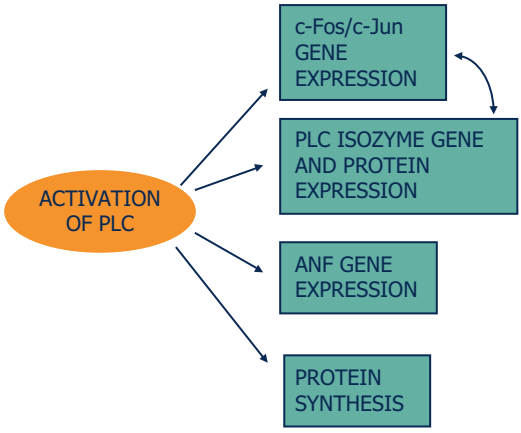

Fig. 18.4 Role of phospholipase C in cardiac hypertrophy. *PLC* phospholipase C, *ANF* atrial natriuretic factor

Acknowledgment Infrastructural support was provided by the St. Boniface Hospital Research Foundation.

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Chapter 19
The Protective Effect of Phospholipase C from Cardiac Ischemia–Reperfusion Injury

Eunhyun Choi, Soyeon Lim, and Ki-Chul Hwang

Abstract Cardiac ischemia–reperfusion is connected with cardiac dysfunction and contributes changes Ca^{2+} homeostasis. Ischemia–reperfusion-injured cardiomyocytes show increasing Ca^{2+} concentrations in both the cytosol and the mitochondria; this is associated with cardiac dysfunction and apoptosis. The mitochondria play an important role in ATP synthesis and heart functions. Ca^{2+} overload by I/R injury in cardiomyocytes induces reactive oxygen species generation and mitochondrial permeability transition pores opening, and sequentially causes cell death. Phospholipase C is known for regulating Ca^{2+}-mediated signaling pathway. The activation of PLC isozymes is induced by G-protein coupled receptors and/or protein tyrosine kinase receptors and is involved in the regulation of cellular Ca^{2+} homeostasis and protein kinase C activities in many cells. During ischemia–reperfusion, the expression and activity of each PLC isozymes are changed, which is related with cardiomyopathy or cardioprotection. Thus, PLC family members are considered as a promising target for cardioprotection from heart damage by oxidative stress. In this chapter, we will discuss the putative roles of PLC isozymes in I/R-injured cardiomyocytes.

Keywords Phospholipase C • Ischemia–reperfusion injury • Heart • Mitochondrial • Permeability transition pores • Calcium ion • Cardioprotection • PLC • mPTP

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_19,

19.1 Introduction

Ischemia–reperfusion (I/R) injury has been well described in a number of articles to cause serious damage in the brain, kidneys, liver, lungs, and heart [1]. Myocardial I/R is associated with cardiac dysfunction and cardiomyocyte death. I/R injury is involved in abnormalities of mitochondrial energetics and cellular Ca^{2+} homeostasis in cardiomyocytes [2–4]. The mitochondria dysfunction due to I/R injury changes protein functions including respiratory complexes and membrane proteins, generates reactive oxygen species (ROS), opens the permeability transition pore (PTP), releases cell death-inducing proteins such as cytochrome *c*, and increases cellular Ca^{2+} concentration in mitochondria and cytosol [5–8]. Myocardial Ca^{2+} homeostasis plays an important role in cardiac function, and alternation of Ca^{2+} homeostasis has been considered as one of the major reasons in heart failure and cell death. For preventing dysfunction and apoptosis of cardiomyocytes during I/R injury, mitochondria-mediated signaling molecules and protein kinases are emerging as therapeutic targets [9]. Among Ca^{2+}-mediated signaling molecules, phospholipase C (PLC) is a key signaling enzyme in heart function [10]. In this chapter, we discuss the Ca^{2+}-mediated cell death during myocardial I/R injury and the cardioprotective roles of PLC isozymes in ischemic-reperfused cardiomyocytes, regulating Ca^{2+} concentration and mitochondria-mediated apoptosis signaling.

19.2 The Role of Mitochondria and Calcium ion in Cardiac Ischemia–Reperfusion Injury

Cardiac mitochondria play an important role in the normal heart function and heart diseases [5, 6, 9, 11, 12]. The mitochondrial metabolism produces ATP and provides the energy to the heart, required for pumping blood through the body [13]. ATP synthesis of mitochondria is dependent on energy demands; however, the levels of ATP in cardiomyocytes remain constant, which is regulated by Ca^{2+} molecules. When increasing workload in the heart, cytosolic Ca^{2+} levels and energy demands are increased. Increasing cytosolic Ca^{2+} concentration is related to uptake of Ca^{2+} into the mitochondria, which induces activation of ATP synthase, dehydrogenase, and nicotinamide adenine dinucleotide hydride (NADH). As a result, these procedures lead to increase of ATP synthesis for meeting the energy requirements [2, 6, 9].

Under pathophysiological conditions, such as cardiac ischemia–reperfusion injury, ATP levels and Ca^{2+} homeostasis are altered, leading to mitochondrial dysfunction and cardiomyocyte death. At ischemia, the decrease in O_2 levels inhibits oxidative phosphorylation and reduces ATP synthesis. Anaerobic glycolysis leads to accumulation of lactate and increases cellular acidification, which decreases the activity of Na^+/H^+ exchanger (NHE) and Na^+/K^+-ATPase. These changes are triggered by Na^+ overload in the cytosol. Subsequently, Ca^{2+} concentration is also a

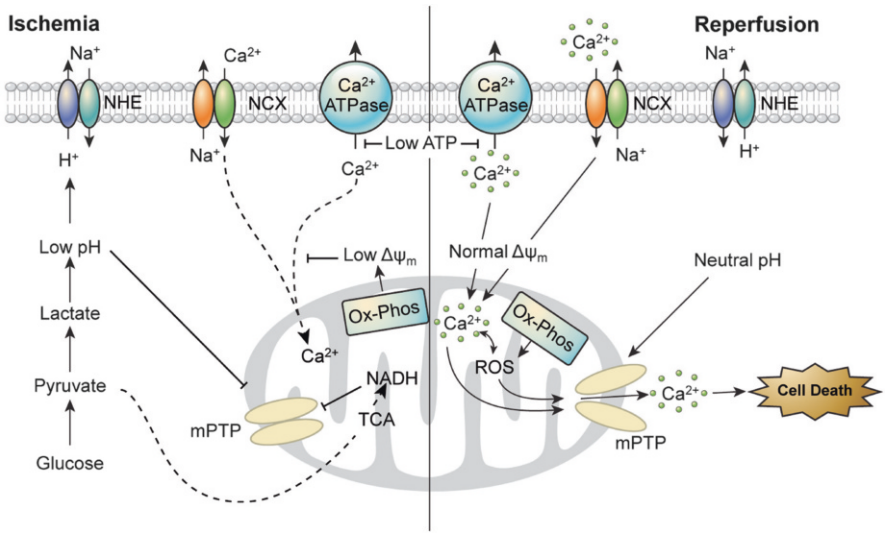

Fig. 19.1 Central roles of mitochondrial Ca^{2+} and mPTP in cardiomyocytes during I/R injury. *NHE* Na^+/H^+ exchanger, *NCX* Na^+/Ca^{2+} exchanger, *mPTP*, mitochondrial permeability transition pore

sustained rise in cytosol via Na^+/Ca^{2+} exchanger (NCX) and abnormal activity of Ca^{2+}-ATPase [2, 9]. Low mitochondrial membrane potential ($\Delta\Psi_m$) induces minimal Ca^{2+} uptake into mitochondria, while acidic pH in the cytosol and NADH accumulation in the mitochondria maintain the mitochondrial permeability transition pore (mPTP), which plays a key role in mitochondria-mediated cell death [8, 14, 15]. Reperfusion is a necessary recovery of ischemic heart, but paradoxically, it induces cardiac damage and apoptosis. At reperfusion, oxidative phosphorylation and $\Delta\Psi_m$ are rapidly re-established. It is associated with a sustained rise in mitochondria Ca^{2+} uptake and a generation of ROS. In addition, the acidic pH is returned to neutral. High levels of Ca^{2+} and ROS, and neutral pH cause opening of the mPTP, leading to ATP loss and cell death (Fig. 19.1) [2, 7, 16–19]. Therefore, alternation of Ca^{2+} homeostasis is involved in I/R injury and cardiomyopathy, as well as cardiomyocyte apoptosis via mitochondrial apoptotic pathway, including mPTP. Despite an attempt of cardioprotection during I/R injury using pharmacological and non-pharmacological therapies, some therapies have less effect on protection and have failed in large animal and human trials [9]. Consequently, more specific and targeted mitochondrial molecules, such as mPTP, have an important consideration for cardioprotection. As mentioned above, Ca^{2+} and mPTP play a key role in myocardial damage and apoptosis, and PLC isozymes are a significant Ca^{2+} regulator. The relation between PLC isozymes and cardioprotective function by regulating Ca^{2+} and mitochondrial function is discussed further below.

19.3 The Phospholipase C Family Members: Function, Structure, and Cellular Distribution

PLC is a cytoplasmic protein that catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces Ca^{2+} release from the endoplasmic reticulum, which is a major regulator of Ca^{2+} concentration in the cytosol, while DAG activates the Ca^{2+}-dependent protein kinase C (PKC), which phosphorylates the downstream signaling molecules that modulate various biological functions [20–23]. Mammalian PLC isozymes are classified into six groups: PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ, and PLC-η; 13 isozymes have been identified. The four different domains consist of PLC structures: the X and Y domains, the EF hands motifs, the C2 domain, and pleckstrin homology (PH) domain (Fig. 19.2a) [21–23]. The X and Y domains have catalytic activity and are highly conserved amino acid regions, which is located between the C2 domain and the EF hands motif. Three other domains are functional domains and enhance the enzymatic activity. Each of the PLC isozymes has different tissue distributions, cellular localizations, and biological functions. The four PLC-β isoforms are activated by the Gβγ subunit and Ca^{2+} and have been found in the nucleus. PLC-β1 is highly expressed in the brain and cardiomyocytes. The two isoforms of PLC-γ are activated by protein tyrosine kinases and play an essential role in differentiation, proliferation, Ca^{2+} flux, and tumorigenesis. PLC-γ1 is expressed in various tissues and PLC-γ2 shows a limited expression in hematopoietic lineage cells.

PLC-δ has three identified isoforms and is activated by Ca^{2+} level in the cytoplasm. PLC-δ1 is found in the brain, heart, lungs, and skeletal muscles. PLC-ε is detected in various tissues, including the heart, brain, lungs, and kidneys and contributes to proliferation and migration. The expression of PLC-ζ is only in the mammalian sperm heads, and PLC-η1 and PLC-η2 isoforms are found in the brain and is sensitive to change in Ca^{2+} concentration [23–25].

19.4 Physiological Roles of Phospholipase C Isozymes in Heart

PLC isozymes regulate cellular physiology and diseases as an effector molecule in diverse signaling transductions. The activation of PLC isozymes is regulated by different receptor tyrosine kinases and/or G-protein coupled receptor (Fig. 19.2b); however, all PLC isozymes require Ca^{2+} for activation [10]. Hydrolysis products of PIP_2 by PLC isozymes, IP_3 and DAG, induce activation of intracellular Ca^{2+} release and PKC. PLC enzymes change both activity and expression under cardiac ischemia or reperfusion [10, 26, 27]. During ischemic conditions, total PLC activities are decreased; on the other hand, its enzyme activities are increased during

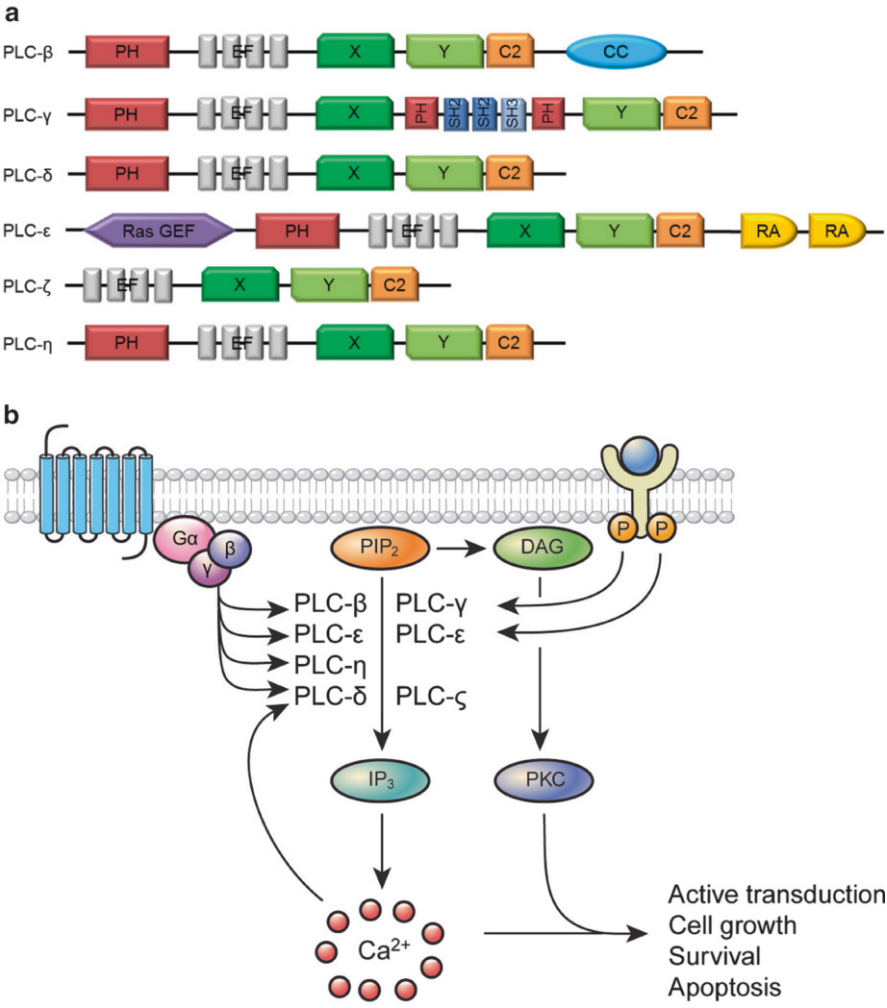

Fig. 19.2 Structure and signaling pathway of phospholipase C family members. *PH* pleckstrin homology domain, *EF* EF hands motifs, catalytic *X* and *Y* domains, *C2* functional domains, *CC* coiled coil; *SH2/SH3* Src homology 2/3, *Ra* Ras association, *Ras* GEF Ras GDP/GTP exchange factor

reperfusion condition, due to the different change of each PLC isozyme activity. In normal cardiac cells, PLC-δ1 and PLC-γ1 are a predominant isoform compared to PLC-β1. Moreover, PLC-δ1 is considered to be a key isoform in cardiac sarcolemma membrane. The activity of PLC-β1 is increased due to the up-regulation of mRNA and protein expressions, and PLC-δ1 and PLC-γ1 are stripped of their activities, under cardiac ischemia. The mRNA level of PLC-δ1 does not change but the

protein content is decreased by degradation. In the case of PLC-γ1, the expression of mRNA and protein is decreased, resulting from diminished tyrosine kinase activity. Conversely, during reperfusion, PLC-δ1 and PLC-γ1 are activated and PLC-β1 is less active [10, 28, 29]. Of the six PLC isozymes, PLC-β, PLC-γ, PLC-δ, and PLC-ε are studied in cardiomyocytes; these are discussed below.

19.5 PLC-β

Despite the fact that PLC-β is a large family member of the PLC isozymes, the connection between PLC-β and ROS or mitochondria-mediated signaling is little known to date. The research of the correlation between cardiac hypertrophy and PLC-β1 is currently going on at Woodcock and Grubb's laboratory [30–32]. The heterotrimeric G protein Gq activates PLC-β1b, which is linked to Gq-mediated cardiac hypertrophy and apoptosis. Overexpression of PLC-β1 causes increased cell size, upregulated hypertrophic marker genes and chamber dilatation, and ultimately induces apoptosis. The heart failure induces the release of catecholamines, such as norepinepnrine and epineprnine that increases the sensitivity of α_1-adrenergic receptors (AR) [26, 33]. The signaling transduction of α_1-AR activates PLC-β1 isozymes by Gαq, which is responsible for cardiac hypertrophy and apoptosis (Fig. 19.3). Since α_1-AR antagonists, including parazosin and carvedilol, decrease PLC-β1 activation and attenuate heart failure, the α_1-AR-Gαq-PLC-β1 signaling pathway may be an important target in cardiomyopathy [10, 34].

19.6 PLC-γ

According to current studies, PLC-γ1 has opposite effects in heart. During oxidative stress, such as H_2O_2, the levels of PLC-γ1 mRNA and membrane protein content are increased, resulting in the inhibition of cardiomyocyte apoptosis via activation of PKC-ε and the phosphorylation of antiapoptotic protein Bcl-2 (Fig. 19.4) [35]. Disruption of PLC-γ1 by U73122 PLC inhibitor causes cardiomyocyte viability. Adversatively, gram-negative bacteria produce endotoxin lipopolysaccarides (LPS) that is responsible for myocardial dysfunction during sepsis. LPS induces TNF-α expression, which mediates contractile depression, while cyclooxygenase-2 (COX-2) regulates cardiac function in ischemic heart [36, 37]. LPS leads to activation and phosphorylation of PLC-γ1, which is connected by IP_3/IP_3R pathway-mediated TNF-α expression and ERK1/2 MAPK signaling-mediated COX-2 expression. Consequently, PLC-γ1 has bifunctional roles in cardiomyocytes, cardioprotection, or cardiotoxicity, which is regulated by a certain physiological condition.

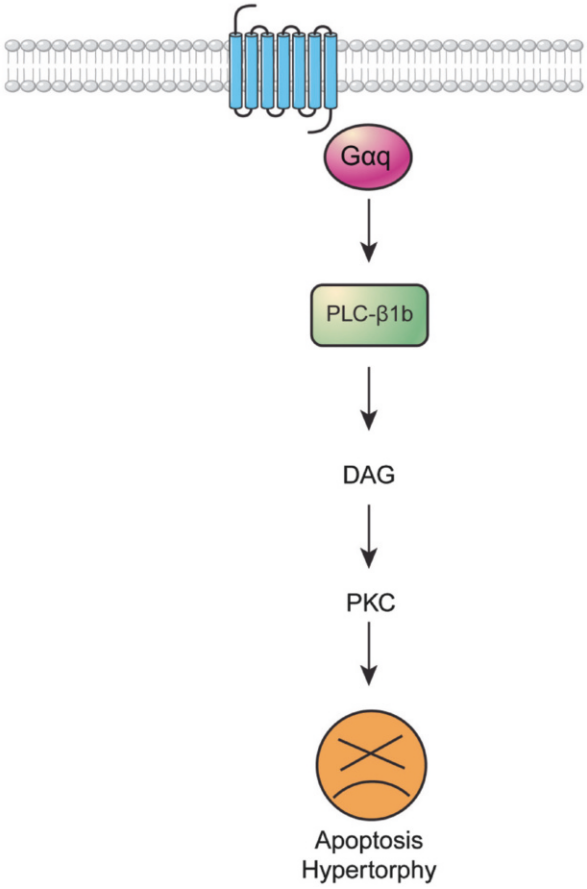

Fig. 19.3 The α_1-AR-Gαq-PLC-β1 signaling pathway in cardiomyopathy

19.7 PLC-δ

Since PLC-δ1 is an abundant isozyme compared to PLC-β1 and PLC-γ1 in the heart and the most sensitive isozyme to Ca^{2+} than other isozymes, many researches for PLC isoforms have been focused on PLC-δ1 regarding cardiac protection from heart diseases. PLC-δ1 plays a critical role in TNF receptor-mediated protection effect against doxorubicin-induced cardiotoxicity. Doxorubicin, trade name adriamycin, is an anticancer chemotherapy drug, but in high doses can cause damage to the heart due to increasing oxidative stress and mitochondrial injury. The protective mechanism from doxorubicin-induced cardiac injury is mediated via tumor-necrosis factor (TNF) receptors [38]. TNF receptor signaling pathways increase NF-κB and activator protein-1 DNA-binding activities, which changes expression of Ca^{2+} homeostasis and mitochondrial functional regulator genes, such as PLC-δ1.

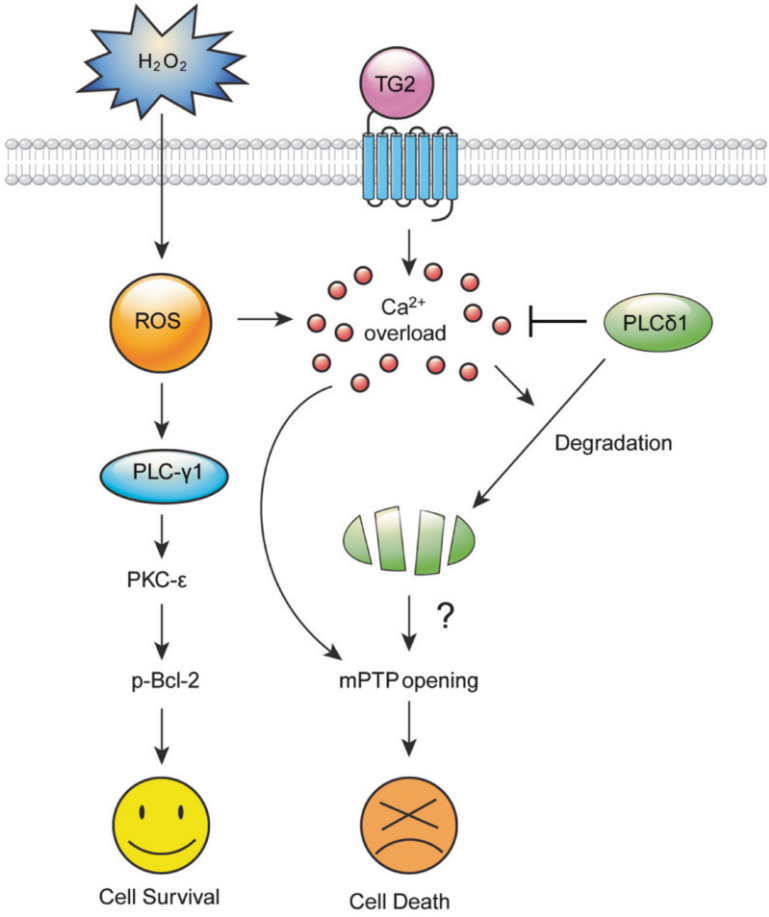

Fig. 19.4 The putative roles of PLC-γ_1 and PLC-δ_1 in cardiac I/R

The inhibition of PLC-δ1 and TNF receptor signaling exacerbates doxorubicin-induced cardiac dysfunction. Our laboratory has been continuing the study of PLC-δ1 function in cardiomyocytes [29]. The expression level of PLC-δ1 is decreased in both the border and scar regions of the infarcted heart and the ectopic overexpression of tissue transglutaminase 2 (TG2; also known as $G\alpha_h$). The hypoxia condition or overexpression of TG2 induces Ca^{2+} overload and PLC-δ1 degradation in cardiomyocytes, leading to cardiac apoptosis [39]. The decrease of PLC-δ1 degradation via treating calpastatin, calcium-dependent cysteine protease inhibitor, or silencing of TG2, protects cardiomyocytes from H_2O_2-induced apoptosis. In our unpublished study, we confirmed that PLC-δ1 rescues I/R heart by the regulation of Ca^{2+} homeostasis and mitochondrial apoptotic pathway. The up-regulation of PLC-δ1 significantly inhibits intracellular Ca^{2+} overload, mPTP opening, and the mitochondrial

membrane potential increase in I/R-injured cardiomyocytes, resulting in the inhibition of apoptosis. These results suggest that PLC-δ1 may be a promising potential target for cardioprotection against I/R injury (Fig. 19.4).

19.8 PLC-ε

The cardiac-physiological function of PLC-ε is studied in PLC-ε knockout mice [10, 40]. Tohru Kataoka's laboratory generated PLC-$\varepsilon^{\Delta x/\Delta x}$ mice by deleting the N-terminal part of the X domain to decrease the enzyme activity, and in this animal, the heart was enlarged by ventricular dilation rather than hypertrophy [41]. The semilunar valve regurgitation was led to chronic volume overload and aortic and pulmonary valves were thickened due to increase in cell numbers. Also, PLC-$\varepsilon^{\Delta x/\Delta x}$ mice observed to have malformation of congenital semilunar valve cell development. Therefore, PLC-ε plays a role in regulating the proliferation and apoptosis of the semilunar valve cells during the late stage of valvulogenesis, which is related to Smad1/5/8 activation. Another group, Alan V. Smrcka's laboratory generated the PLC-$\varepsilon^{-/-}$ mice that are characterized by complete loss of PLC-ε protein [42]. PLC-$\varepsilon^{-/-}$induces hypertrophy by adrenergic stress and showed decreased cardiac function, contractile response, and β-adrenergic receptor-dependent Ca^{2+} transient amplitudes. Together, PLC-ε plays an important role in Ca^{2+}-induced Ca^{2+} release by β-adrenergic receptor stimulation in cardiomyocytes.

19.9 PLC-ζ and PLC-η

PLC-ζ and PLC-η are not related with heart functions due to tissue-specific expression, but these two isoforms are also link to Ca^{2+} signaling and Ca^{2+} homeostasis. PLC-ζ is a sperm-specific PLC isozyme and has a key role in fertilization and embryo development. For egg activation, Ca^{2+} oscillation is required, so PLC-ζ triggers the Ca^{2+} generation and signaling pathway [43]. PLC-η is a neuron-specific isozyme and probably plays an important role in the formation and the maintenance of the neuronal network [44]. The disruption of neuronal Ca^{2+} homeostasis is associated with Alzheimer's disease (AD). PLC-η may contribute to Ca^{2+} accumulation in AD-affected neurons [45].

19.10 Conclusions

PLC family members were discovered in recent years, and the accuracy of the biological mechanism of PLC isozymes is still unrevealed. However, PLC isozymes are involved in regulating diverse cellular signals that effect cell growth, cell

differentiation, cell migration, and cell pathophysiological processes. The cellular Ca^{2+} concentration has an important role in normal and pathological heart functions. In addition, increased Ca^{2+} levels have an influence on mitochondria dysfunctions, including disruption of $\Delta\Psi_m$ and ATP synthesis, and eventually induce cell death. The cardiac I/R injury by oxidative stress induces up-regulating Ca^{2+} concentration in both the cytosol and the mitochondria that leads to increasing ROS generation and opening mPTP. These processes consequently cause cardiac dysfunction and cardiomyocyte apoptosis. The balance of each PLC isozyme activation and expression regulates the maintenance of Ca^{2+} homeostasis and inhibits cardiac cell death by alternation of mitochondria functions from cardiac I/R injury. Therefore, the regulators of Ca^{2+} or mitochondrial proteins, such as mPTP, are considered as a promising therapeutic agent for cardiac protection from I/R injury.

Acknowledgement The authors thank Mr. Dong-Su Jang, Research Assistant, Department of Anatomy, Yonsei University College of Medicine, Seoul, Korea, for his help with the figures.

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Chapter 20
Role of Phospholipase C in Cardioprotection During Oxidative Stress

Paramjit S. Tappia and Naranjan S. Dhalla

Abstract It is well known that myocardial ischemia–reperfusion (I-R) results in contractile dysfunction due to Ca^{2+}-handling abnormalities in cardiomyocytes; these defects are primarily due to oxidative stress. However, very little is known about the nature and mode of the sarcolemmal changes with respect to phospholipase C (PLC)-related signaling events. In addition, the mechanisms involved in protection of heart function in post-ischemic myocardium as well as ischemic preconditioning involving PLC isozymes are not completely understood. This chapter discusses the role of PLC-mediated signal transduction pathway in I-R injury to the heart as well as during treatment of cardiomyocytes with oxidants. The activation of PLC γ_1 seems to play a critical role in the redox-related signal transduction mechanisms for cardioprotection whereas the activation of PLC δ_1 may be intimately involved in the oxidative stress-induced development of intracellular Ca^{2+}-overload and cardiac dysfunction. The evidence available in the literature suggests that specific PLC isozymes may serve as novel targets for cardioprotection against oxidative stress during the development of I-R injury.

Keywords Phospholipase C isozymes • Signal transduction • Ischemia–reperfusion • Oxidative stress • Ischemic preconditioning • Calcium handling • Cardiomyocytes • Cardioprotection • Cardiac dysfunction

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20.1 Introduction

The excessive formation of reactive oxygen species (ROS) as well as oxidants such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) is known to trigger cell death and cardiac dysfunction [1–9]. Oxidative stress is defined as the imbalance between the production of ROS and oxidants and the state of glutathione redox buffer and antioxidant defense system [10–12]. However, both ROS and oxidants can also function as intracellular signaling molecules for cell survival [13–16]. In this regard, low concentrations of ROS and oxidants or exposure of myocardium for a transient period may stimulate the signal transduction mechanisms for cardiomyocyte function as well as expression of genes involved in cell survival, while high concentrations of ROS and oxidants or exposure for a prolonged period produce oxidative stress and result in harmful outcomes [10–12, 17].

It is now well established that oxidative stress is a primary factor in ischemic heart disease [10–12, 18–26]. The adverse effects of ROS and oxidants are, in part, due to the ability of these metabolites to produce changes in subcellular organelles including sarcolemma (SL), sarcoplasmic reticulum (SR), mitochondria, and nucleus, which are intimately involved in the regulation of cardiomyocyte Ca^{2+}-homeostasis [10, 12, 27–30], and cause an intracellular Ca^{2+}-overload and subsequent cardiac dysfunction [10, 27–30]. Accordingly, this article discusses how oxidative stress affects phospholipase C (PLC), a key signaling enzyme known to influence cardiac function, in normal hearts and under conditions of ischemia–reperfusion (I-R). In addition, this review examines the role of specific PLC isozymes in redox signaling and the activation of cell survival pathways. Since intracellular Ca^{2+}-overload and oxidative stress are linked and are the major mechanisms in the pathophysiology of cardiac dysfunction in ischemic heart disease, this chapter will also address the role PLC isozymes play in the regulation of cardiomyocyte Ca^{2+}-handling during oxidative stress induced by I-R. Furthermore, a brief discussion on the role of PLC in ischemic preconditioning and myocardial protection against I-R injury is also provided.

20.2 Myocardial PLC Isozymes

The PLC family consists of seven subfamilies: PLC α, β, γ, δ, ε, ζ, and η [31–38]; however, PLC β_1, δ_1, and γ_1 and γ2 isoforms of PLC ε are the predominant forms expressed in the heart [34, 39]. In fact, PLC δ_1 is considered to be the major cardiac SL PLC isozyme [37, 39–41]. The PLC isozymes display differences in activating mechanisms, but all require Ca^{2+} for their activity [32, 33, 42, 43]. Angiotensin II, α_1-adrenergic agonists, and endothelin-1 are relevant stimulants of PLC β isozymes via the α subunits of the heterotrimeric Gq subfamily [33, 41]; PLC β has also been shown to be activated by Gβγ dimmer [44]. A nontyrosine kinase activation of PLC γ isozymes has been reported [33]. Furthermore, the activation of PLC γ isozymes independent of tyrosine kinase has also been reported [45]. The receptor-initiated

events for the activation of PLC δ isozymes are considered to be mediated via transglutaminase II (G_h), a new class of GTP-binding protein [46, 47]. Although the PLC δ-G_h pathway may be an important player in the signaling pathway that regulates calcium homeostasis and modulates physiological processes [46, 47], the role of transglutaminase as an activator of PLC δ (or indeed as a functional G-protein) is highly questionable. It would appear, however; that this class of PLC isozymes is more regulated by binding of PIP_2 to its pleckstrin homology (PH) domain or by Ca^{2+} [32, 48, 49]. PLC ε isozymes are activated by Ras, Rho, and Rap 2B as well as by Gα12 [34, 50]. The activation of PLC ζ and η is far less characterized; however, it should be noted that PLC ζ [36] is only expressed in sperm and so this PLC isozymes can be excluded as a player in PLC signaling in the heart. Although the distinct functions of each PLC isozyme in cardiomyocytes and the extent of their overlap have yet to be established, the α_1-AR-mediated IP_3 generation in rat neonatal cardiomyocytes has been shown to be mediated by PLC β_1 [51].

20.3 Myocardial Ischemia–Reperfusion

Myocardial I-R injury represents a clinically important problem associated with thrombolysis, angioplasty, and coronary bypass surgery. Injury of myocardium due to I-R results in cardiac contractile dysfunction. Myocardial ischemia produces dramatic changes in cardiac function, metabolism, and ultrastructure. However, the cellular and molecular events leading to contractile dysfunction and derangement of cardiac structure are not completely understood. Although re-institution of coronary flow to the ischemic heart is considered beneficial for recovery of cardiac pump function, reperfusion after a certain period of ischemia has been shown to further aggravate the myocardial abnormalities [10, 12, 52–58]. It is pointed out that cardiac pump failure and changes in cardiac cell ultrastructure due to I-R or hypoxia-reoxygenation involve a variety of complex pathophysiological abnormalities. For example, the beneficial effects of Ca^{2+}-antagonists as well as Na^+–H^+ and Na^+–Ca^{2+} exchanger inhibitors have supported the role of intracellular Ca^{2+}-overload whereas those of antioxidants as well as both α- and β-adrenergic blockers point to the involvement of oxidative stress, mainly due to the production of ROS, in the pathophysiology of I-R injury. Myocardial I-R has also been shown to generate different oxidants such as H_2O_2, peroxynitrite, and HOCl are responsible for the occurrence of intracellular Ca^{2+}-overload associated with I-R injury [59–61].

20.4 Changes in PLC Due to I-R or Exposure to Oxidants

There are a few, but conflicting reports on inositol phospholipid metabolism in I-R due to PLC [62–70]. A 30 min global ischemia has been reported to result in a decrease in IP_3, whereas reperfusion results in a rapid increase in the IP_3 [62, 67]; this

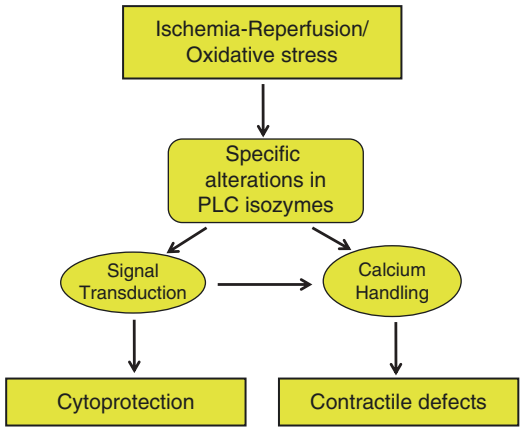

Fig. 20.1 Possible impact of specific PLC isozymes in ischemia–reperfusion and oxidative stress

observation was inferred from assessing the inositol phosphate content of whole ventricular tissue. On the other hand, an increase in IP_3 level has been reported in both ischemia and reperfusion [68]. These contrasting findings could be due to differences in ^{3}H-inositol labeling of cardiac membranes, which is problematic due to the fact that there exists distinct membrane phospholipid pools and microenvironments for PLC isozymes [64]. Ischemia has also been shown to elicit an α_1 adrenoceptor-mediated increase in the mass of total myocardial DAG and was suggested to be due to activation of myocardial PLC [69].

A role for PLC is suggested through the observation that the reperfusion-induced as well as norepinephrine-induced rise in IP_3 could be prevented by neomycin, a PLC blocker [63, 66]. Activation of PLC activity has been evidenced in I-R; however, this was observed in a microsomal fraction that was isolated from whole ventricular tissue [68]. Other investigators have reported a decrease in PLC activity in ischemia and an increase in reperfusion [65]; however, this was conducted in a total membrane preparation from whole ventricular tissue. Similarly, although a recent study has reported that myocardial ischemia induces an increase in PLC activity, measurement of the activity was conducted in left ventricular total homogenate [70]. Thus, studies that do not employ pure SL membrane preparations risk conflict from combining many other subcellular organelles, which may have distinct or unique PLC pathways [71] and therefore confound the data. In the aforementioned studies, no attempt was made to identify specific PLC isozyme changes. However, we believe that I-R and oxidative stress produce remodeling of subcellular organelles [72], which are associated with changes in signal transduction mechanisms and calcium handling in cardiomyocytes leading to either cardioprotection or contractile defects depending upon alterations in specific PLC isozymes (Fig. 20.1).

We were the first to report that while cardiac ischemia was associated with an activation of SL PLC β_1 and decreased SL PLC γ_1 and δ_1 activities, reperfusion of the ischemic heart resulted in the activation of SL PLC γ_1 and δ_1 isozymes, whereas PLC β_1 activity declined progressively [42, 73]. The changes in PLC isozyme

activities observed in the ischemia and I-R may have functional relevance to PKC isozymes, which are activated specifically by PLC-derived DAG [74, 75], and found to be associated with I-R injury [76–78]. Indeed, it has been reported that enhanced protection of the heart can be achieved by administration of PKC-δ inhibitor at the beginning of reperfusion, whereas activation of PKC-ε before ischemia mimics ischemic preconditioning [78]. Therefore, it is possible that the differential changes in PLC isozymes result in specific PKC isozyme activation and that prevention of the I-R-induced activation of specific PLC isozymes in turn precludes the PKC isozyme changes.

The release of catecholamines has been shown to occur in the ischemic heart [79]. In addition, an enhanced sensitivity of the α_1-AR under both ischemia and reperfusion conditions has also been demonstrated [80]. Pretreatment with prazosin, an α_1-AR antagonist, before global ischemia, has been shown to reduce the extent of I-R-induced myocardial injury [81], which may be related to an inhibition of the α_1-AR-induced PLC activation [68]. Since the α_1-AR transduces the signal to PLC β isozymes via Gαq [33], the beneficial effect of prazosin could be due to inactivation of PLC β isozymes. In fact, our preliminary data has shown that the beneficial action of prazosin may be related to the prevention of PLC β_1 activation in the ischemic heart. Carvedilol, a nonselective β-adrenoceptor and α_1-AR blocker, has also been shown to prevent the impairment of hemodynamic function of hearts perfused with H_2O_2 [82]. It was suggested that the cardioprotective effects of carvedilol were, in part, due to the decreased α_1-AR-mediated activation of PLC and production of IP_3, DAG, and Ca^{2+}, which may be due to attenuation of PLC β_1 activation. It should be noted that although the deleterious effects of endogenously released catecholamines during ischemia are well established [83], the specific activation of PLC β_1 in the ischemic heart may have implications for cardiac fibrosis [84] and may contribute significantly to cardiac dysfunction in I-R. Indeed, we have previously proposed a role for the α_1-AR-Gαq-PLC β_1-signaling pathway in myocardial fibrosis [85].

Although exposure of SL membranes and isolated cardiomyocytes to oxidants induces changes in PLC and components of the phosphoinositide pathway [86–88], the effects of oxidants on specific PLC isozymes have not been completely examined. We have been the first to have reported that treatment of cardiomyocytes with H_2O_2 results in an activation of PLC γ_1 [89]. In this study, a concentration-dependent (up to 50 μM) increase in the mRNA level and membrane protein content of PLC γ_1 was observed with H_2O_2 treatment. Furthermore, PLC γ_1 was activated in response to H_2O_2, as revealed by an increase in the phosphorylation of its tyrosine residues. A marked increase in the phosphorylation of the anti-apoptotic protein Bcl-2 by H_2O_2 was also observed, which was attenuated by a PLC inhibitor, U-73122. Although both PKC-δ and PKC-ε protein contents were increased in the cardiomyocyte membrane fraction in response to H_2O_2, PKC-ε activation, unlike PKC-δ, was attenuated by U-73122. Inhibition of PKC-ε with inhibitory peptide prevented Bcl-2 phosphorylation. Moreover, different concentrations of this peptide augmented the decrease in cardiomyocyte viability in response to H_2O_2. In addition, a decrease in cardiomyocyte viability, as assessed by trypan blue exclusion, due to

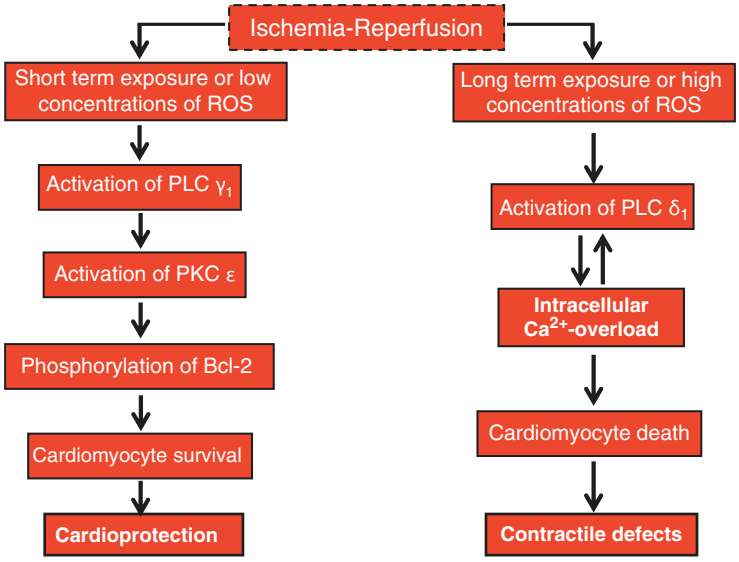

Fig. 20.2 Role of PLC γ_1 and PLC δ_1 under conditions of oxidative stress. *ROS* reactive oxygen species, *PLC* phospholipase C, *PKC* protein kinase C

H_2O_2 was also seen when cells were pretreated with U-73122 and was as a result of increased apoptosis. These observations lead to the suggestion that PLC γ_1 may play a role in cardiomyocyte survival during oxidative stress (Fig. 20.2) via PKC-ε and phosphorylation of Bcl-2. S100A1 is a Ca^{2+}-binding protein of the EF-hand type that belongs to the S100 protein family. It has been reported that S100A1 uptake protects neonatal ventricular cardiomyocytes from 2-deoxyglucose and oxidative stress-induced apoptosis in vitro [90]. It was suggested that S100A1-mediated anti-apoptotic effects involve an ERK1/2 pro-survival pathway, which includes the activation of PLC. Thus PLC may have a role to play in the survival of the myocardium, in vivo, under conditions of oxidative stress.

There is limited information on the mechanisms responsible for and the significance of the changes in specific SL PLC isozyme activities, protein contents, and gene expression with respect to Ca^{2+}-homeostasis and cardiac dysfunction in I-R. However, we have previously shown that verapamil (a L-type Ca^{2+}-channel blocker) partially prevented the increase in SL PLC β_1 activity in ischemia and the decrease in its activity during the reperfusion phase. Furthermore, it elicited a partial protection of the depression in SL PLC δ_1 and PLC γ_1 activities during the ischemic phase and attenuated the increase during the reperfusion period [42]. These changes were associated with an improved myocardial recovery after I-R. Likewise, pretreatment of hearts with U-73122 not only significantly inhibits DAG and IP_3 production in I-R, but has also been shown to enhance the recovery of cardiac function as indicated by measurement of left ventricular end-diastolic pressure (LVEDP), left ventricular diastolic pressure (LVDP), maximum rate of pressure development

(+dP/dt$_{max}$) and decay (−dP/dt$_{max}$). However, verapamil was less effective than U-73122. Accordingly, it was suggested that the inhibition of PLC improves myocardial recovery after I-R.

Perfusion with an increased Ca^{2+} concentration (from 1.25 to 2.55 mM) resulted in an initial increase in inotropy; however, with longer perfusion time (15 min) a severe cardiac dysfunction occurred; this was evidenced by marked increased LVEDP and reduced LVDP, +dP/dt$_{max}$, and −dP/dt$_{max}$, indicating that this concentration of Ca^{2+} is damaging to the heart. Perfusion of hearts with high Ca^{2+} resulted in the activation of all PLC isozymes in heart homogenate to varying degrees; in fact, the activation of PLC δ_1 was most whereas the activation of PLC γ_1 was least. Under these conditions, an increase in PLC γ_1 and δ_1 protein contents in the SL membrane was also observed, suggesting that PLC activation specifically of PLC γ_1 and δ_1 (which occurs in I-R) may be due to the increase in $[Ca^{2+}]_i$. Indeed, the extent of the activation of PLC δ_1 by Ca^{2+} may be due to the higher sensitivity of PLC δ isozymes to Ca^{2+} [32, 42]. Furthermore, it is interesting to note that perfusion of the hearts with low Ca^{2+} revealed a specific decrease in the activity of PLC δ_1. Thus, we believe that the activation of PLC δ_1 may play a contributory role to the occurrence of intracellular Ca^{2+}-overload during I-R. In this regard, the effects of exogenous PLC on $[Ca^{2+}]_i$ have been measured in isolated ventricular cardiomyocytes using fura-2 fluorescence [91]. It was observed that the ratio of rod-shaped cells to all cells decreased in a time- and a concentration-dependent manner, suggesting that PLC causes Ca^{2+}-overload. It was suggested that the activation of PLC may play a role in arrhythmias and cell injury during ischemia/reperfusion. Furthermore, it was also indicated that the increase in $[Ca^{2+}]_i$ during I-R may activate phospholipase, which would further increase $[Ca^{2+}]_i$ to form a vicious cycle.

Although these data provide some information on the role of Ca^{2+} in the activation of PLC isozymes, it should be noted that the role of Ca^{2+} under basal conditions could be different from its role under I-R. In this regard, the increase in PLC β_1 activity seen in the heart upon perfusion with high Ca^{2+} is in contrast to the profile of the activity during I-R. This discrepancy could be explained on the basis that PLC β_1, compared with the other PLC isozymes, is more susceptible to free radical-mediated damage, which occurs during the early reperfusion phase [10–12], rendering PLC β_1 insensitive to Ca^{2+} or as a result of a selective degradation due to activation of proteases which occurs in I-R [92].

In the ischemic heart and hypoxic neonatal cardiomyocytes, PLC δ_1, but not PLC β_1 and PLC γ_1, has been reported to be selectively degraded, a response that could be inhibited by the calpain inhibitor, calpastatin, and by the caspase inhibitor, zVAD-fmk [37]. Furthermore, the overexpression of the PLC δ_1 in hypoxic neonatal cardiomyocytes rescued the occurrence of intracellular Ca^{2+}-overload under ischemic conditions. Accordingly, it was suggested that in the border zone and scar region of infarcted myocardium, and in hypoxic neonatal cardiomyocytes, the selective degradation of PLC δ_1 by the calcium-sensitive proteases may play an important role in intracellular Ca^{2+} regulation under the ischemic conditions. In addition, PLC isozyme changes may contribute to alterations in calcium homeostasis in myocardial ischemia.

It should be noted that the synthesis and the levels of the PLC substrate, PIP_2, in the SL membrane under conditions of oxidative stress have also been examined. We have shown that exposure of the SL membrane to xanthine-xanthine oxidase, an ROS generating system, significantly reduces PI 4 kinase and PI 4P 5 kinase activities, suggesting a reduction in its ability to synthesize PIP_2 [88]. In view of the drastic effects of the ROS on different kinase systems, particularly the rate-limiting PI 4-kinase, which is responsible for the synthesis of SL PIP_2, it is possible that there may occur a severe deficit in the SL amount of this phospholipid. On the other hand, the enhanced production of IP_3 during post-ischemic reperfusion has been reported to be associated with an increased availability of PIP_2 for α_1-AR-activated PLC [93]. Indeed, these investigators have reported that the increased PIP_2 concentration was in the caveolar fraction where PLC β_1 and Gqα are localized, and thus may be critical for the increase of IP_3 generation in early reperfusion of the ischemic heart. We have shown that PI 4 kinase and PI 4P 5 kinase activities are elevated during ischemia, while a biphasic response occurs in reperfusion; where 1 min reperfusion induces a decrease and 5 min reperfusion results in an increase in PI 4 and PI 4P kinase activities. These data would seem to suggest that the SL PIP_2 may be increased in the ischemic phase, decreased in the initial period of reperfusion and progressively increased with prolonged reperfusion. Since the identity of the ROS generated under I-R was not measured [93], it may be difficult to conclude which oxidative species is responsible for the effects on the PIP_2 levels; however, it may be possible that the effect may be due to different concentrations of ROS generated and is dependent upon the time of exposure.

Despite this, the functional importance of PIP_2 cannot be underestimated as its importance is further highlighted by the fact that there are a number of diverse biochemical events regulated by PIP_2 and, therefore, affected by the altered concentration of this lipid in the membrane [94, 95]. Such events can be seen to influence cardiac function. The decreased number of PIP_2 molecules could compromise the contractile performance of the heart by causing depression of the inward rectifier K^+ channels directly [96] as well as suppression of the cardiac SL Na^+–Ca^{2+} exchange and Ca^{2+}-pump activities [94, 97]. This would decrease Ca^{2+}-extrusion from the cardiomyocytes and contribute toward the occurrence of intracellular Ca^{2+}-overload and the development of cardiac abnormalities. It is suggested that a decreased concentration of SL PIP_2 may constitute a further insight into the mechanisms of ROS-induced increase in intracellular Ca^{2+} in cardiomyocytes.

The reduced availability of PIP_2 substrate would be an additional factor in attenuating the PLC-dependent generation of IP_3 and DAG and therefore the cardiac response to agonist stimulation. The reduced PIP_2 could also result in diminished activation of phospholipase D (PLD) isozymes PLD1 and PLD2, which require PIP_2 as a cofactor [98]. These signaling isozymes hydrolyze phosphatidylcholine to produce phosphatidic acid, which increases intracellular Ca^{2+} and cardiac contractility [99]. Such impairment of PLD isozymes would contribute to a depression of cardiac performance. Clinical and experimental evidence has suggested that ROS-mediated oxidative processes are involved in the pathogenesis of congestive heart failure [100] and diabetic cardiomyopathy [101]. In these cardiac pathologies, we have

detected a significant decrease in the SL amount of PIP_2 as a consequence of depressed PI 4-kinase and PI 4-P 5-kinase activities [39, 102, 103], implicating a contributory role of this phospholipid in cardiac dysfunction during conditions of oxidative stress.

20.5 Myocardial Preconditioning

A number of studies have demonstrated that hearts subjected to brief periods of ischemia limit the infarct size as well as cardiac dysfunction due to a sustained period of I-R [104–109]; however, the role of the SL membrane in this phenomenon remains to be determined. PKC is involved in the beneficial effects of ischemic precondioning (IP) [102, 108, 110–112]. The primary step of the signal transduction pathway for the activation of PKC involves the stimulation of PLC, generating the second messenger DAG, suggesting a role for PLC in ischemia preconditioning. Also, adenosine has been reported to be released during cardiac ischemia [104, 113] and can exert important protective functions at the level of cardiomyocytes [114] which may be mediated by PLC signaling pathways [115]. Several studies have shown that the cardioprotective effects of IP may be mediated through α_1-AR [111, 116–118] implicating a role for PLC. Although preconditioning of the isolated perfused heart has been demonstrated to inhibit the reperfusion-induced release of IP_3 [67, 119] and that the blockade of IP_3 receptors can mimic preconditioning [120], the precise role of PLC in ischemic preconditioning remains to be elucidated. Furthermore, it should be noted that the beneficial effect of α_1 -AR stimulation points to a role for PLC β isozymes in the heart. Some of our preliminary studies have addressed the issue of the role of α_1-AR-PLC-mediated signaling and ROS in IP [121]. It was observed that IP attenuated PLC β_1 activation during I-R and was associated with improved post-ischemic contractile recovery of hearts subjected to I-R. While prazosin did not have any effect on the protective effect of IP, mercaptopropionyl-glycine (an ROS scavenger) abolished the protective effect of IP. These data suggest that prazosin protects the heart against I-R injury, but is not an essential component in the mechanism of IP. On the other hand, ROS may be important in mediating the cardioprotective effects of IP. Adenosine, a purine nucleoside, is known to be released by ischemic tissue and is considered as an important trigger of IP that may involve the activation of PLC [122].

20.6 Conclusions

Although little is known about the nature and mechanisms of the changes in PLC-related signaling events due to I-R, the available evidence indicates the possibility that PLC could constitute an important mechanism of cardiac function during conditions of oxidative stress. Our experimental data have suggested that specific PLC

activities might influence $[Ca^{2+}]_i$ and cardiomyocyte contractility. Thus, it is our contention that the activation of PLC δ_1 under conditions of oxidative stress due to I-R may contribute to a self-perpetuating cycle that exacerbates cardiomyocyte Ca^{2+}-overload and leads to cardiac contractile dysfunction. On the other hand, we have suggested that PLC γ_1 may have a role in redox signaling for cell survival in response to short exposure and low ROS and oxidant concentrations. A better understanding of the role played by PLC isozymes in the heart will provide new opportunities for development of therapies that selectively modulate PLC isozymes in ischemic heart disease.

Acknowledgement Infrastructural support was provided by St. Boniface Hospital Research Foundation.

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Part IV
Role of Phospholipase D

Chapter 21
Mammalian Phospholipase D: Structure, Regulation, and Physiological Function of Phospholipase D and its Link to Pathology

Tsunaki Hongu and Yasunori Kanaho

Abstract During the past two decades, structure and functions of mammalian phospholipase D (PLD), which hydrolyzes phosphatidylcholine to produce the signaling lipid phosphatidic acid, has been extensively investigated. Now, it is generally accepted that conventional two PLD isozymes, PLD1 and PLD2, play important roles in diverse cellular functions, such as endocytosis, exocytosis, membrane trafficking, cell growth, differentiation, and actin cytoskeleton reorganization. In addition, phenotypic analyses of mice lacking the *PLD* genes revealed that the disturbance of the PLD-mediated cellular signaling is closely related to several diseases. In this review, we summarize an overview of structures, regulatory mechanisms, and physiological functions of PLD isoforms, and discuss the emerging importance of this protein family in a wide variety of diseases, including tumor growth and metastasis, cardiovascular and cerebrovascular diseases, Alzheimer's disease, and immune responses.

Keywords Phospholipase D • Phosphatidic acid • Lipid signaling • Cancer • Metastasis • Platelet aggregation

21.1 Introduction

The mammalian phospholipase D (PLD) hydrolyzes phosphatidylcholine (PC) to produce choline and the signaling lipid phosphatidic acid (PA) [1] (Fig. 21.1). The first demonstration showing the existence of the PLD activity in mammals was published by Saito and Kanfer using the rat brain in 1975 [2]. After more than 20 years

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_21,

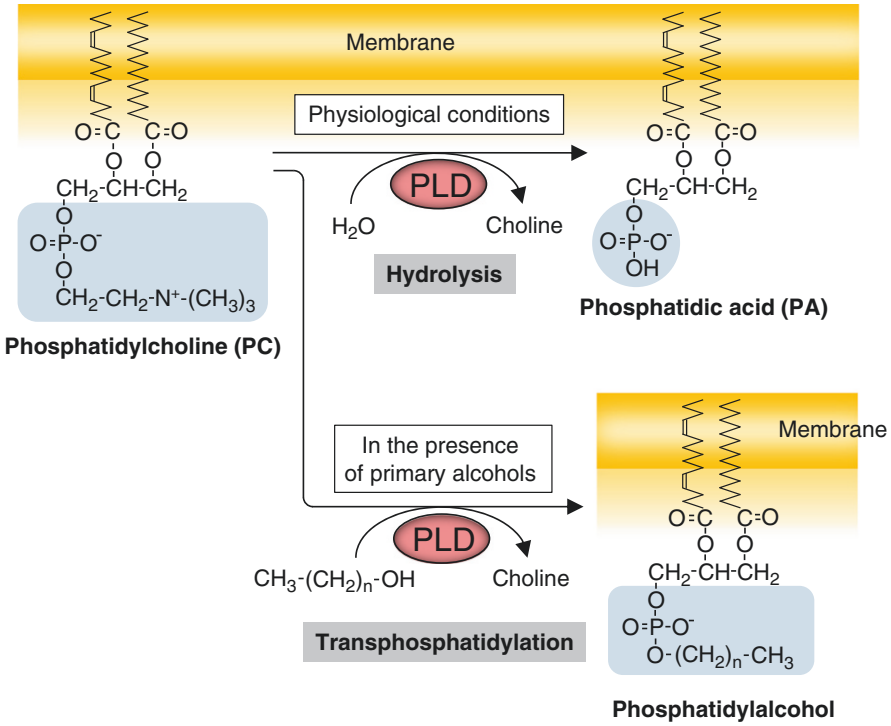

Fig. 21.1 Reactions catalyzed by PLD. Phospholipase D (PLD) catalyzes the hydrolysis of the major membrane phospholipid, phosphatidylcholine (PC), to generate choline and the signaling phospholipid, phosphatidic acid (PA), under physiological conditions. In the presence of primary alcohols, such as ethanol and 1-butanol, PLD preferentially catalyzes transphosphatidylation to produce the non-physiological phospholipids, phosphatidyl alcohols, at the expense of PA production

from the original finding, two independent genes encoding mammalian PLD1 and PLD2 were identified in the mid of 1990s [3, 4]. Utilizing biochemical, molecular biological, and cell biological techniques, the signaling mechanisms through which PLD isozymes regulate diverse cell functions and events have been elucidated. PLDs are ubiquitously expressed in almost all of tissues and cells of mice, and their activity is stimulated in response to various extracellular agonists, such as hormones, neurotransmitters, extracellular matrixes, and growth factors [5]. In general, PLD1 localizes at intracellular compartments, such as the endosome, the Golgi complex, lysosome, and exocytotic vesicles and translocates to the plasma membrane upon agonist stimulation of cells under some experimental conditions [6]. On the other hand, PLD2 predominantly localizes at the plasma membrane [6]. PA generated by PLD at local sites of intracellular compartments functions as a signaling messenger to regulate a wide variety of cell events and functions, including endocytosis, exocytosis, membrane trafficking, cell proliferation, and actin cytoskeleton reorganization [7–10].

In the presence of the primary alcohols, such as ethanol and 1-butanol, PLD also catalyzes a unique reaction referred to as transphosphatidylation to form the non-physiological phospholipid phosphatidyl alcohols by transferring the phosphatidic moiety of PC to the hydroxyl group of primary alcohols at the expense of the production of PA [5, 11] (Fig. 21.1). This characteristic property of PLD has been utilized to assess the PLD activity: although phospholipase C contributes to the production of PA through the production of diacylglycerol (DG) and its phosphorylation by DG kinase, phosphatidyl alcohols are PLD-specific products. Since PA production by the hydrolytic activity of PLD is interfered with in the presence of primary alcohols, ethanol and 1-butanol have been utilized as inhibitors for the PLD-catalyzed PA production to investigate PLD functions at the cellular level. Recently, however, we provided evidence that primary alcohols have potentially nonspecific side effect(s) to modulate PLD-independent cellular signaling pathway(s) [12], warning against the use of primary alcohols as specific inhibitors of PLD-mediated PA formation. Therefore, the functional analysis of mammalian PLD should be reevaluated using more specific strategies such as small interference RNA (siRNA)-mediated knockdown of PLD isozymes or genetic ablation of the *PLD* genes.

The physiological action of mammalian PLDs is to specifically hydrolyze PC to produce PA. As this simple phospholipid-hydrolytic reaction is involved in diverse cellular processes or events, the disruption of the PLD-mediated signaling pathway would cause various diseases. Genetic ablation of the *PLD* genes in mice has provided strong evidence for the implication of PLDs in tumor growth and angiogenesis, cancer metastasis, cardiovascular and cerebrovascular diseases, Alzheimer's disease, and immune response in mice. This review summarizes structures of PLD isozymes, regulatory mechanisms of their enzymatic activity, and their cellular functions and then introduces the increasing knowledge of the pathological linkage of this protein family.

21.2 PLD Isozymes and Their Structures

To date, two conventional mammalian PLD isozymes, PLD1 and PLD2, have been identified [3, 4]. These two isoforms share about 50 % amino acid sequence identity. The clarification of the domain structure of PLDs (Fig. 21.2) has contributed to the elucidation of activation mechanisms and physiological functions of PLD isozymes. Both PLD1 and PLD2 contain the catalytic core regions comprising highly conserved domain I–IV [13]. In domains II and IV, there exist two regions with an HxKxxxxD sequence-designated HKD motif, which are essential for enzymatic activity of PLDs [14]. Point mutations in the HKD motif disrupt the enzymatic activity of PLD [14]. PLD mutants in which the lysine of the HKD motif is replaced by arginine, such as K898R PLD1 and K758R PLD2, lack enzyme activity and are widely employed as dominant negative mutants to investigate functions of PLDs in signal transduction pathways and cell functions.

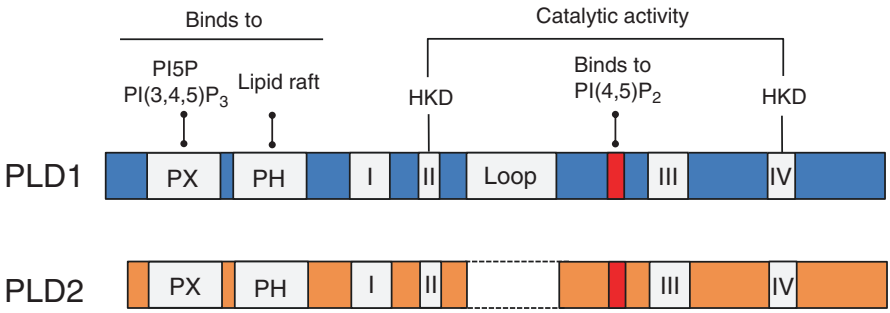

Fig. 21.2 Structures of mammalian PLDs. Both PLD1 and PLD2 contain N-terminal PX and PH domains. These domains bind to polyphosphorylated phosphatidylinositols and the lipid raft and determine their subcellular localization. The catalytic core of PLDs consists of four conserved regions of domain I–IV. Domain II and IV are particularly highly conserved and contain the invariant charged motif, HKD motif. Between domain II and III, PLD contains the $PI(4,5)P_2$-binding polybasic region, binding of $PI(4,5)P_2$ being required for PLD activation. The loop region, which is found in PLD1, but not PLD2, is involved in the auto-inhibition of PLD1 activity

In addition, both PLD1 and PLD2 possess lipid/protein interaction domains, phox (PX) and pleckstrin homology (PH) domain in their N-terminal region [13]. These domains are critical for the subcellular localization of PLDs. Point mutation or deletion of the PX domain causes mislocalization of the proteins in the cell [15, 16]. It has been suggested that this domain specifically binds to phosphoinositides, especially to phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$) and phosphatidylinositol 5-phosphate (PI5P) [17, 18]. The PH domain appears to regulate the association of PLDs with the lipid raft to facilitate the recovery of PLDs from the plasma membrane back to the endosome after translocation of PLDs to the plasma membrane upon agonist stimulation [17]. Another important domain is the region between the domain II and III, which binds to phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) [19]. The binding of $PI4,5P_2$ is absolutely required for the catalytic activity of both PLD1 and PLD2, but does not determine intracellular localization of proteins.

An interesting domain is the “loop domain,” which is found in PLD1, but not in PLD2. The loop domain seems to be involved in the auto-inhibition of enzymatic activity of PLD1, since deletion of this region increases basal activity of PLD1 [20], and insertion of the loop domain into recombinant PLD2 significantly reduces its basal activity [21].

21.3 Regulation of PLD Activity

A wide variety of intracellular regulators of PLD activity have been identified [9, 10, 22]. These include several small GTPases, such as Arf and Rho family GTPases, conventional PKC (cPKC), and phosphoinositides. Arf family of small GTPases, which are composed of six isoforms, Arf1-6, were initially found to

stimulate PLD activity in vitro before PLD isozymes have been identified [23]. After cloning of PLD isoforms, Arfs were identified as activators of PLD1; they activate PLD2 very little, if any [21]. In an in vitro system, there is no significant difference among six Arf isoforms in their abilities to activate PLD1. At the cellular level, it has been demonstrated that Arf1 and Arf6 are major isoforms to activate PLD1 [10]. The PLD1 activity is also regulated by Rho family of small GTPases, RhoA, Cdc42, and Rac1 [22, 24, 25]. Like Arf, RhoA directly activates PLD1 as recombinant RhoA directly interacts with and activates recombinant PLD1 in the in vitro reconstituted system [26–28]. A surface plasmon resonance experiment with purified proteins has demonstrated that the interaction of Rho with PLD1 is independent of Arf binding, consistent with the finding that activation of PLD1 by Rho family of small GTPases is synergistic with Arf [29]. In addition to these PLD1 activators, cPKCs, such as PKCα and PKCβ, have been shown to stimulate PLD1 activity [30–32]. PKCα and PLD1 are co-immunoprecipitated and their interaction can be promoted by phorbol12-myristate 13-acetate, which is a strong activator of PKC, indicating that the interaction of PLD1 with cPKC is dependent on cPKC activation [20, 33]. Interestingly, however, the activation of PLD1 by cPKCs is independent of phosphorylation of PLD1 by cPKC. Other PLD1 activators, such as RalA, Rheb, and cofilin, have also been identified [34–36]. Thus, a wide variety of molecules regulate PLD1 activity.

Catalytic activities of both PLD1 and PLD2 are absolutely dependent on PI(4,5)P_2 binding. PI(4,5)P_2 serves as an essential cofactor for the Arf-dependent activation of PLD1 [23]. The activation of PLD2 by PI4,5P_2 in vitro is much greater than that of PLD1 [37], indicating that PI4,5P_2 is a potential activator of PLD2. Although only PI4,5P_2 was identified as the PLD2 activator to date, several inhibitors of PLD2, including aldolase [38], α-actinin [39], and α- and β-synucleins [40], have been identified. These findings indicate that PLD2 activity is suppressed by these molecules to a low basal activity in the resting state of cells. Considering that PLD2 has extremely high basal activity, it is plausible that PLD2 is activated by releasing an inhibitor(s) from PLD2 in response to agonist simulation.

21.4 Cellular Functions for PLD

Cellular signaling from PLD to a downstream effector(s) is mediated by its product PA, which is a negatively charged phospholipid with a small head group. Functions of PA in the cell are thought as follows. (1) PA serves as a precursor of two well-known signaling lipids, DG and lysophosphatidic acid (LPA), which are generated by PA phosphatase and phospholipase A_2, respectively (Fig. 21.3a): DG activates PKC to couple signals to multiple cellular functions, and LPA acts as an extracellular signaling molecule to transduce its signal through the LPA receptor. (2) PA itself acts as a lipid-signaling molecule through the binding to intracellular downstream effector proteins: in some cases PA functions as a recruiter of signaling molecules and in other cases as a modulator of downstream effectors' activities. Members of potential downstream effectors for PA has been expanding and

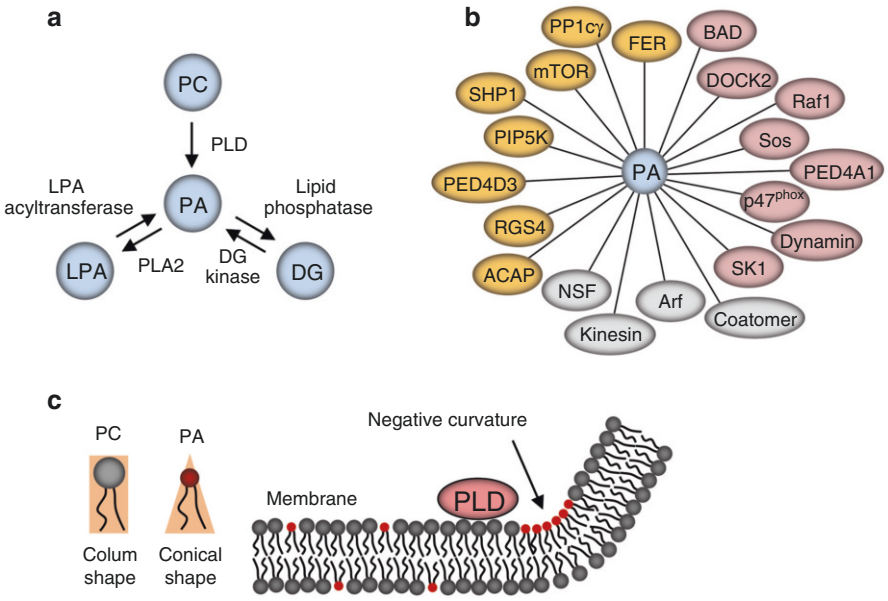

Fig. 21.3 Functions of PA. (**a**) PA generated by PLD can be converted to diacylglycerol (DG) by lipid phosphatase or to lysophosphatidic acid (LPA) by phospholipase A2 (PLA2). PA also can be generated from LPA and DG by LPA acyl transferase and DG kinase, respectively. (**b**) PA can interact with various partner proteins. Proteins colored by orange are modulated in their activities and those colored by pink are regulated in their localization by PA. Proteins, which are not known how PA regulates their functions, are colored by gray. (**c**) PA can serve the formation of membrane negative curvature. PA is a conical-shaped phospholipid with the small polar head group, therefore making a negative curvature stable. PA-induced membrane curvature is thought to facilitate membrane fusion or fission step in the membrane trafficking events

their functions at the cellular level are widespread [41] (Fig. 21.3b). (3) PA physically forms membrane curvature at the cytoplasmic leaflet. Since PA has a small head group and two fatty acyl chains, it has a conical shape that induces the negative membrane curvature, therefore facilitating fusion and budding of membranes [42] (Fig. 21.3c).

21.4.1 Membrane Trafficking

PLD is involved in widespread cellular events through the molecular function(s) of the PLD product PA described above. One of the prominent functions of PLD at the cellular level is to regulate membrane trafficking such as endocytosis, exocytosis/secretion, and endosomal recycling. It has been demonstrated that PLD2 plays a crucial role in the internalization of several types of receptors. The overexpression of a catalytically inactive mutant of PLD2 or knockdown of endogenous PLD2 by

siRNA inhibits the internalization of the epidermal growth factor receptor (EGFR) [43], μ-opioid receptor [44], angiotensin II receptor [45], and glutamate receptor [46], indicating that PLD2 activity is essential for the regulation of receptor endocytosis. In the cellular signaling pathway of endocytosis for various types of receptors, downstream molecule(s) of the PLD2 product PA has not yet been clearly identified. Recent study suggested that type 4 phosphodiesterase (PDE4) is involved in the ligand-independent internalization of EGFR at the downstream of PA [47]. An intriguing new model of EGFR endocytosis mediated by PLD was provided by Lee and colleague [48]. They found that both PLD1 and PLD2 bind to dynamin through their PX domains and stimulate its GTPase activity. In addition, they showed that expression of PX domain enhances internalization of EGFR, and knockdown of both PLD1 and PLD2 suppresses EGFR endocytosis, which is rescued by expression of either wild-type or catalytically inactive PLDs, indicating that PLDs regulate EGFR endocytosis in its lipase activity-independent manner. On the contrary of this report, it has been shown that the enzymatic activity of PLD affects the rate of EGFR internalization [43]. Interestingly, Lee and colleague have provided a model of PLD-mediated EGFR endocytosis [49]. They found that at a lower concentration of EGF (0.2 nM: a physiological concentration), the lipase activity of PLD is not essential, although PLD itself is required, for EGFR internalization, while at higher concentration of EGF (20 nM: a concentration observed at some specific site, e.g., tumor tissues), PLD activation is substantive for the EGFR endocytosis. These observations suggest that PLD regulates endocytosis of EGFR through the GTPase activating protein (GAP) activity of its PX domain for dynamin at the lower concentrations and through PA produced by PLD at the higher concentrations.

PLD also appears to function as a regulator of exocytosis/secretion. PLD1, rather than PLD2, is most frequently associated with exocytosis and secretion, such as secretion of hormones from endocrine and neuroendocrine cells [50, 51], release of neurotransmitters from neurons [52], mast cell degranulation [53], and release of IL-8 from epithelial cells [54]. The key evidence supporting the notion for the involvement of PLD1 in exocytosis has been provided by a series of studies using adrenal pheochromocytoma PC12 cells [8]. In this cell line, PLD1 is a predominantly expressed isoform and exclusively localizes at the plasma membrane. Overexpression of an inactive mutant of PLD1 or knockdown of endogenous PLD1 by siRNA inhibits hormone release from PC12 cells, whereas a kinase-dead inactive mutant of PLD2 had no effect. During the secretion, PLD1 is activated by Arf6, Rac1, and RalA at the plasma membrane. Furthermore, ribosomal S6 kinase 2 (RSK2), which is a regulator of the calcium-dependent exocytosis, physically interacts with PLD1, phosphorylates it at Thr-147, and thereby activates PLD1. Phosphorylation of PLD1 by RSK2 is essential for the high potassium-stimulated secretion of growth hormone from PC12 cells. In this event, it is speculated that PA produced by the activated PLD1 forms negative membrane curvature of the lipid bilayer at the plasma membrane-granule docking site to stimulate the fusion of the plasma membrane with granules.

Several lines of evidence have been provided that PLDs are also required for other types of intracellular membrane trafficking. An effector domain mutant of

Arf6, N48I, which is still activated by Arf6 guanine nucleotide exchange factor (GEF) and inactivated by GAP, lacks the ability to activate PLD [55]. However, this mutant of Arf6 can still activate another Arf6 effector, phosphatidylinositol 4-phosphate 5-kinase (PIP5K). These observations indicate that this Arf6 mutant is useful to specifically interfere with the PLD-mediated cellular signaling pathway. Using this Arf6 mutant, it was demonstrated that in HeLa cells, expression of the N48I Arf6 mutant inhibits the recycling of major histocompatibility protein class I (MHCI) from the endosome back to the plasma membrane, indicating that PLD1 activation by Arf6 is essential for endosomal recycling of this protein [56]. It has also been reported that PLD2 and its product PA play an important role in the generation of COPI vesicle at the Golgi [57]. Electron microscopic observation revealed that siRNA-mediated PLD2 depletion inhibits the late stage of COPI vesicle fission and disrupts Golgi morphology, indicating that PLD2 functions in Golgi maintenance through COPI vesicle formation.

21.4.2 Actin Cytoskeleton Reorganization and Plasma Membrane Dynamics

PLD is also involved in actin cytoskeleton reorganization and membrane dynamics. The PLD product PA supports the Arf-dependent activation of PIP5K, a lipid kinase responsible for the production of the versatile signaling phospholipid PI4,5P_2 [58]. Under physiological settings, PA produced by PLD2, but not by PLD1, participates in Arf6-dependent activation of PIP5K: in HeLa cells, PLD2 translocates to peripheral ruffling membranes in response to EGF stimulation and colocalizes with PIP5K, while PLD1 does not alter its perinuclear localization [59]. Since the PIP5K product PI4,5P_2 can reorganize the actin cytoskeleton via its regulation of actin-binding proteins, it is plausible that PLD plays an important role in actin cytoskeleton reorganization via PIP5K activation. Recent study demonstrated that both PLD1 and PLD2 are required for the PDGF-dependent dorsal ruffle formation [60]. Embryonic fibroblasts isolated from *PLD1*/*PLD2* double knockout mice show defect in the dorsal ruffle formation. In this case, two isoforms of PLD appear to cooperate, since *PLD1* or *PLD2* single knockout does not interfere with the dorsal ruffle formation. DOCK1, which is an atypical Rac1 GEF, has been shown to be a putative downstream molecule of PA in this phenomenon.

Reorganization of actin cytoskeleton is absolutely required for membrane shape changes. Since PLDs regulate the actin cytoskeleton reorganization as described above, it is suggested that PLDs are involved in a wide variety of cell events requiring the plasma membrane dynamics through actin cytoskeleton remodeling. It has been reported that PLD plays important roles in phagocytosis [61], neuronal outgrowth [62], cell spreading, and migration [10], via regulation of actin cytoskeleton and membrane dynamics.

21.5 Pathological Role of PLD

As mentioned above, signaling pathways mediated by PLD are very complicated, and therefore its physiological functions are diverse. It has also been reported that the disruption of the PLD signaling closely links to a wide variety of diseases [63]. These include cancer, cardiovascular and cerebrovascular disease, neurodegenerative disease, and immune responses (Fig. 21.4).

21.5.1 Cancer

PLD has been increasingly recognized as a critical regulator of cancer progression. The PLD expression and its activity are up-regulated in various types of human cancers, such as colon, gastric, kidney, and thyroid cancers [64]. In colorectal carcinoma, tumor size and patient survival highly correlate with the PLD2 expression level [65]: the size of tumor with higher expression of PLD2 is larger than that of tumor with lower expression of PLD2, and the patients of carcinoma, in which PLD2 is highly expressed, show significantly poor survival rate. It has also been reported that PLD activity is elevated in cells transformed by oncogenes, such as *v-Src*, *v-Ras*, *v-Fps*, and *v-Raf* [66], and that up-regulation of PLD expression stimulates anchorage-independent growth and cell cycle progression of fibroblasts [67]. Thus, PLD frequently associates with oncogenic signaling pathways.

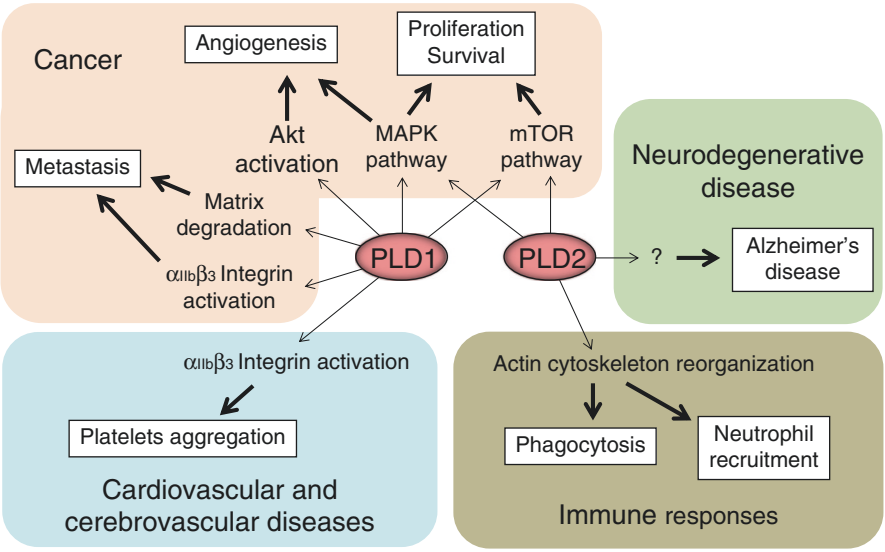

Fig. 21.4 Implication of PLDs in a wide variety of human diseases. PLD is involved in a wide variety of diseases, such as cancer, cardiovascular and cerebrovascular diseases, neurodegenerative disease, and immune responses. See text for details

One of the molecular mechanisms through which PLD is involved in cancer is the recruitment of Sos, which is a GEF for the oncogene product Ras, to plasma membrane through the PLD product PA, leading to the activation of Ras, which in turn induces cell transformation [68]. In addition, PA generated by PLD interacts with and recruit the mitogen-activated protein kinase kinase kinase (MAPKKK) Raf1 to plasma membrane, where it can activate MAPK cascade [69]. Ras and Raf1 are the key molecules involved in the mitogenic-signaling pathway. Therefore, the recruitment of the Ras GEF, Sos, and Raf1 by the PLD product PA is implicated in proliferation and anti-apoptosis of cancer cells. Another critical downstream target of PLD in cancer cells is the mammalian target of rapamycin (mTOR), which is a serine/threonine kinase and known as a key regulator in cell growth and survival signaling pathways [66]. Since PA binds to and activates mTOR [70], overexpression of PLD1 or PLD2 stimulates mTOR activity, which was monitored by the phosphorylation of the mTOR enzymatic substrate S6 kinase in breast adenocarcinoma or rat fibroblasts, through PA production [71, 72]. PLD activation also induces c-Myc expression, which is regulated by mTOR, in breast adenocarcinoma, indicating the implication of PLD-mTOR signaling pathway in cancer cell growth and survival signal [73]. The mTOR inhibitor, rapamycin, has been used as an anti-cancer drug. However, rapamycin-based therapeutic strategies are unsuccessful in some cancer patients. Interestingly, it has been demonstrated that PA competes with rapamycin in mTOR regulation, and activation of PLD inhibits the effect of rapamycin in human breast cancer cell line [66]. Therefore, inhibition of PLD may provide the strategy for the suppression of the survival signal of rapamycin-resistant cancer cells.

It has also been demonstrated that PLD regulates cancer cell metastasis. The PLD activity closely correlates with migration and invasion activities of human cancer cell lines [74]. Moreover, it has been reported that PLD plays an important role in the invasion of glioma cells, as overexpression of PLD1 or PLD2 facilitates the invasion of glioma cells [75]. Related to the function of PLD in exocytosis described above, PLD1 is required for secretion of matrix metalloprotease (MMP) 9 and MMP2 in colorectal cancer cells and glioma cells, respectively [75, 76]. In addition, PLD has been shown to be involved in the release of microvesicles from melanoma cells, which contain the membrane type of MMP1, thereby contributing to the degradation of extracellular matrix [77].

Phenotypic analysis of *PLD1* knockout mice, which are otherwise viable and grossly normal, revealed that PLD1 expressed in tumor microenvironment plays important roles in tumor growth and metastasis [78]. The tumor microenvironment consists of various types of cells such as vascular and lymphatic endothelial cells, mesenchymal cells, and immune cells [79]. The soluble factors, signaling cues, extracellular matrix, and mechanical cues provided by the tumor microenvironmental cells can promote tumor progression by supporting tumor growth and invasion and by protecting the tumor from host immunity attack. Neovascularization of tumors, which is required to supply oxygen and nutrients, is one of the major aspects of tumor microenvironment contributing to the tumor progression; inhibition of angiogenesis in tumor prevents tumor growth. Interestingly, the growth of tumors formed by subcutaneously transplanted B16 melanoma and Lewis lung carcinoma

cells is impaired in *PLD1* knockout mice, due to the reduction of tumor-induced angiogenesis [78]. PLD1 is expressed in vascular endothelial cell, and its deficiency decreases the activation of Akt and p38 MAP kinase signaling induced by vascular endothelial cell growth factor (VEGF), thereby suppressing the adhesion of endothelial cells. The siRNA-mediated PLD1 depletion inhibits endothelial cell migration and proliferation stimulated by VEGF [80], suggesting that a PLD1-mediated signaling pathway is essential for VEGF-induced tumor neoangiogenesis.

In addition to the roles of PLD1 in tumor angiogenesis described above, it has also been demonstrated that PLD1 regulates tumor metastasis through the modulation of platelet function [78]. Ablation of *PLD1* in mice incurs damage in lung metastases of intravenously injected melanoma cells. Platelet can aggregate with tumor cells, which is mediated by multiple types of receptors, and tumor cells thus coated by platelet are vital for lodging of the tumor cells in the lung [81]. $\alpha_{IIb}\beta_3$ integrin in platelets contributes to the interaction with tumor cells, since blockage of $\alpha_{IIb}\beta_3$ integrin by a specific antibody inhibits the interaction between platelets and tumor cells and suppresses the lung metastasis of tumor cells [78]. Intriguingly, platelets prepared from *PLD1* knockout mice are impaired in the $\alpha_{IIb}\beta_3$ activation [82] and in their aggregation with tumor cells, resulting in the decreased seeding of tumor cells into the lung parenchyma [78]. Thus, PLD1 in the tumor microenvironmental cells is required for both primary tumor growth and metastasis.

21.5.2 Cardiovascular and Cerebrovascular Disease

Platelets aggregation is also a critical event in the thrombus formation, which causes cardiovascular and cerebrovascular diseases such as ischemic myocardial and brain infarction and stroke. It is noteworthy that *PLD1* knockout mice show resistance to pulmonary emboli and arterial occlusion induced by vascular injury and to neuronal damage induced by focal cerebral ischemia, without significant effects on normal hemostasis [82]. Platelets prepared from *PLD1* knockout mice display the impaired $\alpha_{IIb}\beta_3$ integrin activation and the defect in aggregate formation on collagen under high shear flow conditions, but not under low or intermediate shear conditions. Platelets adhesion and aggregation strictly depend on the interaction between platelet surface glycoprotein Ib-V-IX (GPIb) and von Willebrand factor (vWF) [83], which binds to subendothelial collagen, suggesting that PLD1 in platelets plays a role in a signaling pathway downstream of vWV-GPIb interaction to regulate $\alpha_{IIb}\beta_3$ integrin activation.

Interestingly, mutations in PLD2 have been identified as hypertensive risk factors by large-scale genetic screening [84]. Moreover, it has been reported that PLD2 is involved in arterial wall thickening, which cause hypertensive disease [85]. It has been shown that PLD2 can also catalyze the hydrolysis of lysophosphatidylcholine to generate another type of lipid second messenger, cyclic PA (cPA) [85]. Moreover, cPA directly inhibits the function of the nuclear hormone receptor, PPARγ, which plays essential roles in regulation of lipid and glucose homeostasis via its transcriptional activity. The activation of PPARγ has a direct impact in

atherosclerosis by promoting the uptake of low-density lipoprotein (LDL) in macrophage, leading to foam cell formation at arterial wall, which induces arterial wall thickening. PLD2 activity suppresses PPARγ activation, and its unconventional product cPA attenuates arterial wall thickening induced by PPARγ agonist in a rat in vivo model [85]. Taken together, these findings indicate functional importance of PLD2 in the regulation of blood pressure.

21.5.3 Other Pathological Functions of PLD

It has been demonstrated that PLD is involved in Alzheimer's disease (AD). Elevation of PLD activity was detected in oligomeric amyloid β (Aβ)-treated cultured neuronal cells and in the brain prepared from mice overexpressing with the amyloid precursor protein, which constitute a genetic model for AD [86]. Interestingly, genetic ablation of *PLD2* in AD model mice, which are normal in their viability and fertility, rescues the impaired learning and memory [86]. Furthermore, Aβ-induced suppression of long-term potentiation in hippocampal slices was improved by *PLD2* ablation, suggesting that PLD2 plays a critical role in the synaptotoxic action of Aβ.

PLDs also play roles in immune cell functions. Genetic ablation of either *PLD1* or *PLD2* inhibits the uptake of opsonized beads or bacteria by macrophages with the defect in actin cytoskeleton organization, inducing the abnormal phagosomal cup formation [61]. Neutrophils also have important functions in bacterial infections and inflammation. It has also been demonstrated that *PLD1*-deficient neutrophils exhibit defect in chemoattractant-dependent migration. In addition, neutrophils recruitment to the pancreas following acute pancreatitis is impaired in *PLD1* knockout mice [61]. Collectively, these findings suggest that PLD-driven processes in immune cells are important for the host defense against bacterial infection and for inflammation.

21.6 Conclusions

Many works have expanded our understanding of molecular details of mammalian PLD in their structures, the regulatory mechanisms, and their functions in the cell. In addition, generation of *PLD* knockout mice allows us to provide insight into the implication of PLD functions in several diseases, such as cancer, cardiovascular and cerebrovascular disease, neurodegenerative diseases, and inflammation. Noteworthy, recently developed PLD-specific inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), can effectively prevent tumor growth and metastasis in mouse model [78]. Thus, growing evidence showing involvement of PLD in human health and disease strongly suggests that PLD or molecules involving PLD signaling pathways are valuable targets for therapeutic intervention.

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Chapter 22
Emerging Roles of Phospholipase D in Pathophysiological Signaling

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Abstract Phospholipase D (PLD) is a phospholipid-hydrolyzing enzyme that generates phosphatidic acid (PA) as a lipid second messenger by hydrolyzing phosphatidyl choline (PC). Various extracellular signals have been reported to activate PLD, which acts as a key mediator of many cellular functions through the generation of PA and the interactions of PLD and PA with their binding partners. Currently, about 60 PLD-binding partners, including proteins and phospholipids, are known, and PA has been found to interact with about 50 proteins. Although the interactions

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8_22,

of binding molecules with PLD and PA are complex and multilayered, the unique interactions between them are important for their unique intracellular functions. Here, we address the interrelationships between PLD and PA and their binding partners in several key signaling pathways, such as the EGFR–ERK signaling axis, nutrient/growth signaling axis, and cytoskeletal reorganization machinery axis. These interrelationships demonstrate dynamic interactions and cooperative regulation, which mediate special intracellular functions. Furthermore, we describe the regulation and functions of PLD in mediating normal and pathological signaling. Additionally, we summarize the roles of PLD as determined in animal studies (*Drosophila,* zebrafish, and mice) and changes in the PLD expression level in disease states. These findings provide new insight into the functions of PLD under pathophysiological conditions.

Keywords Phospholipase D • Signaling pathway • Pathophysiology • EGFR signaling • Growth signaling • Cytoskeletal reorganization

22.1 Introduction

Phospholipase D (PLD) hydrolyzes phosphatidyl choline (PC) to generate choline and phosphatidic acid (PA), which acts as a second messenger [1]. In mammals, two PLD isozymes (PLD1 and PLD2) are known to hydrolyze PC (Fig. 22.1) [2–4]. They show about 50 % sequence identity and have several conserved domains, including the phox homology (PX) domain, pleckstrin homology (PH) domain, and two HKD (so-called because they contain the HxKxxxxD motif) domains (Fig. 22.1b) [5, 6]. The PX domain of PLD is reportedly involved in interactions with phospholipids, such as phosphatidylinositol 5-phosphate (PtdIns5P) and phosphatidylinositol 3,4,5-phosphate (PtdIns(3,4,5)P_3), and proteins, including dynamin and RhoA [7–11]. The PLD–PH domain also interacts with phosphatidylinositol 4,5-phosphate (PtdIns(4,5)P_2) to regulate its intracellular localization and with Rac2 to serve as a guanine nucleotide exchange factor (GEF) [12, 13]. The PLD–HKD domain has a conserved catalytic motif (the previously mentioned HxKxxxxD) to directly mediate PC-hydrolysis [1]. The main difference in the primary structure of PLD isozymes is the presence of a loop region: PLD1 has a loop region and shows low basal activity, while PLD2 lacks this region and exhibits high basal activity [3, 14–16]. Therefore, the loop region of PLD1 may have an autoinhibitory function. In addition, PLD1 was reported to be localized mainly in perinuclear regions such as the endoplasmic reticulum, Golgi apparatus, endosomes, and lysosomes, and PLD2 to be localized predominantly in the plasma membrane [17–19]. Recently, however, PLD1 was identified in the plasma membrane and PLD2 was found to be localized in the Golgi apparatus [20–22].

Various extracellular signals activate PLD to generate PA [23]. PLD activity can be regulated by dynamic interactions with multiple classes of binding proteins (GTP-binding proteins, protein kinases, and structural proteins) and phospholipids

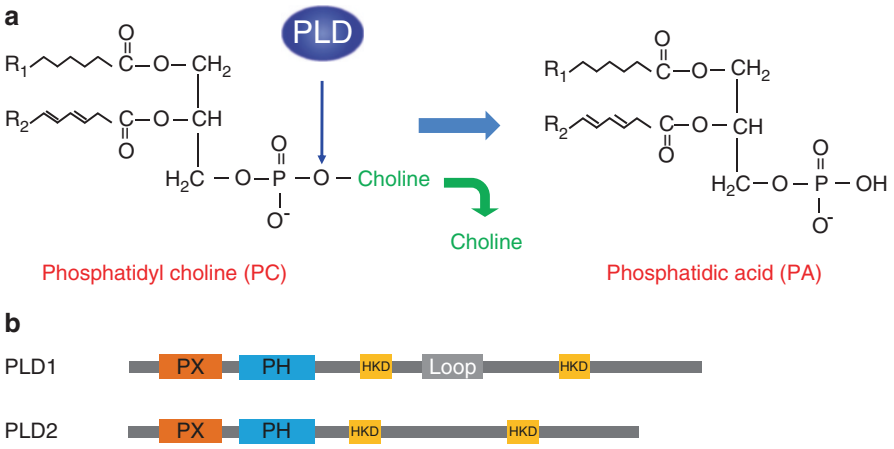

Fig. 22.1 Phosphatidyl choline (PC) hydrolysis by PLD and its isozymes. (**a**) PLDs hydrolyze the phosphodiester bond of phosphatidyl choline (PC) in membranes to yield free choline and phosphatidic acid (PA). (**b**) Two isoforms of PC-hydrolyzing PLD were identified in mammalian systems, PLD1 and PLD2. These isoforms share ~50 % sequence identities and are composed of conserved domains. Two HKD domains, which contain the HxKxxxxD sequence, comprise the catalytic motifs mediating the hydrolysis of PC. The phox homology (PX) and pleckstrin homology (PH) domains located in the N-terminus mediate the interaction of PLD with lipids or proteins. Only PLD1 has a loop region, which functions as a negative regulatory element; PLD2 thus has a higher basal activity than PLD1

(PA, PtdIns5P, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3) [24, 25]. Furthermore, PA as a second messenger is known to have a variety of binding partners (PtdIns(4)P 5-kinase, mammalian target of rapamycin (mTOR), Raf, Rac, and SOS) to mediate cellular functions such as cell growth, proliferation, differentiation, migration, exocytosis, endocytosis, and cytoskeletal reorganization [24]. Multiple cellular functions mediated by PLD and PA have been revealed mainly by cell-based analyses. Recently, however, the pathophysiological roles of PLD have been demonstrated using animal models, such as *Drosophila*, zebrafish (*Danio rerio*), and *Pld* knockout (KO) mice [26–28]. In this chapter, we summarize the dynamic interactions and cooperative regulation between PLD and PA and their binding partners (proteins and phospholipids) in several major PLD–PA pathways/networks and address their functions in pathological and physiological processes.

22.2 PLD in the EGFR–ERK Signaling Axis

The EGF signaling pathway, which is representative of receptor tyrosine kinase pathways [29], is one of the most important in determining cell fate [30, 31]. Activation of the EGF receptor (EGFR) by autotyrosine phosphorylation can recruit multiple downstream molecules (PtdIns 3-kinase, phospholipase

Cγ (PLCγ), Src, Grb2, Shc, PTP-1B, and SHP-1) to docking sites (phosphotyrosine residues). The recruitment of the molecules to EGFR transmits signals to a unique pathway and/or complex network. Eventually, EGFR is internalized by endocytosis, and EGF signaling is turned off though the activation of phosphatase and the process of negative feedback to terminate interactions between EGFR and its binding partners [32].

PLD is considered to be one of the key mediators of EGF signaling [25], and PLD can be activated by EGF signals in a wide range of cell types, including HEK 293 [33], HeLa [34], osteoblastic [35], C2C12 [36], liver [37], immortalized epithelial [38], and pancreatic cells [39]. EGF-induced PLD activation can be mediated by various upstream binding partners, such as small GTP-binding proteins (Rac1, Arf4, and Ras), protein kinase C (PKC), and cyclin-dependent kinase 5 (Cdk5). However, the mechanism of PLD activation by EGF depends on the cell type and cell context. For example, the activation of PLD by EGF was found to be mediated by PKC in Swiss 3T3, HEK 293, and 3Y1 cells [40–42]. PKC, however, reportedly was not involved in EGF-induced PLD activation in A431 cells [43]. Furthermore, Rac1, but not PKC, was shown to mediate PLD activation by EGF in Rat1 fibroblasts [44].

Currently, about 15 binding partners are known to have interrelationships with PLD and PA to mediate EGF signaling (Fig. 22.2) [25]. They can be categorized into several classes such as GTP-binding proteins, protein kinases, inhibitory proteins, and phospholipids. Many reports have suggested that small GTPases, including the Rho, Arf, and Ras families, directly bind to PLD and activate it in vitro [4, 6, 14, 45]. Furthermore, dominant-negative mutant studies have reported that Rac1, Arf4, RalA, and Ras mediated EGF-induced PLD activation in vivo [41, 44, 46]. Recently, PLD was found to interact with dynamin as a large GTPase in an EGF-dependent manner, and their interaction is important for PLD activation by EGF [47]. PKC has also been reported to be a major PLD activator in vitro and in vivo [40, 48, 49]. In vitro, PKC can directly activate PLD in a phosphorylation-independent manner [6]. However, multiple residues of PLD1 (S2, T147, and S561) were reportedly phosphorylated by PKCα and EGF-induced phosphorylation and activation of PLD1 by PKCα in vivo [50, 51]. Cdk5 was also found to mediate EGF-induced phosphorylation (S134) and activation of PLD2, but not PLD1 [52]. Although both Src and EGFR can also phosphorylate PLD, these tyrosine phosphorylations were not involved in PLD activation by EGF [53]. Furthermore, Grb2 and PLCγ1 that were recruited into EGFR by EGF also interacted with PLD, and these interactions contributed to EGF-induced PLD activation [54, 55]. Since munc-18 is an inhibitor of PLD, it basally binds to PLD to inhibit PLD activity. EGF triggers the dissociation of munc-18 from PLD to activate PLD [56]. In addition to proteins, phospholipids also affect PLD activation. PtdIns(4,5)P_2 is an essential cofactor for PLD activation [57]. PtdIns(3,4,5)P_3 was found to interact with the PX domain (R179) of PLD1, and this interaction was required for PDGF-induced PLD1 activation [9]. PA generation by PLD can activate many downstream molecules. PtdIns(4)P 5-kinase can be recruited to be activated by PA [58]. EGF-induced PtdIns(4,5)P_2 generation was

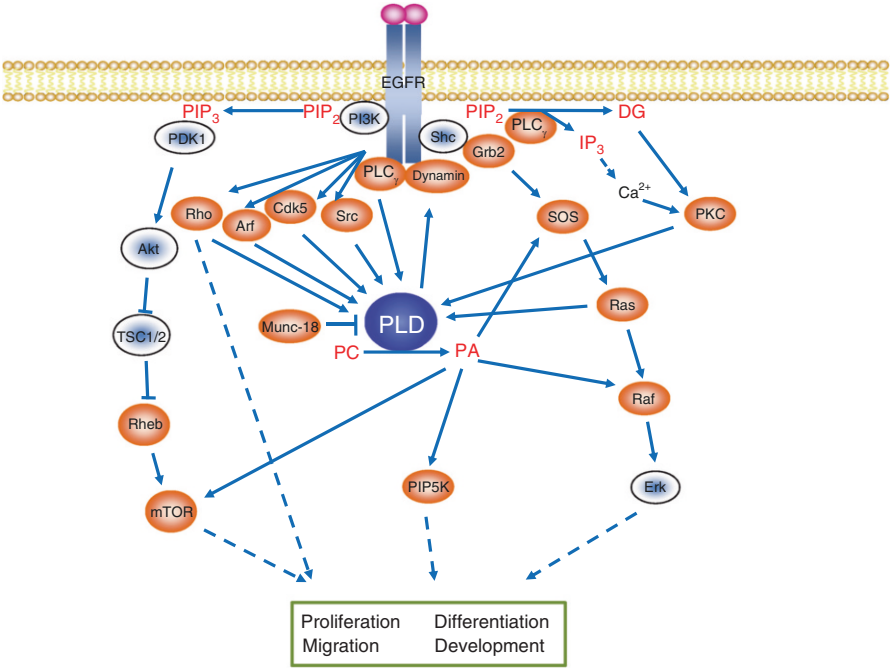

Fig. 22.2 PLD in the EGFR–ERK signaling axis. PLD is involved in the EGF signaling pathway through the generation of PA and direct interactions with several molecules. In its basal state, munc-18 binds to the PX domain of PLD1. When EGFR is activated, munc-18 is rapidly dissociated from PLD. PLC**γ**1 is recruited to the phosphotyrosine residue of EGFR and interacts with PLD. This interaction is important for EGF-induced activation of PLC**γ**1 and PLD. PLC**γ**1 generates DAG and inositol 1,4,5-trisphosphate (IP_3) from PtdIns(4,5)P_2 (PIP_2). DAG and IP_3-induced calcium secretion activate PKC. Activated PKC phosphorylates PLD1 and contributes to PLD1 activation. Another protein kinase, Cdk5, and Src can phosphorylate PLD. However, Src-induced phosphorylation of PLD is not required for PLD activation. Small GTPases such as Rho, Arf, and Ras are well-known upstream binding partners that mediate EGF-induced activation of PLD. The adaptor protein Grb2 interacts with PLD2, and this interaction is essential for EGF-induced activity and intracellular localization of PLD2. PA generated by PLD contributes to EGF signaling through binding to several proteins. SOS interacts with PA and is translocated to the membrane, mediating EGF-induced Ras signaling. PA recruits PtdIns(4)P 5-kinase to generate PIP_2, which binds with dynamin, a large GTPase involved in EGFR endocytosis. PLD has the GAP property for dynamin. Therefore, PLD and PA generation serve as important regulators of EGFR-induced endocytosis. EGF activates the PtdIns-3-kinase (PI3K)/Akt signaling pathway through recruitment of PI3K to the plasma membrane, PtdIns(3,4,5)P_3 generation by PI3K, and the sequential activation of Akt and mTOR. In this signaling pathway, binding of PLD and PA with mTOR increases the activity of mTOR. Finally, PLD contributes to EGF-mediated cellular functions—such as survival, migration, differentiation, and development—through several interrelationships with various components in the EGFR signaling pathway. These roles of PLD and PA depend on the cell type and cell context. The orange circle indicates the binding partner of PLD and/or PA. This figure presents a simplified pathway of the role of PLD and PA and does not provide a complete signaling pathway

mediated by activation of PtdIns(4)P 5-kinase by PA. Recently, PA generation by EGF signaling was reported to recruit *son of sevenless homolog* (SOS) into membranes, and this recruitment was critical for mediating the Ras–Raf–ERK cascade in EGF signaling [59].

As noted above, PLD has a variety of binding partners. Among them, many (munc-18, PLCγ1, munc-18, dynamin, PKCα, Grb2, and PtdIns(3,4,5)P_3) interact with the PX domain of PLD to mediate EGF signaling. In its basal state, munc-18 interacts with PLD resulting in blocking of its activation, and EGF stimulation triggers a dissociation of munc-18 from PLD [56]. EGFR can recruit PLCγ1, and PLD can bind and activate PLCγ1, which acts as a GEF for dynamin and as a generator of IP_3 and diacylglycerol (DAG) for activation of PKCα [55]. Then, PLD can be activated by interacting with GTP-loaded dynamin and phosphorylation by PKCα [47, 51]. At the same time, PLD serves as a GTPase-activating protein (GAP) for dynamin, which mediates EGFR endocytosis to remove EGFR from the cell surface [10]. PA generation by PLD activation can recruit SOS, which acts as a GEF for Ras [59]. Eventually, GTP–Ras sequentially activates the MAP kinase cascade, a key pathway in EGF signaling. These dynamic interactions between the PLD–PX domain and its binding molecules may mediate spatiotemporal activation of EGF signaling and regulate signaling strength and duration.

EGF as a representative of survival/proliferation signaling is known to mediate various cellular physiological functions, such as proliferation, survival, migration, differentiation, and development [30]. PLD has also been reported to be critically involved in EGF-induced cell proliferation [53, 60]. In the case of vesicle trafficking, PLD has been implicated in EGFR endocytosis [10] and in EGF-induced insulin secretion (exocytosis) of pancreatic islets and a pancreatic beta cell line [61]. In another study, PA as a product of PLD activation was found to mediate EGF-dependent cell motility in Madin–Darby canine kidney cells [62]. Furthermore, the recruitment and activation of PtdIns(4)P 5-kinase by PA was required for EGF-mediated cell spreading through the production of PtdIns(4,5)P_2 [58]. In addition to these cellular physiological functions, EGF signaling has been implicated in pathological functions such as tumorigenesis. Many different types of cancer exhibit dysregulation of EGF signaling through changes in the expression level and mutation of key mediators in the EGF signaling pathway [63, 64]. Furthermore, several cancer cells such as gastric, breast, and colon cancer show an increase in PLD activity and expression [65–67]. Also, the elevated expression level of PLD and EGFR contributes to cellular transformation in 3Y1 fibroblasts [68]. PA generation by PLD activation was found to potentiate the transforming activity of Ras through the recruitment of SOS to membranes in NIH3T3 cells [59]. In addition to the transformation activity of PLD, it is known to be required for matrix metalloproteinase (MMP)-9 and MMP-2 secretion in colon cancer cells and glioma cells, respectively [69, 70]. These findings suggest that PLD is a key factor in regulation of the pathophysiological function of EGF signaling.

22.3 PLD in the Growth/Nutrient Signaling Axis

Cells perceive a change in environmental condition (e.g., nutrient level and growth signals) and regulate cell growth and energy homeostasis through accurate and efficient signaling. Key players regulate cell growth and energy homeostasis in intracellular signaling: the mTOR is a Ser/The protein kinase that plays key roles in signaling by integrating many kinds of incoming signals, including growth factors, amino acids, and glucose level [71–74]. mTOR forms mTOR complex 1 (mTORC1) with several proteins, such as regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL), proline-rich AKT substrate 40 kDa, and DEP-domain-containing mTOR-interacting protein (Deptor) [71]. Raptor recruits substrates for phosphorylation by mTOR [75]. The two best-characterized targets of mTORC1 are S6 kinase 1 and eukaryotic initiation factor-4E-binding protein 1 (4EBP1), which regulate protein synthesis (Fig. 22.3) [76]. Rapamycin binds to the FKBP12–rapamycin-binding (FRB) domain of mTOR as a complex with FKBP12 and inhibits mTORC1 activity (Fig. 22.3) [77]. The Ras homolog enriched in brain (Rheb), a small GTPase, is a well-known direct activator of mTORC1 (Fig. 22.3) [78]. Rag GTPases [79, 80] and class III phosphatidylinositol 3-kinase (hVPS34) [81, 82] are also required for the regulation of mTORC1 by amino acids (Fig. 22.3). In addition, glucose transporters (GLUTs) and AMP-activated protein kinase (AMPK) have also been known to play a key role in regulating glucose homeostasis and energy balance [83]. In this part, we will describe the molecular mechanism and roles of PLD in nutrient/growth signaling.

In growth signaling, both PLD and PA can affect the regulation of mTORC1 [72, 84]. PA can directly bind to the FRB domain of mTOR and compete with rapamycin for binding to mTOR (Fig. 22.3) [85, 86]. Furthermore, PA is also required for the formation and stabilization of mTOR complexes [87]. PLD is also involved in mTORC1 signaling. The PLD2 PH domain binds to Raptor of mTORC1 [88]; this PLD2-Raptor binding is required to activate mTORC1 [88]. Note that Rheb interacts with and activates PLD to activate mTORC1 in a GTP-dependent manner (Fig. 22.3) [29]. In other words, PLD appears as an effector of Rheb in mTORC1 signaling. These findings suggest that PLD and PA may form a functional complex with Rheb and mTORC1 to transduce mitogenic signals. mTORC1 also senses nutrient (amino acids and glucose) signals [71, 73]. Moreover, PLD and PA mediate nutrient-induced activation of mTORC1 as well as the mitogenic activation of mTORC1 (Fig. 22.3) [72, 84]. However, amino acids have no impact on the signals that regulate Rheb [78, 89]. Nutrient-induced PLD and mTORC1 activation are dependent on the GTPases RalA and ARF6 (Fig. 22.3) [90], which are known to be the binding partners of PLD [91]. hVps34 is required for amino acid-dependent PLD activation, and hVps34-produced PtdIns3P interacts with the PX domain of PLD and activates PLD (Fig. 22.3) [90].

Glucose is the most fundamental source of energy for all eukaryotic cells [92]. The transport proteins, called GLUTs, serve as shuttles to transport glucose across the cell surface (Fig. 22.3) [93]. This glucose homeostasis is a physiologically

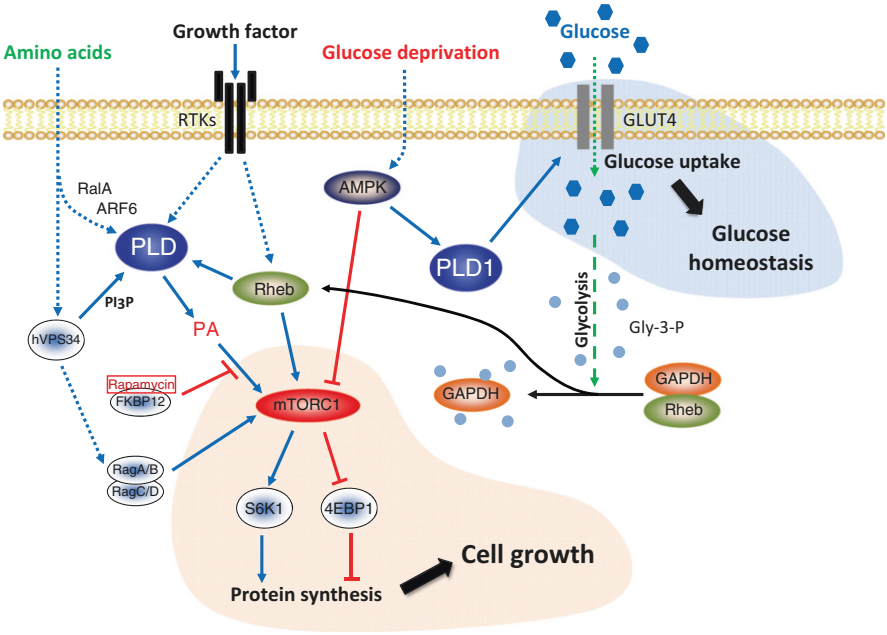

Fig. 22.3 PLD in the growth/nutrient signaling axis. PLD plays critical roles in growth and nutrient signaling and is activated by growth factors, amino acids, and glucose. PLD activation by growth factors and nutrients enhance the activity of mammalian target of rapamycin (mTOR) complex 1 (mTORC1), which is a key regulator of cell growth. mTORC1 phosphorylates S6 kinase 1 (S6K1) and eukaryotic initiation factor (4EBP1), which regulate protein synthesis. Rapamycin binds to mTORC1 and inhibits mTORC1 activity. PA can bind to and activate mTORC1 and competes with rapamycin. Ras homolog enriched in brain (Rheb) is required for mTORC1 activation by growth factors and activates PLD1. RalA and ARF6 mediate the PLD activation by nutrients. The class III phosphatidylinositol 3-kinase (hVPS34) can activate PLD through its product (PI3P). AMP-activated protein kinase (AMPK) stimulates PLD1 activity during glucose deprivation and regulates glucose uptake through GLUT4. Furthermore, AMPK can also decrease mTORC1 activity. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) basally binds to and inhibits Rheb. The GAPDH–Rheb interaction is disrupted in a substrate-dependent manner (glyceraldehyde 3-phosphate; Gly-3-P)

well-balanced mechanism and an essential process for cell survival and growth [92]. AMPK is known to play a critical role in the regulation of energy balance [92]; it has been reported to stimulate PLD1 activity through the phosphorylation of serine 505 induced by glucose deprivation and to play a key role in the regulation of glucose uptake through GLUT4 translocation in muscle cells [94]. These findings suggest that the AMPK–PLD1 pathway may contribute to the control of glucose homeostasis (Fig. 22.3). Glucose deprivation has been reported to lessen mTORC1 signaling through AMPK [95], but is suggested to active PLD signaling [90]. As noted above, PLD signaling can activate mTOR signaling by mitogens and nutrients [72, 84]. The relationship between PLD and mTORC1 seems to be dependent on conditions that include the cell type and signal. Therefore, further

characterization is required to determine the relationships among glucose availability, PLD, and mTOR signaling.

As stated previously, glucose deprivation can induce the activation of PLD in muscle cells, but can inhibit PLD activity in human cancer cells [90]. PLD activity decreased when the medium lacked glucose [90]. Glucose signals can increase the activity of both PLD and mTORC1 [90], and glycolytic flux can also regulate mTORC1 signaling through glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [96]. GAPDH (a PLD-binding protein) can basally bind to Rheb and inhibit its activity (Fig. 22.3) [96], and the GAPDH–Rheb interaction is disrupted in a substrate-dependent manner by a glucose influx [96]. The GAPDH–Rheb pathway functions independently of AMPK [96]. Moreover, hydrogen peroxide reportedly induces an association between GAPDH and PLD2 to facilitate PLD2 activation in PC12 cells [97]. However, how the GAPDH–PLD2 interaction contributes to mTORC1 signaling remains unclear.

Elevated PLD activity and/or levels have been identified in a large number of human cancers and are reported to enhance survival [65, 67, 98, 99]. As mentioned above, PA contributes to activation of mTOR in a competitive manner with rapamycin, and elevated PLD activity confers rapamycin resistance on human cancer cells [86]. Because PLD activity is elevated in a large number of human cancers, elevated levels of PA in cancer cells may hinder successful treatment with rapamycin [84, 100]. Therefore, suppressing PLD activity and reducing PA levels may increase sensitivity to rapamycin [84]. Furthermore, AMPK-mediated PLD1 activation is essential for glucose uptake when glucose levels in muscle cells are low [94], implying that PLD signaling is important for glucose homeostasis and is associated with metabolic syndrome (e.g., diabetes). The interaction between AMPK and PLD1 may facilitate resolution of difficult issues in metabolic diseases.

22.4 PLD in the Cytoskeletal Reorganization Machinery Axis

In response to multiple signals and stimuli, cells must change their shapes or locations. These tasks are achieved by reorganizing their filamentous system, the cytoskeleton. Reorganization of the cytoskeleton is accompanied by dynamic changes in the polymerization/depolymerization of actin and/or tubulin, and a variety of proteins and phospholipids participate in the process [101, 102]. In particular, integrin, which can mediate both outside-in and inside-out signaling, is a representative signal that contributes to cell adhesion, spreading, and migration [103]. Also, Rho family GTPases, such as RhoA, Rac, and Cdc42, are master regulators of actin cytoskeletal rearrangements [104]. Rho induces the formation of stress fibers, while Rac is required for lamellipodia formation and Cdc42 regulates filopodia generation. In migrating cells, Rho is activated at the rear of the cell, and Rac and Cdc42 are stimulated at the leading edges. In addition, WASP and WAVE are important proteins essential for actin polymerization. GTP–Rac directly and indirectly can

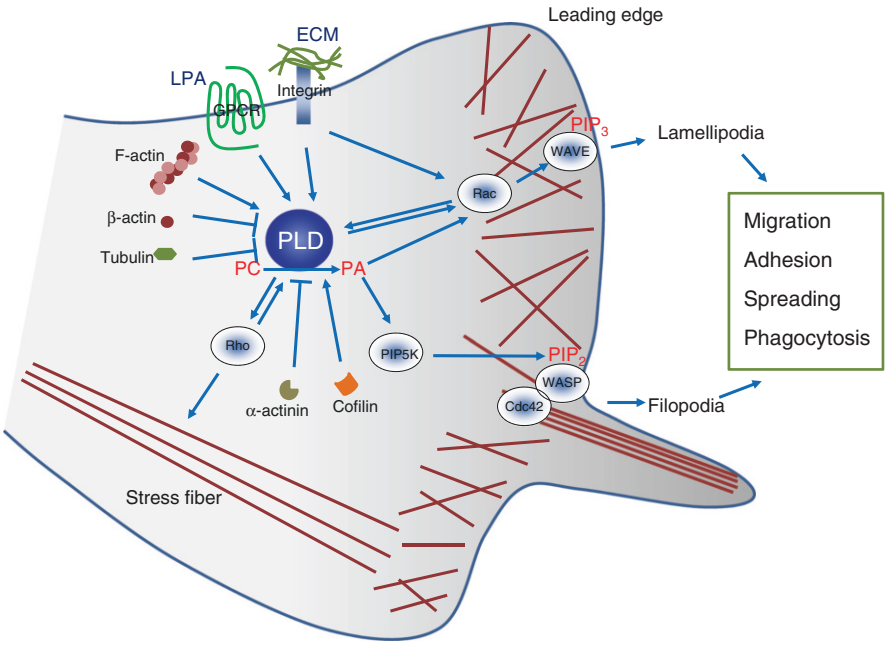

Fig. 22.4 PLD in the cytoskeletal reorganization machinery axis. PLD is implicated in cell migration, adhesion, spreading, and phagocytosis through regulation of the cytoskeleton. The function of PLD in cytoskeletal reorganization is conferred mainly by interactions with several small GTPases, such as Rho, Rac, and Cdc42, and cytoskeletal proteins. In response to several extracellular stimuli, PLD can activate small GTPases and cytoskeletal-related proteins and be activated by them. PA generation by PLD activation mediates the dissociation of Rho-guanine nucleotide dissociation inhibitor (GDI) and recruits Rac to the plasma membrane. Rac activates the WAVE complex, which mediates lamellipodia formation. Cdc42 induces WASP complex formation and filopodia generation. PA induces PtdIns(4)P 5-kinase (PIP5K) recruitment and PtdIns(4,5)P_2 (PIP_2) generation, which binds to the WASP complex. Furthermore, PLD activates Rho via its GEF activity. Activated Rho induces stress fiber formation. Cytoskeleton proteins and cytoskeletal-related proteins also contribute to the regulation of PLD activity. Filamentous actin (F-actin) and cofilin increase PLD activity, while monomeric β-actin, tubulin, and α-actinin have been known to inhibit PLD activity. Although PLD and PA are clearly important regulators of cytoskeletal reorganization, their exact role depends on the cell type, cellular context, and stimuli

activate the WAVE complex, and GTP–Cdc42 directly forms a complex with WASP [105]. Also, PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 interact with the WAVE and WASP complexes, respectively, to induce actin polymerization [105].

PLD has been implicated in signaling to regulate cytoskeletal dynamics (Fig. 22.4) [106]. As described in the section on the EGFR–ERK signaling axis, PLD is activated by small GTPases (Rho, Rac, cdc42, Arf, and Ras) that can induce cytoskeletal reorganization. PA generation through integrin-mediated PLD activation has been reported to recruit GTP-loaded Rac1 to the plasma membrane and to dissociate Rho-guanine nucleotide dissociation inhibitor (GDI) from Rac1, with PA generation eventually being required for cell spreading and migration [107].

Also, integrin-mediated PA generation was found to recruit and activate PtdIns(4)P 5-kinase to generate PtdIns(4,5)P_2, which can form a complex with WASP to induce actin polymerization [58]. Recently, PLD2 was reported to directly activate some Rho GTPases by acting as a GEF. Jeon et al. reported that PLD2 can act as a GEF for RhoA and that its GEF activity for RhoA was required for the formation of lysophosphatidic acid (LPA)-induced stress fibers [11]. In addition, PLD2 has been identified to have GEF activity for Rac2, and its Rac2–GEF activity was found to increase chemotaxis and phagocytosis [108, 109]. Furthermore, PLD can also be regulated by actin, tubulin, and actin-binding proteins. Monomeric β-actin was found to inhibit PLD activity directly in vitro, but filamentous actin (F-actin) was reported to activate PLD in vitro [110, 111]. Moreover, monomeric tubulin can interact with PLD to inhibit its activity, and their interaction is required for the transient activation of PLD by carbachol [112]. Also, α-actinin as an actin-binding protein was found to inhibit PLD [113], but in the case of cofilin, another actin-binding protein, PLD activity was increased by interactions with phosphorylated cofilin to mediate the formation of carbachol-induced stress fibers [114]. Although cytoskeletal proteins (β-actin, tubulin, α-actinin, and cofilin) can directly regulate PLD activity, further studies of the interrelationships between PLD and cytoskeletal proteins are required to reveal the detailed mechanism of cytoskeletal dynamics.

Cytoskeletal reorganization in cells is required to mediate essential cellular functions such as cell polarization, adhesion, spreading, migration, intracellular vesicle trafficking, and cell division (cytokinesis), which are involved in pathophysiological phenomena, including morphogenesis, development, neurite outgrowth, inflammation, and metastasis [115, 116]. PLD was also reported to be involved in cytoskeleton-mediated cellular functions such as phagocytosis, neurite outgrowth, and cytokinesis [26, 117, 118]. As described above, PLD can be activated by several cytoskeletal proteins and their regulators and activate them to mediate many cellular functions through cytoskeletal rearrangements. LPA-mediated stress fiber formation was induced by the GEF activity of PLD for RhoA [11]. Furthermore, PA generation by PLD activation was reported to be important for migration of many cell types, such as fibroblasts, cancer cells, and epithelial cells [107, 119, 120]. Meanwhile, PLD's GEF activity for Rac2 has been implicated in MCSF-induced cell migration (chemotaxis) in RAW264.7 macrophages [109]. These findings suggest that PLD-mediated PA or PLD itself can transmit the signals for stress fiber formation and migration in an extracellular stimuli- and/or cell context-dependent manner. Additionally, reports of PLD-mediated cell spreading are contradictory. Du et al. reported that PLD2 negatively contributed to fibronectin-induced cell spreading in Chinese hamster ovary cells [121], but Chae et al. suggested that PLD2 facilitated cell spreading by fibronectin in human ovarian cancer (OVCAR-3) cells [107]. Therefore, to understand the exact roles of PLD in cytoskeletal dynamics, global approaches that integrate and consider many different conditions (cell types, cell contexts, and extracellular signals) are required. In addition to its physiological roles in cytoskeletal rearrangement, PLD has been implicated in pathological states such as metastasis/invasion, thrombosis, and ischemic brain infarction. Over-expression of PLD or increased PLD activity has been reported to enhance cell invasion and

metastasis in several cell types including glioma cells, MDA-MB-231 breast cancer cells, and EL4 mouse lymphoma cells [70, 122, 123]. Also, defects in PLD1 were recently reported to impair integrin-mediated adhesion and aggregation of platelets, and eventually show protective effects against thrombosis and ischemic brain infarction [28]. This issue will be addressed in detail in the following section.

22.5 Pathophysiological Function of PLD In Vivo

Although several cellular functions of PLD have been studied, its in vivo functions at an organismal level remain poorly investigated. Changes in the expression patterns and activity of PLD under pathophysiological conditions could provide clues as to what to expect regarding its functions in vivo. In rodents, the expression level of PLD during developmental stages was reported in some tissues such as the brain, eye, lung, testis, and heart. PLD2 mRNA has been detected in all brain regions investigated and increases progressively during postnatal development of the rat brain [124]. Dynamic expression patterns of PLD1 in several cell types were reported during retina development [125]. PLD1 expression was reported in the heart at embryonic day 17 [126]. In postnatal development, both PLD1 and PLD2 expressions were detected in the lung and testis [127, 128]. Those of expression patterns could suggest a role for PLD in developmental stages. Under pathological conditions, especially cancer, increased expression of PLD was observed in several human cancers. PLD1 expression is elevated in breast, renal, and colorectal cancers [65–67]. In colorectal cancer, the expression level of PLD2 was significantly correlated with tumor size and the survival rate of patients. PLD is implicated in the pathogenesis of Alzheimer's disease (AD) [129]. Amyloid precursor protein (APP) stimulates PLD activity and PLD1 expression is increased in the brain of patients with AD. The role of PLD2 under pathological conditions induced by hypoxia in the rat brain was reported; during apoptosis of the cerebellum and brain stem induced by focal cerebral hypoxic–ischemic injury, PLD2 transcript levels transiently decrease [130]. PLD2 expression was increased in a hypoxic hippocampus induced by four-vessel occlusion, and PLD2 over-expression was found to inhibit cultured neuronal cell death induced by hypoxia [131]. These reports suggest that PLD has important functions in brain disease. Increased PLD1 mRNA expression, but not that of PLD2, in rat β-cells in response to IL-1β, a cytokine involved in the physiological and pathological functions of β-cells, suggests a role for PLDs in metabolism [132].

Recently, in vivo pathophysiological roles of PLD have been reported in several organisms such as *Drosophila*, zebrafish, and mice (Table 22.1). The first evidence of in vivo functions of PLD comes from *Drosophila melanogaster*. Pld-deficient *Drosophila* showed delayed cellularization during embryogenesis [26]. Cellularization is the specialized process of converting the syncytial *Drosophila* embryo into thousands of distinct cells during development. Pld expression peaks prior to cellularization and localizes to small cytoplasmic vesicles during the

Table 22.1 In vivo pathophysiological function of PLD

Isotype	Genetic manipulation	Species	Phenotype	Reference
PLD	Knockout	*Drosophila*	Delayed cellularization during embryogenesis	[26]
			Decreased light sensitivity of photoreceptor cells and heightened susceptibility to retinal degradation	[133]
PLD1	Antisense morpholino oligonucleotide	Zebrafish	Impaired intersegmental vessel development	[27]
PLD1	Knockout	Mouse	Impaired $\alpha_{IIb}\beta_3$ integrin activation and protection from ischemic brain infarction	[28]
			Decreased starvation-induced macroautophagy in the liver	[134]
PLD2	Knockout	Mouse	Improved learning and memory in Alzheimer's disease	[135]

process. Genetic ablation of *Pld* reduces viability in early embryogenesis and leads to delayed cellularization with altered morphology of the Golgi apparatus and defective vesicle trafficking. Over-expression of Pld also reduces the viability of the embryo, indicating that maintaining the proper amount of Pld is necessary for normal development. The role of Pld in the adult *Drosophila* eye has also been reported [133]. In response to light stimuli, rhodopsin in the photoreceptor rhabdomeric microvilli is converted to metarhodopsin, and then G-protein and Plc are sequentially activated. DAG converted from PIP_2 by Plc mediates the opening of several classes of cation channels and induces depolarization. The roles of Pld in the phototransduction cycle were demonstrated by genetic approaches using several mutant flies. Pld localizes to the cell body and subrhabdomeral cisternae and regulates the phototransduction responsiveness of photoreceptor cells by maintaining the PIP_2 substrate pool. Moreover, Pld also functions in maintaining photoreceptor viability in the absence of ongoing phototransduction events.

The function of PLD in vertebrate development has been studied in the zebrafish. During early development, Pld1 is expressed dynamically in the notochord, the somites, and the liver. Inhibition of Pld1 expression in the embryo via injection of antisense morpholino oligonucleotides [49] results in vascular defects, especially in the intersegmental vessels, with edema and slower heartbeat. Transplantation experiments provided evidence that the function of Pld1 in the developing notochord is important for intersegmental blood vessel formation [27]. In both *Drosophila* and zebrafish, a lack of Pld revealed its developmental function, although developmental defects have not been reported in PLD-deficient mice. Both *Pld1* and *Pld2* KO mice are viable and fertile and seem to be healthy under normal conditions, suggesting that a deficiency of one isoform can be compensated for by the other isoform or by another PA-regulating enzyme such as DAG kinase, PA phosphatase, or LysoPA acetyltransferase. Under pathological conditions, *Pld* KO mice showed major

differences from wild-type mice such as impaired platelet aggregation and improved cognitive defects in AD. Therefore, the developmental function of PLD should be carefully evaluated in detail.

The role of PLD1 in integrin activation and aggregation of platelets was investigated [28]. The activation of integrin is an essential process for stable platelet adhesion and aggregation. In particular, $\alpha_{IIb}\beta_3$ is the principal receptor for platelet adhesion to the ECM in vivo. Integrin activation and agonist-induced binding of fibrinogen decreased in PLD1-deficient platelets without defects in degranulation. In in vitro experiments, platelets from PLD1-deficient mice aggregated unstably under high-shear conditions. In vivo thrombus formation also decreased in PLD1-deficient mice both in the ferric chloride injury model and aorta occlusion model, and PLD1-deficient mice were resistant to neuronal damage after focal cerebral ischemia in the transient middle cerebral artery occlusion model. PLD1 is also involved in macroautophagy in vivo. Macroautophagy is an essential catabolic mechanism that mediates the lysosomal degradation of defective cytosolic compartments through the formation of autophagosomes. This process is associated with several human diseases and physiologies, such as neurodegeneration, infectious diseases, and cancer. In HeLa cells and human cervical cancer cells, PLD1, but not PLD2, co-localizes with the autophagosome marker protein LC3 upon nutrient deprivation. PLD1 transferred to the outer membrane of amphisomes through the endosomal pathway, not the lysosomal pathway or through autophagosome formation. In the liver from *Pld1* KO mice after starvation for 24 h, the size and surface area of the LC3-positive compartment failed to increase. Although direct data regarding pathological phenotypes are lacking, increased aggregation of Tau and p62 in brain slices from human Tau transgenic mice upon treatment with the PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) suggests a role for PLD1 in autophagy-related pathogenesis [134]. PLD2 deficiency exerted protective effects on memory deficits exhibited in AD [135]. Amyloid β (Aβ) accumulation is one of the causes of AD. Oligomeric Aβ42 (oAβ42) treatment induces localization of PLD2 at the plasma membrane in a Ca^{2+}-dependent manner. PLD activity is increased in cultured neurons by oAβ42 treatment, but not in neurons from *Pld2* KO mice. Moreover, oAβ42-induced synaptic dysfunction and impaired long-term potentiation in the CA hippocampal region is suppressed in *Pld2* KO brain slices. Ablation of PLD2 ameliorates the defects of learning and memory in a transgenic AD model. This protective effect is mediated by restoring the synaptic protein level without altering the APP or Aβ levels.

The function of PLD in neutrophils is controversial. The roles of PLD in several functions of neutrophils through generation of reactive oxygen species (ROS) have been suggested. Most of the functions were investigated with primary alcohol as a PLD inhibitor. However, a primary alcohol such as 1-buthanol cannot discriminate isotypes of PLD, and off-target effects have been reported by several recent studies. Specifically, two reports described contradictory results regarding the previously reported functions of PLD in neutrophil physiology using the KO model and newly developed isotype-specific inhibitors of PLDs. One of them suggested that ROS generation in neutrophils induced by several stimuli such as phorbol 12-myristate

13-acetate, fibrinogen, polyvalent integrin ligand surface (pRGD), IgG-opsonized sheep red blood cells, and IgG immobilized as immune complexes are PLD1-dependent rather than PLD2-dependent [136]. However, neither PLD1 nor PLD2 are necessary for the migration and adhesion ability of neutrophils, which were previously reported to be PLD-dependent. They used freshly isolated neutrophils from wild-type and *Pld2* KO mice with a PLD dual inhibitor and a PLD1-specific inhibitor. The other report, however, suggests that both PLD1 and PLD2 are dispensable for ROS generation and degranulation induced by *N*-formyl-methionyl-leucyl-phenylalanine in neutrophils isolated from *Pld1* and *Pld2* KO mice [137], but that ethanol can suppress ROS generation and degranulation in *Pld1* and *Pld2* double-KO neutrophils. These results suggest off-target effects of primary alcohol. Although some systemic differences exist between previous reports and recent works, these two reports showed the importance of reliable models and isotype-specific inhibitors for investigating the physiological functions of PLDs. In addition, the validation of previously reported functions of PLDs might be required in vivo using genetic models and new inhibitors.

22.6 Conclusions

PLD as a key mediator of intracellular signaling is known to be activated by a variety of extracellular signals, including growth factors (EGF and PDGF, and VEGF), a hormone (insulin), and bioactive lipids (LPA and sphingosine 1-phosphate), and to mediate multiple pathophysiological functions, such as growth/proliferation, survival, migration, vesicle trafficking, tumorigenesis, metastasis, inflammation, thrombosis, and ischemic brain infarction. These functions are mediated primarily by PA as a product of PLD activation or by PLD itself without PA. As shown in Figs. 22.2, 22.3, and 22.4, PLD and PA are associated with a complex pathway/network and have many binding partners, and a highly complex and dynamic interrelationship between PLD and PA and interacting molecules existed in mediating downstream signaling. That is, PLD may act as a modulator that mediates the fine-tuning of intracellular signals and the cross-talk among multiple signaling networks. However, to verify this complex PLD signaling network, studies to determine further detailed mechanisms and a global analysis of the PLD signaling/network are needed. Furthermore, as shown in Table 22.1, *PLD* KO animal studies (*Drosophila*, zebrafish, and mice) have recently revealed in vivo pathophysiological roles of PLD. Further animal studies are also needed to gain novel insights into the functions of PLD. Recently, in addition to genetic models, a pan-PLD inhibitor (FIPI) and PLD isozyme-selective inhibitors have verified the roles of PLD in several functions, such as spreading, chemotaxis, and invasion, of breast cancer cells. Therefore, these studies will lead to further opportunities to better understand the pathophysiological function of PLD at a mechanistic level and to its use as a therapeutic agent for pathological processes such as tumorigenesis and autoimmunity.

Acknowledgments This work was supported by the grants (No. 2012R1A2A1A03010110 and NRF-M1AXA002-2010-0029764) of National Research Foundation funded by the Ministry of Education Science and Technology of Korea.

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Chapter 23
Alterations in Phospholipase D During the Development of Myocardial Disease

Paramjit S. Tappia and Naranjan S. Dhalla

Abstract Phospholipase D (PLD) produces phosphatidic acid, which is converted to diacylglycerol (DAG) by phosphatidate phosphohydrolase (PAP). Since both these lipid signaling molecules regulate Ca^{2+}-movements, they also influence cardiac contractile function. In this article, we discuss the importance of PLD in relation to the production of lipid signaling molecules and regulation of cardiac function under various pathophysiological conditions such as ischemic heart disease, diabetic cardiomyopathy, and congestive heart failure. In fact, marked alterations in PLD activities have been reported to occur in ischemic heart, diabetic heart, and failing heart. While the mechanisms of changes in PLD activities in heart disease may be of complex nature, oxidative stress seems to play a critical role in the activation of PLD. From the evidence provided it is suggested that impairment in this phospholipid signal transduction pathway results in cardiac dysfunction during the development of different myocardial diseases.

Keywords Phospholipase D • Signal transduction • Diabetic cardiomyopathy • Congestive heart failure • Ischemia-reperfusion injury • PLD-mediated signal transduction

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23.1 Introduction

The hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) produces phosphatidic acid (PA), which in turn is converted to 1, 2 DAG by the action of phosphatidate phosphohydrolase (PAP) [1, 2]. Both PLD and PAP are thus considered to modulate myocardial levels of PA and DAG. Different hormones such as norepinephrine, endothelin-1, and angiotensin II (Ang II) have been shown to increase formation of PA in cardiomyocytes [3, 4] and stimulate sarcolemmal (SL) and sarcoplasmic reticular (SR) Ca^{2+}-transport systems [5, 6]. Furthermore, PA has been reported to increase the intracellular concentration of free Ca^{2+} in adult cardiomyocytes and to augment cardiac contractile activity of the normal heart [5, 7]. DAG can also influence cardiac function through phosphorylation of myocardial proteins, including ion channels, via activation of protein kinase C (PKC) isozymes [8]. These PLD-mediatd signal transduction events are summarized in Fig. 23.1.

Two mammalian PLD isozymes, PLD1 and PLD2, have been cloned [9]. While PLD1 is localized to the Golgi apparatus and nuclei [10], PLD2 is the major myocardial PLD isozyme specifically localized to the SL membrane [11]; other subcellular localizations of PLD2 have also been reported [12, 13]. Interestingly, a transient expression of PLD1 during heart development in rats has been demonstrated [14]. In this regard, the level of PLD1 protein increased transiently from 0 to 3 days postpartum and declined gradually beginning 7 days after birth. This suggested that PLD1 protein in the heart is strongly associated with the early postnatal development of the heart in rats [14].

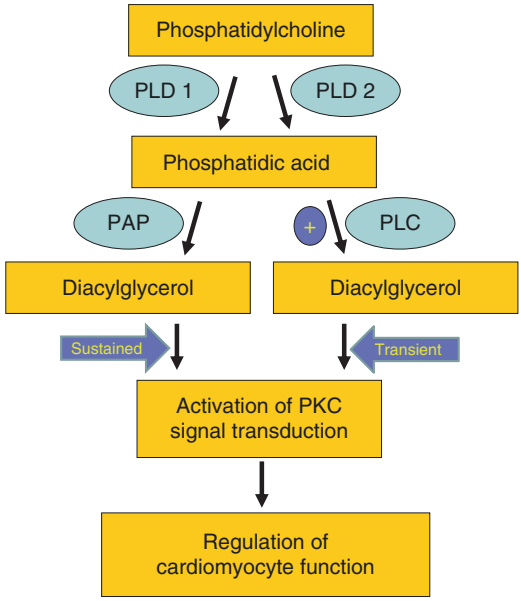

Fig. 23.1 Myocardial phospholipase D signal transduction. *PLD1* phospholipase D1, *PLD2* phospholipase D2, *PAP* phosphatidate phosphohydrolase, *PLC* phospholipase C, *PKC* protein kinase C; +, stimulation

PLD1 requires phosphatidylinositol 4, 5-bisphosphate (PIP_2) for its activity, which is stimulated by PKC and Rho small G-protein family members [9, 15–24]. PLD2 also requires PIP_2 for its activity [11], but, unlike PLD1, PLD2 is activated by unsaturated fatty acids [2, 16, 17, 25, 26] and is insensitive to the PLD1 activating factors [27]. It should be noted that PLD isozymes contain N-terminal PH (pleckstrin) and PX (phox) homology domains. Both these domains also interact specifically with distinct phosphoinositide ligands [28]. Both the PH and PX domains are important for PLD function by controlling the dynamic association of the enzyme with the plasma membrane. Thus, there are two modes of PLD regulation by phosphoinositides; stimulation of activity mediated by the polybasic domain and dynamic regulation of membrane targeting mediated by the PH and PX domains [28].

Some studies have shown that both receptor- and non-receptor coupled tyrosine kinases are involved in the regulation of PLD activity, in addition to serine/threonine kinases, Ca^{2+}-calmodulin-dependent protein kinase, and cAMP kinases [29–31]. G-proteins, Gα12 and Gα13, have also been reported to activate PLD [32]. Another important regulator of PLD is ARF; ARF directly activates PLD1 and has also been shown to activate PLD2 [25, 33–36]. In fact, PLD2 has been reported to be selectively activated by ARF6 [12]. It is interesting to note that U73122, a known phospholipase C inhibitor, is a potent inhibitor of myocardial PLD by a PIP_2-dependent mechanism and thus PLD may be involved in some of the effects ascribed to PLC [37]. While there is some information on the posttranslational mechanisms of regulation of the myocardial PLD isozymes, this is not completely understood.

The increased formation of reactive oxygen species (ROS) is generally associated with oxidative stress and subsequent cardiovascular injury and cardiac dysfunction [38–40]. Since ROS and oxidant molecules such as H_2O_2 are implicated in the pathogenesis of cardiac dysfunction, this article is intended to describe the role of oxidative stress in relation to myocardial PLD and cardiac dysfunction under different myocardial diseases such as diabetic cardiomyopathy, congestive heart failure, and ischemic heart disease.

23.2 Impairment of PLD Activities During Diabetes

Oxidative stress has been implicated in the pathogenesis of diabetic cardiomyopathy [41–47]. As a consequence of the effects of oxidative stress on the cardiomyocytes, it would be expected that oxidants and ROS could have an impact on the PLD activity during diabetes. In fact, SL PLD activities have been shown to be significantly depressed in diabetic animals [48, 49], resulting in a marked reduction of PLD-derived PA. It has been suggested that this could lead to an impairment of cardiac function in chronic diabetes [48, 49].

It is pointed out that enhanced tissue Ang II levels have been reported in diabetes and might lead to cardiac dysfunction through oxidative stress [50]. Recently Ang II-induced NADPH oxidase has been shown to be involved in hyperglycemia-induced cardiomyocyte dysfunction, which might play a role in diabetic cardiomyopathy [51] and may be related to impaired PLD activities due to superoxide

generation. Impaired PLD activation has been shown to be involved in the damaging effects of oxidative stress in other cells as well. Decreased superoxide generation by neutrophils in insulin-dependent diabetics is, in part, due to impaired activation of PLD [52], and is solely due to high glucose concentrations. The suppressive effect of glucose on diabetic neutrophils is associated with a reduction in PLD activation, which improves when diabetic neutrophils are placed in a normal glucose environment. Glucose causes a reduction in PLD activation, leading to a decrease in second messenger generation and incomplete activation of the respiratory burst [52]. It is interesting to note that we have reported a decrease in the SL amount of PIP_2, due to depressed activities of the phosphatidylinositol (PI) kinases in the diabetic heart [53], likely as a result of oxidant-mediated depression in the PI kinase activities [54]. In this regard, the depressed SL PLD activity during diabetes [45, 46] may also be explained on the basis of a reduced SL PIP_2 level. While direct information on the redox regulation of PLD isozyme activities and the functional consequences of changes in PLD activities in diabetic cardiomyopathy remains to be established, it is reasonable to assume that the depressed PLD activities in the heart during diabetes may be due to oxidative stress.

23.3 Abnormal PLD Activities During Cardiac Hypertrophy and Heart Failure

It is well known that heart failure is a major cause for significant morbidity and mortality; however, the pathophysiological events have not been fully elucidated. There is growing evidence that oxidative stress is implicated in the cardiac dysfunction leading to CHF [55–58]. Oxygen-free radicals can affect heart SL [59–62], SR [63], and mitochondrial functions [64], thus affecting signal transduction mechanisms that are possibly involved in cardiac remodeling and subsequent CHF. Since oxidative stress has significant effects on the SL membrane during CHF, it can be assumed that the oxidative stress will also exert detrimental effects on PLD activities.

The mRNA expression levels of both PLD1 and PLD2 have been reported to be markedly enhanced in ventricular pressure-overload hypertrophy subsequent to aortic banding in rats [65]. A similar induction of PLD mRNA and protein expression has also been reported in hypertrophied human hearts of individuals who had died from noncardiac causes [65]. These authors suggested that PLD activation by α-adrenoceptor and PKC plays a significant role in cell signaling in hypertrophy due to pressure overload [65]. Ventricular fibrosis is promoted by many factors that activate PLD and induce cardiac dysfunction and heart failure. In a hypertensive heart failure model using Dahl-Iwai salt-sensitive rats, PLD activity was seen to be increased with progressive ventricular fibrosis, leading to myocardial stiffening and heart failure [66]. Inhibition of PLD activity with administration of *N*-methylethanolamine decreased collagen content, prevention

of myocardial stiffening, attenuation of ventricular hypertrophy as well as hemodynamic deterioration [66].

We have previously shown that PLD activities are differently altered in CHF subsequent to myocardial infarction induced by the occlusion of the coronary artery [67]. While SL PLD1 activity was decreased, an increase in PLD2 activity was observed in the viable left ventricular tissue. Although the specific role of cardiac PLD isozymes is not fully established, an oleate-dependent PLD activity has been shown to be drastically increased during apoptosis of Jurkat T cells [68], whereas increased PLD2 activity has been shown to reduce hypoxia-induced death of PC12 cells [69]; these studies suggest that PLD2 may play a role in cellular apoptosis. It is interesting to note that Ang II activates NADPH oxidase [70, 71], which can be prevented by imidapril, a known angiotensin converting enzyme inhibitor. Activation of the renin-angiotensin system is the hallmark of CHF [72]. In addition, increased myocardial NADPH oxidase activity in CHF has been reported [73, 74]. We have earlier shown that imidapril normalizes the augmented PLD2 activity in CHF [75]. It is possible that this may be due to a blockade of NADPH oxidase and ROS-mediated activation of PLD2. However, while extensive studies need to be conducted to fully determine the functional significance as well as the mechanisms of impaired PLD1 and PLD2 activities in CHF, it is likely that PLD isozymes are altered due to oxidative stress and may influence cardiomyocyte function of the failing heart through impaired Ca^{2+}-handling.

23.4 Alterations in PLD Activities During Cardiac Ischemia-Reperfusion

A decrease in the blood supply to the heart due to atherosclerosis, thrombosis, or coronary artery spasm is known to induce myocardial ischemia. Although reperfusion of the ischemic myocardium during early stages is essential to prevent cardiac damage, reperfusion of the ischemic heart, after a certain critical period, exerts deleterious effects. These are represented by contractile dysfunction, an increase in infarct size, ultrastructural damage, and changes in myocardial metabolism, which at a later stage leads to cell necrosis [76]. During ischemia, mitochondrial carriers are in a reduced state, due to the degradation of the adenine nucleotide pool. Thus, the interaction of molecular oxygen trapped within the inner membrane of the mitochondria with the leakage of electrons from the respiratory chain leads to the formation of ROS [77]. The deleterious effects of oxidative stress in myocardial I-R are well documented and strongly correlated with cardiac dysfunction [78], a decrease in the antioxidant defense mechanism [79, 80] as well as an increase in lipid peroxidation [80, 81], leading to increased membrane permeability. PLD has been shown in many cases to contribute to the deleterious effects due to oxidative stress in I-R injury. For example, lipid oxidation products such as oxidized LDL have been considered prime candidates for inducing cellular necrosis. Oxidized LDL stimulates PLD [82], implicating a role for PLD in cellular necrosis. Cardiac SL sodium-hydrogen (Na^+–H^+)

exchanger is critical for the regulation of intracellular pH and its activity contributes to I-R injury. Incubation of porcine cardiac SL vesicles with exogenous PLD results in an inhibition of Na^+–H^+ exchanger [83]. It was concluded that PLD-induced changes in the cardiac SL membrane phospholipid environment alter Na^+–H^+ exchanger activity.

While some investigators have reported that the activation of PLD is associated with an improvement of post-ischemic functional recovery and attenuation of cellular injury [84], other investigators, as well as work from our laboratory, have found that PLD is not activated in the ischemic heart [85–88]. Furthermore, our studies also revealed that the increase in the SL PLD2 activity in early reperfusion of the 30 min ischemic heart was associated with an increase in V_{max}, indicating that the PLD2 activation may be due to posttranslational modifications as a result of oxidative stress. On the other hand, we have reported that a Ca^{2+}-independent phospholipase A_2 (cytosolic PLA_2) and subsequent mobilization of the unsaturated fatty acid has been shown to modulate the activity of PLD in heart SL [89]. Interestingly, the cytosolic PLA_2 is also activated by H_2O_2 [90], which could provide a mechanism of an indirect regulation of the SL PLD2 activity by H_2O_2. It should be noted that we also observed a decrease in the SR PLD2 activity after 5 min of reperfusion. Although the K_m value of the SR PLD2 was reduced (increased substrate affinity), the depressed V_{max} value would seem to imply a defect in the catalytic domain of this enzyme; it was suggested that a reversible oxidation may occur since the PLD2 activity was recovered after 30 min reperfusion. In fact, SR PLD activity, in vitro, has been reported to be inhibited by nonradical oxidants, H_2O_2 and HOCI, through reversible modification of associated thiol groups [18]. Thus, the enzyme may be controlled by the GSH redox status of the cardiac cell. In this regard, in the isolated perfused rabbit heart, an ischemic period results in a progressive reduction of tissue glutathione content and of the GSH/GSSG ratio [91], while post-ischemic reperfusion has been shown to lead to a further decrease in the GSH/GSSG ratio [91]. However, a similar response has also been demonstrated for the SL enzyme [92], which is not consistent with the increase in its activity. This inconsistency could be explained on the basis that the functional thiol groups of the SL PLD2 in the isolated perfused heart are not as readily accessible by oxidants as these are in the isolated SL preparation. Such differences may exist between the sensitivity of the SR and SL PLD to different concentrations of oxidant molecules as well as ROS.

Ischemic preconditioning (IP) involving a brief period of ischemia, prior to a prolonged period of ischemia, has been shown to improve myocardial function and diminishes the infarct size. Activation of PLD due to I-R injury as well as in the preconditioned hearts has been documented [93–95]. Agonists of PLD simulate the effects of IP, whereas the inhibition of PLD blocks the beneficial effects of IP as evidenced by the increased incidence of ventricular arrhythmias [85]. The inhibition of PLD can be seen to reduce the amount of DAG and PA as well as significantly inhibit the stimulation of PKC. Thus, PLD may play a role in the myocardial protection afforded by IP. Indeed, this protective effect may be due to ROS generation during the IP [96, 97], which may also be related to the activation of PLD, thus providing a mechanism of action of IP and protection against I-R injury. In addition,

myocardial adaptation to ischemia (IP) is considered to occur through the activation of several tyrosine kinases [98]. The phosphorylation of tyrosine kinases has been shown to be linked with the activation of PLD leading to the activation of multiple kinases [93, 94] including PKC isozymes [99], therefore suggesting that PLD may be a component in the redox signaling designed to protect the heart during IP. While the exact consequences of the changes in PLD1 and PLD2 activities in the heart remain to be determined, PLD isozymes could emerge as an important target for protection against injury during cardiac I-R.

23.5 Conclusions

From the aforementioned discussion, it is evident that impairment of myocardial PLD activities is associated with cardiac dysfunction under different myocardial diseases, while PLD isozyme specific activation may provide cardioprotection (Fig. 23.2). Although significant advancements have been made, more is required to define the role of PLD in different cardiac pathologies. While oxidative stress appears to be a major factor in causing PLD abnormalities, the targeting of PLD, more specifically, modulation of membrane PA levels, may offer a potential for drug development. Defects in other phospholipid-mediated signaling pathways (PLC and PLA2) are also implicated in different myocardial diseases, and in view of the cross-talk and complexities between these pathways (Fig. 23.3), lipid products

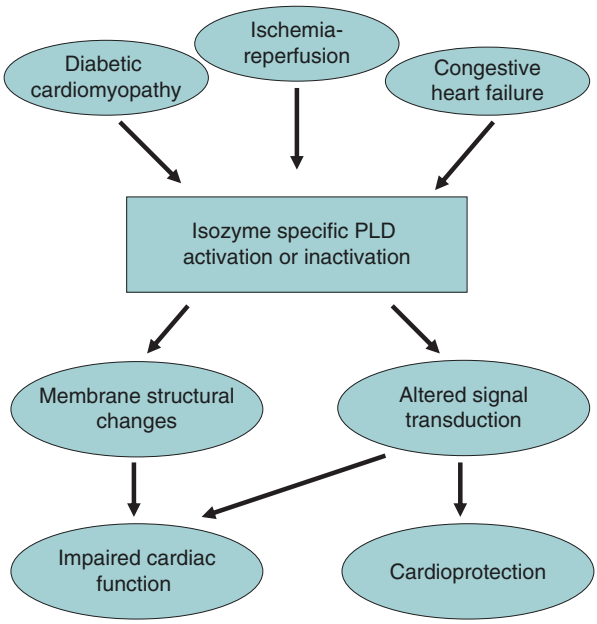

Fig. 23.2 Differential changes and impact of phospholipase D during different cardiac pathologies. *PLD* phospholipase D

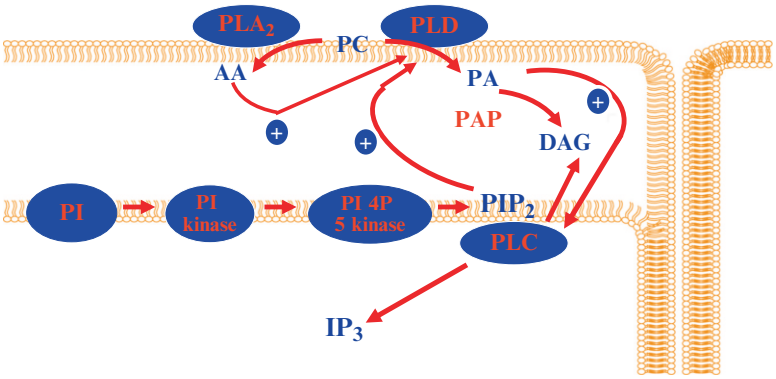

Fig. 23.3 Complexities of phospholipid-mediated signal transduction pathways. *PLA_2* phospholipase A_2, *PLD* phospholipase D, *PAP* phosphatidate phosphohydrolase, *PLC* phospholipase C, *PC* phosphatidylcholine, *DAG* diacylglycerol, *PA* phosphatidic acid, *AA* arachidonic acid, *PI* phosphatidylinositol, *PI4P* phosphatidylinositol-4 phosphate, *PIP_2* phosphatidylinositol,-4,5-bisphosphate, *IP_3* inositol-1,4,5-trisphosphate; +, stimulation

generated through their activities may not only alter signal transduction processes, but also modulate the lipid microenvironment of membrane-associated proteins. Thus, alterations in the PLD activities can be seen to influence cardiac function and may constitute additional therapeutic targets for drug discovery [100–102] for the treatment of heart disease due to different etiologies.

Acknowledgment Infrastructural support was provided by the St. Boniface Hospital Research Foundation.

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Descriptive Blurb

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8,

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P.S. Tappia and N.S. Dhalla (eds.), *Phospholipases in Health and Disease*, Advances in Biochemistry in Health and Disease 10, DOI 10.1007/978-1-4939-0464-8,