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CHAPTER 12

Glycerolipids in signal transduction

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1. Introduction

Glycerolipids have emerged as essential molecules for the regulation of cell functions by hormones, neurotransmitters, growth factors, and inflammatory cytokines. Membrane phospholipids are acted upon by a host of phospholipases, lipid kinases and phosphatases to generate signaling lipids. Fig. 1 illustrates the major enzymes involved in the production of phospholipid-derived second messengers. In general, formation of signaling lipids is initiated by ligand binding to a specific cell-surface receptor, which leads to the activation of phospholipases and/or lipid kinases. The lipid-derived second messengers generated then act on specific target proteins to influence cell function. Signaling lipids and inositol phosphates, like most second messengers, are quickly metabolized to limit the response or to generate another signaling molecule.

The first glycerolipid pathway known to be associated with cell signaling was the turnover of phosphatidylinositol (PI), which leads to changes in the levels of

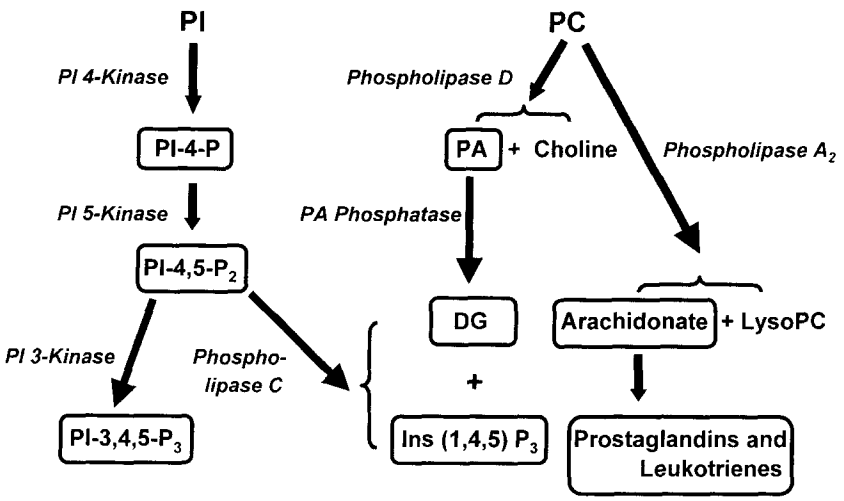

Fig. 1. Overview of phospholipase and lipid kinases that participate in signal transduction mechanisms. Abbreviations: PI, phosphatidylinositol; PI-4-P, PI-4-phosphate; PI-4,5-P_2, PI-4,5-bisphosphate; PI-3,4,5-P_3, PI-3,4,5-trisphosphate; PC, phosphatidylcholine; PA, phosphatidic acid; DG, diacylglycerol; Ins(1,4,5)P_3, inositol trisphosphate.

various phosphoinositides and the generation of diacylglycerol (DG) and inositol-1,4,5-trisphosphate (Ins-1,4,5-P_3). Both of these products are important second messengers. The turnover of phosphatidylcholine (PC) also generates second messengers: phosphatidic acid (PA), by the action of phospholipase D (PLD), and arachidonic acid and lyso-PC (LPC), by the action of phospholipase A_2. PA has an emerging role as a second messenger and also can be converted to lyso-PA (LPA) by certain phospholipases A. Arachidonic acid also has direct intracellular targets as a second messenger, but primarily is metabolized to prostaglandins and leukotrienes (Chapter 13). Alkyl species of lyso-PC are converted to platelet-activating factor (Chapter 9). PA and DG are interconvertible by the action of the enzymes PA phosphatase and DG kinase. DG, PA, Ins-1,4,5-P_3 and the phosphoinositides primarily have intracellular sites of action. In contrast, prostaglandins, leukotrienes, platelet-activating factor, LPC and LPA exit the cell and act as autocrine, paracrine, or circulating hormones by binding to specific cell-surface heptahelical receptors on the same, neighboring, or distant target cells.

This chapter describes the mechanisms by which lipid-derived second messengers regulate various cell functions. Current information about the pathways that regulate the formation and metabolism of signaling lipids and inositol phosphates, as well as the progress in identifying protein targets for the second messengers, will be discussed. Excluded is coverage of arachidonic acid-derived signaling lipids and platelet-activating factor, as they receive extensive discussion in other chapters (Chapters 9 and 13).

2. *Inositol phosphates*

2.1. Mechanisms of generation and metabolism

2.1.1. Phosphoinositide-hydrolyzing phospholipase C

Mabel and Lowell Hokin were the first to observe (in 1953) that the turnover of PI increased greatly in response to cholinergic stimulation of pigeon pancreatic slices [1]. This began the study and elucidation of the PI cycle, illustrated in Fig. 2. A series of two lipid kinases (PI 4-kinase and PI 5-kinase) sequentially add phosphates to positions 4 and 5 of the inositol ring to form PI-4,5-bisphosphate (PI-4,5-P_2). PI-4,5-P_2 is the major substrate for the phosphoinositide-hydrolyzing phospholipase C (PLC) family of enzymes, which are activated in response to hormone or other agonist stimulation. Hydrolysis of PI-4,5-P_2 by PLC produces the second messengers DG and Ins-1,4,5-P_3. The function of these messengers is discussed below (Sections 2.2, 3.2, 3.3). Both products are metabolized further and used for the resynthesis of PI, as shown in Fig. 2. The situation is more complex than shown, since the action of additional kinases and phosphatases results in the formation of inositol phosphate species phosphorylated on various combinations of up to all six hydroxyls of the inositol ring. Many of the over 60 potential species have not been isolated from biological sources, but several inositol tetrakisphosphates, Ins-1,3,4,5,6-P_5 and Ins-1,2,3,4,5,6-P_6, are present in animal cells [2]. Known and potential functions of these molecules are described in Section 2.2.

The mammalian PLC family consists of 11 isozymes divided into four subgroups (β, γ, δ, ε), all of which catalyze the hydrolysis of PI-4,5-P_2 to Ins-1,4,5-P_3 and DG in

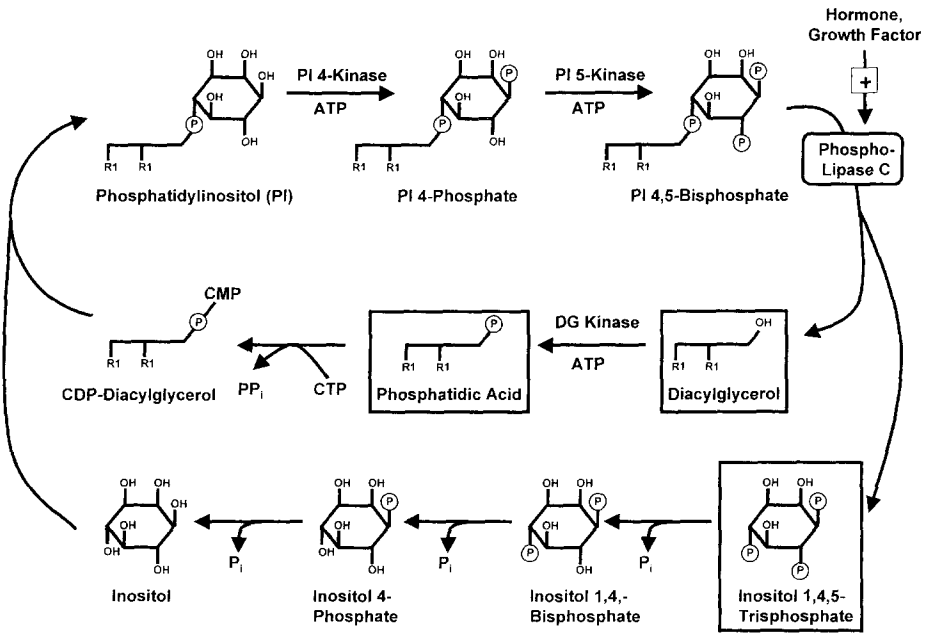

Fig. 2. Phosphatidylinositol cycle. See text for details. Shown in bold boxes are the signaling molecules derived from the PI cycle. DG kinase, diacylglycerol kinase; PI, phosphatidylinositol.

response to agonist binding to over 100 cell-surface receptors [3]. The four subgroups all contain similar catalytic X and Y domains, but differ in size and in their regulatory regions (Fig. 3). The divergent regulatory regions are responsible for differences in the types of receptors and the mechanisms by which members in each subgroup are activated. Three of the subgroups (δ, β, γ) contain three regulatory regions in common:

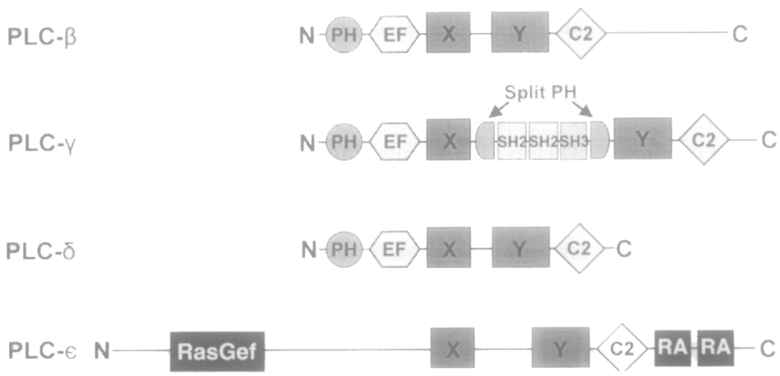

Fig. 3. Domain structures of the four isoforms of phosphatidylinositol-specific phospholipase C (PLC). The X and Y catalytic regions are conserved among all the isoforms. PH, pleckstrin homology domain; EF, EF hands; SH2 and SH3, Src homology domains 2 and 3; C2, C2 domain; RasGEF, Ras guanine nucleotide exchange factor-like domain; RA, Ras-binding domain. See text for discussion.

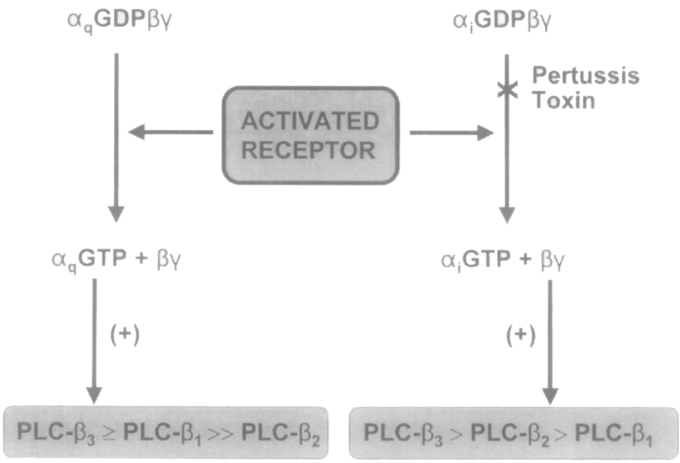

Fig. 4. Scheme for activation of phospholipase C-β (PLC-β) isoforms by G protein α and βγ subunits. The left side of the figure shows the pertussis toxin-insensitive activation of PLC-β isoforms by $G_q\alpha$ subunits. The right side of the figure shows the pertussis toxin-sensitive activation of PLC-β isoforms by βγ subunits derived from a G_i type of G protein.

an N-terminal pleckstrin homology (PH) domain, an EF hand domain and a C-terminal C2 domain. PH domains bind certain phosphoinositides and/or proteins (see Section 5.2.1), EF hand domains usually bind calcium [4], and C2 domains usually bind anionic phospholipids in the presence of calcium (see Section 3.2) [5]. The PLC-γ subgroup contains a second PH domain that is split by two Src homology 2 (SH2) domains and a Src homology 3 (SH3) domain. SH2 regions usually interact with phosphorylated tyrosine residues in other proteins and SH3 domains bind specific polyproline motifs in target proteins. The PLC-ε subgroup contains a C2 domain, as well as an N-terminal Ras guanine nucleotide exchange factor-like domain and one or two C-terminal Ras binding domains. The crystal structure of PLC-δ (minus the PH domain) has been solved (Chapter 11), which clearly illustrates the domain structure of the enzyme.

Each PLC subgroup is regulated by different mechanisms, triggered by agonist binding to specific receptors and involving recruitment of the soluble PLC to the plasma membrane. The PLC-β subgroup is regulated by interaction with α and βγ subunits of certain heterotrimeric G proteins [3] (Fig. 4). Agonist binding to heptahelical receptors triggers the exchange of GTP for GDP on the α subunit of heterotrimeric G proteins, causing the α and βγ subunits to dissociate. Both free subunits then interact with effector enzymes to regulate intracellular signaling. G protein α subunits hydrolyze the bound GTP back to GDP and the subunits reassociate to terminate the signaling process. There are four subfamilies of G protein α subunits, and one of these ($G\alpha_q$) interacts with the C2 domain and the extended C-terminal region of PLC-β to aid in membrane recruitment and induce enzyme activation. The βγ subunit released by activation of the G_i subfamily also can recruit and activate PLC-β by interaction with the PH domain. Treatment of cells with pertussis toxin can determine which G protein family is responsible for PLC activation. Pertussis toxin inhibits the activation of the G_i family of G proteins, but has

no effect on the G_q family. Different isozymes in the PLC-β subfamily demonstrate differing abilities to respond to G protein α and βγ subunits. For example, PLC-β1 is quite sensitive to $G\alpha_q$ and poorly responsive to Gβγ. Finally, PI-3-P, generated by a PI 3-kinase, binds the PH domain of PLC-β1 and aids in recruitment of the enzyme to the membrane.

PLC-γ is activated by a large group of cell-surface receptors, including receptor protein tyrosine kinases, antigen and immunoglobulin receptors, and heptahelical receptors [3]. The basic mechanism used by all receptors involves tyrosine phosphorylation of PLC-γ and targeting of the enzyme to the plasma membrane. Receptors with intrinsic tyrosine kinase activity (e.g. growth factor receptors) first recruit PLC-γ to phosphorylated tyrosine residues on the receptor by interaction with the N-terminal SH2 domain. This allows the receptor tyrosine kinase to phosphorylate PLC-γ, inducing enzyme activation. Recruitment of PLC-γ to the membrane also involves the interactions of PI-3,4,5-P_3 with the C-terminal SH2 domain and the PH domain. Regulatory roles for the EF hand, SH3 and C2 domains of PLC-γ are not known. Activation of PLC-γ by antigen involves intracellular non-receptor protein tyrosine kinases that are either recruited to the receptor or activated by other pathways. These tyrosine kinases phosphorylate PLC-γ and, along with PI-3,4,5-P_3, induce enzyme activation. With heptahelical receptors, a non-receptor tyrosine kinase phosphorylates and activates a growth factor receptor, which then recruits and phosphorylates PLC-γ, as described above.

The pathways used by membrane receptors to activate the PLC-δ isozymes are unclear [3]. PLC-δ is more sensitive to Ca^{2+} than the other subgroups, so an increase in intracellular Ca^{2+} levels induced by receptor-mediated activation of one of the other PLC subtypes may be responsible for PLC-δ activation. Also, a newly discovered type of GTP-binding protein, G_h (high-molecular-weight G protein) may regulate PLC-δ after stimulation of cells through α_1-adrenergic, oxytocin, or the α subtype of thromboxane A_2 receptors.

The newly discovered PLC-ε appears to be regulated by two mechanisms [3]. Growth factor receptors lead to activation of the small GTPase Ras, which binds to PLC-ε via the C-terminal Ras binding domain and activates the enzyme. The N-terminal Ras guanine nucleotide exchange factor-like domain may prolong PLC-ε activation by reactivating exchange of GTP for GDP on Ras. PLC-ε is also activated by the α subunit of the heterotrimeric G protein G_{12}, which couples to LPA receptors, which are members of the large heptahelical (G protein-coupled) receptor family.

2.1.2. Inositol phosphate kinases and phosphatases

The initial product of PLC, Ins-1,4,5-P_3, acts as a second messenger for the release of Ca^{2+} from intracellular stores (Section 2.2.1). However, Ins-1,4,5-P_3 is rapidly metabolized by sequential phosphatase action to free inositol or by Ins-1,4,5-P_3 3-kinase to inositol-1,3,4,5-tetrakisphosphate (Ins-1,3,4,5-P_4). Inositol polyphosphate phosphatases comprise a family of enzymes, which use both inositol phosphates and the phosphoinositides as substrates to varying degrees [6]. Group I inositol polyphosphate 5-phosphatases act on Ins-1,4,5-P_3 and Ins-1,3,4,5-P_4 and appear to be key enzymes for dampening Ca^{2+} signaling. Inositol polyphosphate 1-phosphatase hydrolyzes Ins-1,4-P_2 and Ins-1,4,5-P_3 and may be the target of lithium inhibition during therapy for manic depression.

Ins-1,4,5-P_3 3-kinases, which also remove Ins-1,4,5-P_3, are activated by the Ca^{2+}-binding protein calmodulin as a feedback mechanism when Ins-1,4,5-P_3 causes a rise in Ca^{2+} levels [2]. Other members of the inositol phosphate kinase multi-gene family include inositol phosphate multikinases, which can phosphorylate Ins-1,4,5-P_3 to form Ins-1,3,4,5,6-P_5, and $InsP_6$ kinases, which can form Ins-1,2,3,4,5,6-P_6 from multiple inositol phosphate substrates [2].

2.2. Cellular targets

2.2.1. Control of intracellular calcium levels by inositol-1,4,5-trisphosphate

Ca^{2+} is an important intracellular signaling molecule, which regulates a host of cell functions [7]. These include contraction, proliferation, fertilization, secretion, vesicle trafficking, apoptosis, and intermediary metabolism. In keeping with its signaling role, the cytosolic level of calcium is very low (~100 nM), but can rise to ~1000 nM when cells are activated by agonist–receptor interaction. Receptor activation triggers pathways leading to the opening of Ca^{2+} channels in the endoplasmic reticulum and the plasma membrane, causing the marked elevation in intracellular Ca^{2+} concentration. The increase is transient because the second messengers acting to open Ca^{2+} channels are rapidly metabolized and Ca^{2+} pumps and exchangers remove Ca^{2+} from the cytosol to restore the resting state. A major pathway for increasing Ca^{2+} levels is the formation of Ins-1,4,5-P_3 by activation of PLC (Section 2.1.1). Ins-1,4,5-P_3 binds to its receptor, which is a Ca^{2+} channel in the endoplasmic reticulum. The role of Ins-1,4,5-P_3 may be to increase the sensitivity of the receptor to stimulation by cytosolic Ca^{2+}, rather than directly causing the channel to open.

2.2.2. Targets for other inositol phosphates

More highly phosphorylated inositol phosphates also may regulate cell function [2]. Ins-1,3,4,5-P_4 has a higher affinity than Ins-1,4,5-P_3 for hydrolysis by inositol polyphosphate 5-phosphatase and can prolong the lifetime of Ins-1,4,5-P_3, thus enhancing increases in Ca^{2+} concentration. Ins-1,3,4,5-P_4 directly activates Ca^{2+} channels in the plasma membrane in some cells, also leading to enhanced Ca^{2+} levels. Ins-3,4,5,6-P_4 appears to inhibit plasma membrane Ca^{2+}-regulated chloride channels. Inositol hexakisphosphate ($InsP_6$), also known as phytic acid, has several proposed functional targets. One is a protein kinase that phosphorylates pacsin/syndapin I, a protein involved in vesicle trafficking. A second is a DNA-dependent protein kinase that regulates DNA end-joining. $InsP_6$ also may regulate mRNA transport out of the nucleus.

3. Diacylglycerols

3.1. Mechanisms of generation and metabolism

3.1.1. Hydrolysis of phospholipids

The initial step for DG generation in response to agonist–receptor engagement is the hydrolysis of PI-4,5-P_2 by PLC (Section 2.1.1). A PC-hydrolyzing PLC activity has also

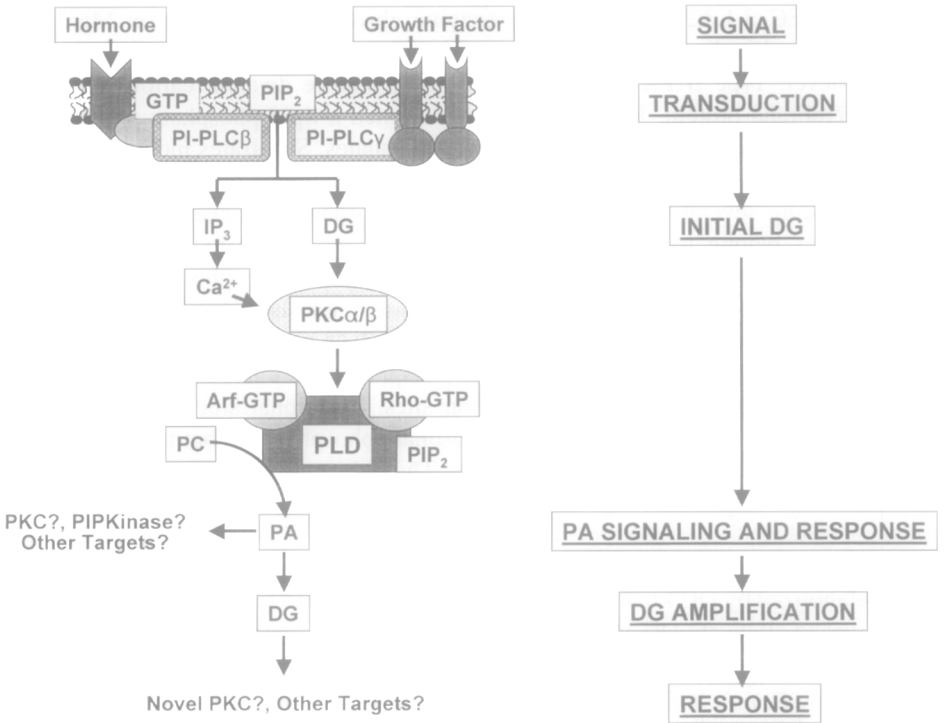

Fig. 5. Phospholipase cascade model for activation of phospholipase D (PLD) and amplification of diacylglycerol generation. Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; DG, diacylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid; PKC, protein kinase C; PIP_2, PI-4,5-bisphosphate; PIPkinase, PI phosphate kinase.

been described (e.g. [8]), but is not yet identified at the molecular level. In many cell types (e.g. human neutrophils [9]), two 'waves' of DG production take place caused by a phospholipase cascade (Fig. 5). PI-4,5-P_2-hydrolyzing PLC is responsible for the initial rapid wave of DG production. The second, more sustained wave of DG production is from the activation of PC-hydrolyzing PLD to generate PA, followed by conversion of the PA to DG by a PA phosphatase [10,11]. PA has its own roles as a second messenger (Section 4.3). Depending on the molecular species of the PC pools acted upon by PLD, a mixture of diradylglycerols (diacylglycerols, alkylacylglycerols, alkenylacylglycerols) may be formed. Clearly, the fatty acid composition of the DG species derived from PI-4,5-P_2 and PC differs [11], suggesting their functional roles are not the same. Indeed, the DG and other diradylglycerol species derived from PC may not activate protein kinase C (PKC) — the major target of DG in cells [11]. However, DG binds to proteins other than PKC (Section 3.3), raising the possibility that PC-derived diradylglycerols have other targets. Alternatively, the diradylglycerols derived from PA phosphatase may enter lipid biosynthetic pathways (Fig. 2).

The PA phosphatases that convert PA to DG comprise a family of enzymes, which function in both general lipid metabolism and glycerolipid signaling [10]. Type I PA

phosphatase (EC 3.1.3.2) is a cytosolic enzyme absolutely dependent on Mg^{2+} and is likely involved in lipid biosynthetic reactions. It has not been characterized at the molecular level. Type II PA phosphatase does not require divalent cations and is an integral membrane protein. It is more properly termed a lipid phosphate phosphatase, since it hydrolyzes a variety of signaling lipid phosphates. Substrates include PA, LPA, ceramide 1-phosphate, sphingosine 1-phosphate, and diacylglycerol pyrophosphate. The cDNAs encoding several Type II lipid phosphate phosphatases have been cloned and these enzymes belong to a phosphatase superfamily. Superfamily members include bacterial non-specific acid phosphatases, yeast lipid phosphate phosphatases, fungal haloperoxidases, mammalian glucose-6-phosphatase, and several others. All family members share three highly conserved domains that likely comprise the active site. The mechanisms that regulate the lipid phosphate phosphatases are not clear, but several of the enzymes contain putative phosphorylation sites. Additional studies are needed to determine the functional roles of the lipid phosphate phosphatases, but they clearly can degrade a number of lipid second messengers. Thus, their primary function may be to attenuate cellular responses to agonist–receptor interaction.

3.1.2. Diacylglycerol kinases

DG is metabolized by DG kinases, which phosphorylate DG to form PA [12]. This action likely serves to attenuate DG-mediated responses, but the generated PA may be another second messenger (Section 4.3). Mammalian DG kinases comprise a family of nine isoenzymes (α, β, γ, δ, η, ε, ζ, ι, θ), differing in their primary structure (Fig. 6), substrate specificity and tissue distribution. All contain a conserved catalytic domain and two cysteine-rich (C1) domains. C1 domains in PKC and other proteins bind DG (Sections 3.2 and 3.3); however, the role of C1 domains in DG kinase isoforms is not known. The presence of other protein : protein and protein : lipid interaction domains (e.g. PH, EF hand, proline-rich region, ankyrin repeats) divides the isoenzymes into five classes

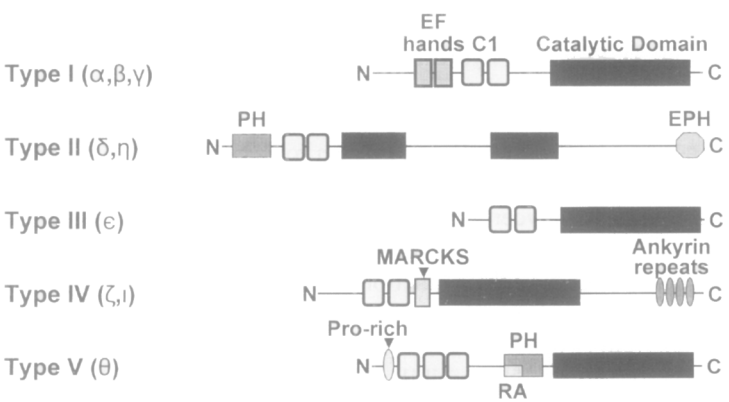

Fig. 6. Domain structures of mammalian diacylglycerol (DG) kinases. Domains are labeled from top of figure. PH, pleckstrin homology; EPH, ephrin C-terminal tail homology; MARCKS, sequence homologous to the myristoylated alanine-rich C-kinase substrate (MARCKS) phosphorylation site domain; Pro-rich, proline-rich domain; RA, Ras-associating domain.

(Fig. 6), suggesting that each class may participate in different signaling pathways. Many of the isoenzymes also have putative phosphorylation sites. Thus, regulation of DG kinases involves phosphorylation reactions and specific protein : protein and protein : lipid interactions, resulting in alterations in localization and activity of the enzymes. The diversity and complexity of the DG kinase family suggests that, like PKC (Section 3.2) and PLC (Section 2.1.1), these enzymes are crucial participants in lipid-mediated signaling processes. However, specific roles for DG kinases in the regulation of cell functions await elucidation.

3.2. Protein kinase C is an important target for diacylglycerol

PKC was the first lipid-regulated protein kinase to be described and its discovery by Nishizuka and colleagues [13,14] initiated a new era of lipid-mediated signal transduction. DG and Ca^{2+} were quickly identified as the physiological activators of PKC, placing PKC as a primary downstream participant in the hormonally activated PI pathway described by the Hokins [1] (Section 2.1.1). PKC regulates almost all cell functions, including cell differentiation, proliferation, metabolism, and apoptosis. The discovery that PKC is the target of the tumor-promoting phorbol diesters illustrated its importance for the regulation of carcinogenesis [15].

PKC is now known to be a family of twelve mammalian isoforms, divided into four classes based on structural differences and cofactor dependence [15,16] (Fig. 7). All PKCs are a single polypeptide chain, consisting of an N-terminal regulatory region and a C-terminal catalytic domain connected by a protease-sensitive hinge region. The conserved catalytic domain consists of the C3 ATP-binding region and the C4 substrate-binding domain. The conventional isoforms (α, βI, βII, γ) are activated by

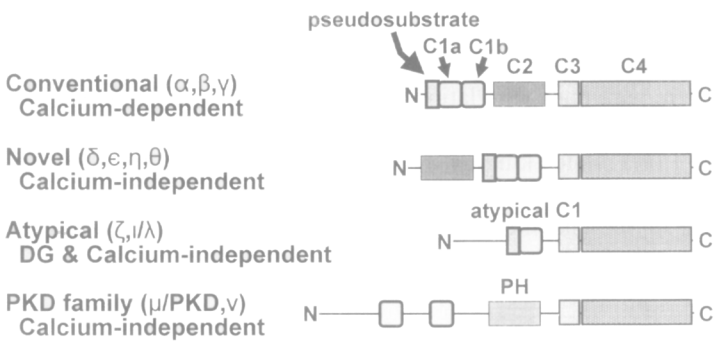

Fig. 7. Domain structure of protein kinase C (PKC) isoforms. The N-terminal half of the protein contains regulatory regions (C1, C2), while the C-terminal half contains the catalytic regions (C3, C4). The conventional isoforms contain tandem C1 domains and one C2 domain, which bind diacylglycerol (C1), Ca^{2+} (C2) and anionic phospholipids (C2). Adjacent to the C1 domains is a pseudosubstrate sequence involved in maintaining the auto-inhibitory conformation of the PKCs. The novel isoforms contain an altered C2 domain, which no longer binds Ca^{2+}. The atypical isoforms lack a C2 domain and have one atypical C1 domain, accounting for their lack of activation by diacylglycerol (DG) and Ca^{2+}. The PKD family is larger in size, has two non-adjacent C1 domains and a pleckstrin homology (PH) domain instead of a C2 domain. It is Ca^{2+}-independent, but is activated by DG and anionic phospholipids.

Ca^{2+}, DG, and phosphatidylserine. The C2 region binds phosphatidylserine and Ca^{2+}, while DG binds to the two C1 domains. A pseudosubstrate region, containing sequences resembling phosphorylation sites in PKC substrates but without a phosphorylatable residue, is at the N-terminal end of the first C1 domain. In the inactive state, PKC is folded such that the pseudosubstrate region interacts with the C4 substrate binding domain, blocking access of exogenous substrates to the active site. Binding of DG, phosphatidylserine and Ca^{2+} to the regulatory domain disrupts this interaction, leading to PKC activation.

The novel PKC isoforms (δ, ε, θ, η) have two DG-binding C1 domains and an atypical C2 region near the N-terminus, which binds phosphatidylserine, but not Ca^{2+}. These isoforms are Ca^{2+}-independent and are activated by phosphatidylserine and DG. The atypical PKCs (ζ, ι/λ) lack the C2 region and have an atypical C1 region that does not bind DG. Thus, these isoforms are Ca^{2+}- and DG-independent, but can still be activated by phosphatidylserine. Other mechanisms of activating the atypical isoforms likely exist. Members of a fourth subclass (μ, ν) are larger than the other isoforms and have two separated C1 regions and a PH domain in the extended regulatory region [17]. These isoforms are activated by phosphatidylserine and DG, but may have additional mechanisms of regulation.

It has become clear that regulation of PKC involves more than binding of activators to induce conformational changes in the enzyme. The lipid activators of PKC also target the enzyme to membranes, which regulates full activation of the enzyme, access of the enzyme to substrates, and proteolytic degradation of the enzyme [16]. Membrane targeting involves the cooperation between lipid binding to the C1 and C2 domains and probable interactions of other regions with membrane lipids. Protein : protein interactions also regulate PKC localization and function [15]. PKC isoforms interact with a wide variety of proteins, including RACKs (receptors for activated C kinase), STICKs (substrates that interact with C kinase), cytoskeletal proteins, and scaffolding proteins. These interactions function either to target specific PKC isoforms to sites of activator generation and/or substrates or to integrate PKC into other signaling pathways. Finally, all PKC isoforms require phosphorylation during maturation of the enzyme for catalytic competence. Phosphorylation within the activation loop by phosphoinositide-dependent protein kinase-1 or a related kinase is required for enzyme activity. Also, two sites in the C-terminal tail of PKC must be autophosphorylated for proper localization and catalytic competence of the enzyme. The multiplicity of PKC isoforms and the complexity of their regulation affirm that this family of enzymes plays critical roles in various signaling pathways.

3.3. Non-PKC targets of diacylglycerol

Most of the PKC isoforms are targets of DG and mediate much of the signaling by this lipid second messenger. However, other proteins contain C1 domains that may bind DG. Some of these appear to be atypical C1 domains, similar to the C1 domain in PKC-ζ [16]. These include DG kinase isoforms, the protein kinase Raf, and the Rac guanine nucleotide exchange factor Vav. In contrast, DG and phorbol diesters have been found to bind to several non-kinase proteins, including the chimaerins, which are Rac GTPase-

activating proteins, Unc-13 and related proteins, which may be scaffolding proteins, and RasGRP, a guanine nucleotide release protein for Ras [15]. The main function of DG may be to recruit these proteins to membrane sites where they regulate other enzyme activities (e.g. RasGRP activates Ras). Thus, DG may have other functional roles beyond activation of PKC.

4. Phospholipase D and the generation of phosphatidic acid

4.1. Discovery and molecular nature of phospholipase D

PLD (EC 3.1.4.4) was discovered in plants over 50 years ago as an enzyme that catalyzed the hydrolysis of PC to form PA and choline [18]. However, PLD was not of interest in the field of signal transduction until mammalian PC-hydrolyzing PLD was shown to be activated by extracellular stimuli in the late 1980's (reviewed in [18]). Mammalian PLD is activated by a wide variety of hormones, neurotransmitters, cytokines, and growth factors and plays essential roles in the regulation of numerous cellular functions [18,19]. A number of other eukaryotic PLD activities have been described, including oleate-activated PLD, phosphoinositide-specific PLD, phosphatidylserine/phosphatidylethanolamine-hydrolyzing PLDs, *N*-acyl-phosphatidylethanolamine PLD, lyso-PLD, and glycosylphosphatidylinositol-PLD. With the exception of the glycosylphosphatidylinositol-PLD, which is a serum enzyme and has no signaling role, none of these other enzymes have been identified at the molecular level. Thus, this section will focus on the molecularly characterized PC-hydrolyzing PLDs that clearly function in glycerolipid signaling.

PC-PLD enzymes are members of a diverse gene superfamily that includes cardiolipin and phospholipid synthases, endonucleases and certain viral proteins, which share a common 'HKD' motif involved in catalysis (E.V. Koonin, 1996; C.P. Ponting, 1996). PC-PLDs are present in most organisms, including bacteria, plants, flies, yeast, worms, and mammals [18]. Some organisms express only one PC-PLD gene (e.g. yeast), while others express multiple genes (plants, mammals). The domain structure of representative PC-PLDs from various organisms is shown in Fig. 8. All have four conserved domains (I–IV), which comprise the catalytic core of the enzyme. The exact functions of Domains I and III are unknown. Domains II and IV contain the short conserved sequence $HxKx_4Dx_6GG/S/TxN$ termed the 'HKD motif' or the phosphatidyltransferase motif, present in all members of the PLD superfamily. PLD activity requires both HKD motifs and Domains I–IV may form a bilobed, 'dimer' structure, such that both HKD motifs are present in the active site (I. Leiros, 2000). The histidines are thought to attach the phosphatidyl moiety to the enzyme, forming a phosphohistidine intermediate. This is a hallmark of the 'transphosphatidylation' reaction catalyzed by PC-PLD and other members of the PLD superfamily. The phosphatidyl moiety is transferred to a H_2O molecule to form PA, or it can be transferred to an alcohol acceptor to form a phosphatidylalcohol. This property is the basis for a commonly used PLD assay, in which cells are incubated in the presence of a short-chain primary alcohol (e.g. 1-butanol, ethanol) and the formation of the phosphatidylalcohol (which is poorly metabolized) is measured.

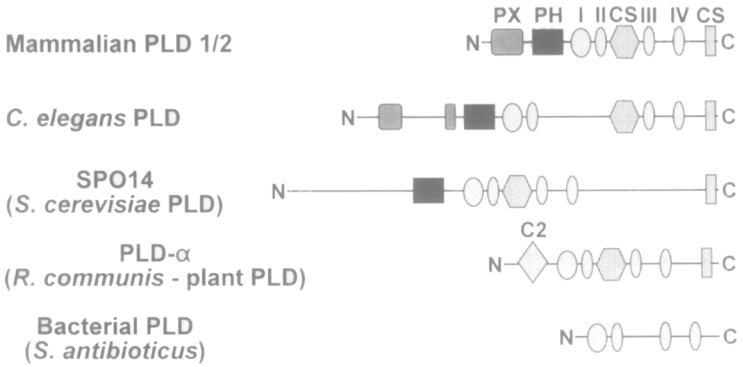

Fig. 8. Comparison of domain structures of phospholipase D (PLD) isoforms from various organisms. Domains are labeled at the top of the figure, except for the C2 domain found only in plant PLD isoforms. All isoforms contain four conserved regions involved in catalysis (I, II, III, IV). All isoforms except the bacterial enzymes contain two conserved regions (CS) of unknown function. PX, phox homology domain; PH, pleckstrin homology domain. The PX domain in *C. elegans* PLD is split.

Incubation of cells with primary alcohols can be used to reduce the production of PA by PLD, thus interfering with PLD-dependent regulation of cellular functional responses.

Most of the PC-PLDs from higher organisms are inactive in unstimulated cells and are regulated by the interaction of intracellular signaling proteins and lipids with domains present in the various proteins [18] (Fig. 8). The active site of PLD is likely blocked by the folded structure of the protein and requires a conformational change to release the autoinhibition. Mammalian and *Caenorhabiditis elegans* PLDs each contain an atypical PH domain and a PX (phox homology) domain in the N-terminal half of the molecule. The *Saccharomyces cerevisiae* enzyme contains only the PH domain. The PH domain in human PLD1 binds PI-4,5-P_2 (M.N. Hodgkin, 2000), but it is controversial whether or not this binding is responsible for the PI-4,5-P_2 dependence of PLD activity. PX domains in other proteins were recently shown to target to membranes by binding selected phosphoinositides [20]. The function of the PX domain in PLDs is not known. The plant PLDs lack PH and PX domains, but have a C2 domain in the N-terminal region (Fig. 8). The C2 domain binds Ca^{2+} and anionic phospholipids and is likely responsible for the dependence of the plant enzymes on Ca^{2+} for activity. All eukaryotic PC-PLDs also contain conserved regions between catalytic Domains II and III and at the C-terminus. The functions of these regions are unknown.

4.2. Localization and regulation of phospholipase D

Numerous receptor agonists induce activation of mammalian PC-PLD in vivo [19]. Activation does not involve direct interaction of occupied receptors with PLD, but instead uses intracellular signaling intermediates. Evidence is strong that PLD is regulated in vivo by PKC, the ARF, Rho, and Ras/RalA families of small GTPases, and the intracellular levels of PI-4,5-P_2. The cDNAs for two mammalian PLD isoforms (PLD1, PLD2) have been cloned and each gene can be alternatively spliced to yield two products. In vitro assays of the recombinant PLD enzymes show that PLD1a and PLD1b

require PI-4,5-P_2 for activity and are synergistically activated by PKC-α or -β, ARF1, and RhoA (see Fig. 5). It is not known if such synergism between multiple activators takes place in intact cells. In contrast, PLD2a and PLD2b require PI-4,5-P_2, are only slightly activated by ARF1 and are insensitive to PKC and RhoA. For the most part, the functional binding sites on the PLD molecule for these regulators are unknown. The PI-4,5-P_2-binding site may be the PH domain or the conserved region between Domains II and III (Fig. 8). The ARF-binding site has not been identified, despite intensive effort. The PKC-α/β binding site appears to be in the N-terminal third of PLD1 and the RhoA-binding site is in the C-terminal third, but the specific motifs involved have not been defined.

PLD1 and PLD2 usually are differentially localized in cell types that have been examined [18]. PLD1 is associated with intracellular membrane compartments, most likely endosomal/lysosomal membranes. PLD2 is associated with the plasma membrane and has been found in caveolae. Given the in vitro differences in the regulation of PLD1 and PLD2 and their differential localization, it is likely that each has unique functions in cellular regulation.

4.3. Functions regulated by phospholipase D and phosphatidic acid

Studies that selectively manipulate PLD1 and PLD2 levels and activity in intact cells, in order to identify PLD isoform-dependent downstream signaling elements and cellular functions, are still in the early stages. Similarly, while PA regulates a number of proteins in vitro, it is not clear whether PA has the same role in vivo for most of the proteins. However, pharmacological approaches and PLD localization studies have strongly implicated PLD and PA as important regulators of several cell functions.

4.3.1. Membrane trafficking, secretion and cell proliferation

Both PLD1 and PLD2 have been implicated in membrane trafficking events, including budding of vesicles from the Golgi apparatus and the *trans*-Golgi network and ARF-dependent clathrin coat assembly in the endosomal/lysosomal system [18]. The production of PA by PLD may promote the recruitment of adapter and coat proteins to sites of vesicle budding. PA also can induce activation of Type I PI 4-phosphate kinases, leading to increased levels of PI-4,5-P_2 and additional protein recruitment and PLD activation. Scission of vesicles is mediated by the GTPase dynamin or its analogues, which are also regulated by anionic phospholipids. Thus, the increased levels of PA and PI-4,5-P_2 in the budding membrane are thought to mediate recruitment of the proteins necessary to complete the budding process.

Evidence also implicates PLD in the regulation of stimulated secretory and degranulation responses, such as the insulin-stimulated translocation of GLUT4 to the plasma membrane, release of granule contents from mast cells and neutrophils, exocytosis from chromaffin cells, and the release of matrix metalloproteases from cancer cells [18]. The mechanisms by which the PLD product PA mediates secretion are not known, but also may involve recruitment of particular proteins to secretory vesicle and granule membranes.

PA is thought to have a similar recruitment role in the regulation of Raf1, a key

protein kinase that regulates cell proliferation (M.A. Rizzo, 2000). Raf1 is a target of the small GTPase Ras and triggers cell proliferation through activation of the mitogen-activated protein kinase cascade. PLD activation is required for the activation of Raf1 in intact cells and a PA-binding site in Raf1 has been identified. However, PA has no direct effect on Raf1 activity. Mutation of the PA-binding site prevents recruitment of Raf1 to the membrane and inhibits activation of the downstream mitogenic kinase cascade. This suggests that the role of PA is to recruit Raf1 to the membrane where Ras is located.

4.3.2. NADPH oxidase

Pharmacological data strongly indicate that PLD activation is needed for receptor-mediated activation of the phagocyte multi-component enzyme NADPH oxidase [21]. This enzyme functions in host defense against infection and in tissue damage during inflammatory diseases. Study of the processes regulating this enzyme is providing insight into the complex mechanisms by which signaling lipids mediate functional responses. Activation of NADPH oxidase requires recruitment of four cytosolic proteins, including the small GTPase Rac, to assemble with heterodimeric flavocytochrome b_{558} in the membrane. The flavocytochrome then undergoes a presumed conformational change, allowing it to transfer electrons from NADPH to O_2 to form superoxide anion. The cytosolic proteins exist in inactive complexes and must be dissociated or undergo conformational changes to move to the membrane. The entire process involves phosphorylation of components (by lipid-activated protein kinases, such as PKC), lipid- and phosphorylation-regulated SH3 region-mediated protein : protein interactions, and direct interaction of signaling lipids (PA, DG, arachidonic acid) with various oxidase proteins. PA and arachidonic acid each cause conformational changes in a cytosolic oxidase component, $p47^{phox}$, and the membrane-bound flavocytochrome. PA (plus DG) or arachidonic acid (alone) induce phosphorylation-independent oxidase activation in purified cell-free systems containing only oxidase proteins, presumably by their direct interactions with $p47^{phox}$ and flavocytochrome b_{558}. Two of the cytosolic oxidase components, $p47^{phox}$ and $p40^{phox}$, have N-terminal PX domains, which bind phosphoinositides and likely participate in the recruitment process [20]. New data indicate that PA binds to the $p47^{phox}$ PX domain with greater affinity and efficacy than phosphoinositides (L.C. McPhail, unpublished results). Thus, PA may be the physiological ligand for this particular PX domain and may regulate NADPH oxidase activation partially through its PX domain interaction. Studies are ongoing, but it is clear that PA and other signaling lipids induce activating conformational changes in NADPH oxidase components and regulate membrane localization of oxidase proteins by both direct binding and protein kinase-dependent mechanisms.

5. Phosphoinositides

The phosphoinositides comprise a large group of inositol-containing phospholipids, with phosphates attached to positions D3, D4, or D5 (or various combinations of these positions) of the inositol ring (Figs. 9 and 10). They are present at low concentrations in cells: PI, the precursor phospholipid, is present at less than 10% of total membrane

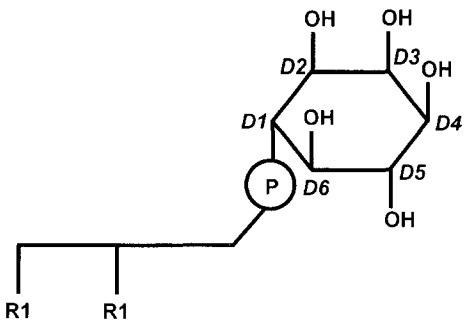

Fig. 9. Numbering of the inositol ring. Phosphates can be attached at positions D1 through D6 on the inositol ring. Shown is phosphatidylinositol, in which inositol is attached to the glycerol backbone by a phosphate in position D1.

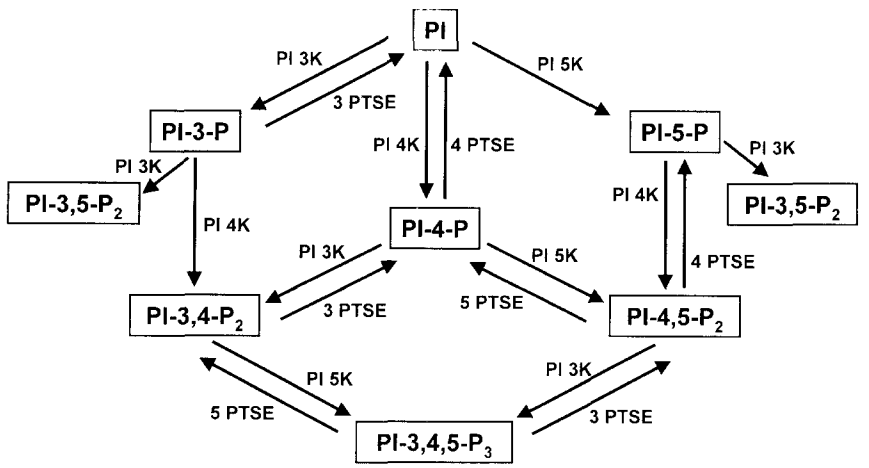

Fig. 10. Interconversion of phosphoinositides. The pathways by which phosphoinositide kinases and phosphatases synthesize and degrade various phosphoinositides are shown. Abbreviations: PI, phosphatidylinositol; PI-4-P, PI-4-phosphate; PI-4,5-P_2, PI-4,5-bisphosphate; PI-3,4,5-P_3, PI-3,4,5-trisphosphate; PI 3K, PI 3-kinase; 3 PTSE, PI 3-phosphatase.

lipid; PI-4,5-P_2, the substrate for PLC, is about 5%; phosphoinositides phosphorylated at the 3 position are less than 0.25% of the total inositol-containing lipids [22]. The phosphoinositides regulate a large number of signaling pathways and cellular functions by binding to specific sites on proteins to alter their localization or activity (Section 5.2). A complex set of PI kinases and phosphatases is involved in the interconversion (Fig. 10) (Section 5.1), and considerable progress in identifying these enzymes at the molecular level has been made. Phosphoinositide-metabolizing enzymes are regulated by receptor-triggered signaling processes, which results in highly localized changes in the levels of specific phosphoinositides to coordinate temporal and spatial regulation of cellular functions.

5.1. Phosphoinositide kinases and phosphatases

5.1.1. Phosphatidylinositol 4- and 5-kinases

The enzymes that phosphorylate positions D4 and D5 of the inositol ring fall into two major categories, i.e. those that classically act on PI-4-P and those that utilize PI [23]. The PI 4-kinases convert PI to PI-4-P and are divided into two types (II and III), based on biochemical differences (sensitivity to detergents and adenosine). The cDNA for a Type II enzyme has recently been cloned and is suggested to establish a novel family of PI 4-kinases because it lacks the phosphoinositide kinase (PIK) domain (S. Minogue, 2001). The PIK domain is found in the Type III PI kinases and PI 3-kinases (Section 5.1.2) and has an unknown function. No homology to other known proteins was found in the Type II enzyme. Several Type III enzymes have been identified molecularly in yeast and mammals and are categorized as α and β isoforms, based on sequence homology. All contain the PIK domain and a conserved catalytic region. The mammalian α isoform additionally contains ankyrin-like repeats, a SH3 domain, and a polyproline motif. Although it is likely that the PI kinases are regulated by extracellular signals, the mechanisms involved are not known.

The enzymes that act on monophosphorylated forms of PI (PIP) are divided into two families, based on their preferred site of phosphorylation [23,24]. The PIP 5-kinases (also known as Type I) phosphorylate position 5 and PIP 4-kinases (Type II) phosphorylate position 4 of the inositol ring. The PIP 5-kinases are more promiscuous in substrate specificity and phosphorylate both PI-4-P (to form PI-4,5-P_2) and PI-3-P (to form PI-3,5-P_2 or PI-3,4-P_2). In addition, they can add two phosphates to PI-3-P in vitro to form PI-3,4,5-P_3. The PIP 4-kinases prefer PI-5-P as substrate and form PI-4,5-P_2. Multiple isoforms of each have been found in plants and animals. The two groups share homology in their kinase domain, which resembles only slightly the kinase domains in other phosphoinositide and protein kinases. The Type II enzymes contain polyproline sequences, indicating possible interaction with SH3 domains. The two groups do not possess other known domains or motifs. A crystal structure of a PIP 4-kinase shows the enzyme exists as a dimer, forming an extended basic flat face that interacts with membranes containing anionic phospholipids (V.D. Rao, 1998).

Regulation of the PIP 5- and 4-kinases by receptor-mediated signals is under intensive study, but physiologically relevant mechanisms are unclear [23,24]. Some of the Type II enzymes associate with cell-surface growth factor and cytokine receptors. The Type I enzymes are markedly stimulated by PA in vitro and this also may occur in intact cells (D.R. Jones, 2000). The Type I enzymes may be regulated by GTP-binding proteins, since the non-hydrolyzable GTP analog GTPγS increases PIP 5-kinase activity in cells.

5.1.2. Phosphoinositide 3-kinases

Phosphoinositide 3-kinases catalyze the addition of phosphate to the D3 position of the inositol ring of phosphoinositides. Phosphoinositide 3-kinases comprise a large family of enzymes divided into three classes (I, II, and III), with each class showing differences in structure (Fig. 11), substrate specificity, and regulation [23]. The class I enzymes are heterodimeric proteins, consisting of a 110 kDa catalytic subunit and a 50–101 kDa adaptor/regulatory subunit. The adaptor/regulatory subunit inhibits catalytic activity

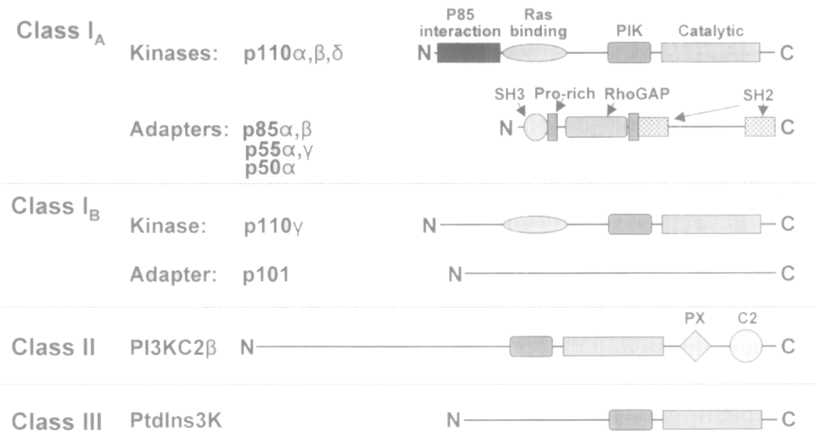

Fig. 11. Classes of mammalian phosphoinositide 3-kinases. Class I enzymes consist of a kinase subunit and an adapter (regulatory) subunit. Functional domains are labeled at the top of the figure or above each isoform. Domains include: p85 interaction domain; Ras binding domain; phosphoinositide kinase (PIK) domain; catalytic domain; Src homology 2 (SH2) and 3 (SH3) domains; proline-rich region (Pro-rich); Rho-GTPase-activating protein (RhoGAP) domain; phox homology (PX) domain; and C2 domain.

and recruits the enzyme to specific subcellular sites by regulated interaction with other proteins. In vitro, the class I enzymes use PI, PI-4-P, and PI-4,5-P_2 as substrates. However, the major products in vivo are PI-3,4-P_2 and PI-3,4,5-P_3, so intracellular regulatory mechanisms must limit substrate specificity.

The class I enzymes are further subdivided into two classes (IA and IB), based on differences in sequence and receptor regulation (Fig. 11). The class IA enzymes are regulated by receptor protein tyrosine kinases and consist of three 110 kDa catalytic subunits (α, β, δ) from different genes and at least five regulatory subunits (p85α, p85β, p55α, p55γ, p50α), which can interact interchangeably. The p55α and the p50α proteins are alternatively spliced products of the p85α gene. By convention, all of these subunits are referred to as p85. The class IB enzyme is activated by G protein-coupled receptors and is made up of one p110 catalytic subunit (γ) and a 101 kDa regulatory subunit, with no homology to the class IA adaptor/regulatory subunits. The p110γ subunit cannot interact with the class IA regulatory subunits, because it lacks an N-terminal interaction region present in p110α, β, and δ (Fig. 11). The p110 subunits share a number of other functional domains, including the C-terminal catalytic domain, a PIK domain shared with the Type III PI 4-kinases, and a Ras-binding domain. When activated by receptor agonists, the small GTPase Ras can bind to p110 and increase catalytic activity, indicating that the class I enzymes are Ras effectors.

The class IA p85 subunits also share a number of protein-interaction domains (Fig. 11), whose function is to recruit the holoenzyme to specific cellular locations by interaction between the domains and specific target proteins. All p85 proteins contain two SH2 domains, which bind to specific motifs containing phosphorylated tyrosine residues. Tyrosine phosphorylation occurs in many receptor complexes (e.g. receptor tyrosine kinases such as the epidermal growth factor receptor) in response to

extracellular stimuli. Binding of the SH2 domains of p85 to phosphotyrosine motifs in the receptor complex mediates the recruitment of p110 to the membrane, where it can access substrates and undergo additional activation by Ras. The longer forms of p85 also contain a SH3 domain in the N-terminal region, which binds to polyproline motifs in target proteins, and either two (p85α) or three (p85β) polyproline (Pro-rich) motifs. The shorter p85 isoforms contain only one polyproline motif. Only a few binding partners (e.g. the GTPase dynamin for the SH3 region, the Src family of protein tyrosine kinases for the polyproline motifs) for the N-terminal region are known. The longer forms of p85 contain a Rho-GTPase-activating protein homology domain, which mediates interaction of the Rho family of GTPases (e.g. Rac, Cdc42) with p85. Whether these different protein-interaction motifs cooperate to localize and activate the enzyme, antagonize each other under certain conditions, or allow the enzyme to participate in different signaling pathways is not clear.

The class IB catalytic subunit (p110γ) is activated by interaction with βγ subunits of heterotrimeric G proteins. It shares the catalytic, PIK, and Ras-binding domains with the class 1A p110 subunits (Fig. 11). The function of the p101 adaptor/regulatory subunit is not clear, since it has no homology to the p85 subunits. However, p101 appears to be necessary for the activation of p110γ by βγ subunits.

The class II phosphoinositide kinases (α, β) are single polypeptides, sharing the catalytic and PIK domains with the class I p110 subunits. These kinases also contain an atypical C2 domain, which may mediate the binding of the protein to anionic phospholipids in membranes. The C2 domain lacks residues that are important for Ca^{2+} binding, so Ca^{2+} most likely does not play a role in phospholipid binding. The two mammalian isoforms both have a C-terminal PX domain, which may bind PI-4,5-P_2 (X. Song, 2001). Both the C2 and the PX domains are likely to regulate membrane recruitment and activation of the class II enzymes. In vitro, the class II enzymes prefer PI and PI-4-P as substrates, thus forming PI-3-P and PI-3,4-P_2. Their specificity in vivo is not known.

The class III phosphoinositide 3-kinases share only the catalytic and PIC domains with the enzymes in the other two classes. The first class III enzyme was identified in yeast, where it is essential for transport of proteins. These enzymes phosphorylate only PI and are thought to be the major contributors to the synthesis of PI-3-P. A putative adaptor/regulator for the class III enzymes has been identified, which is a protein serine/threonine kinase. It was thought that the class III enzymes are not subject to receptor-mediated regulation, but recent work suggests that they regulate the process of phagosome maturation in professional phagocytic cells (O.V. Vieira, 2001). Since several protein domains have been shown to bind PI-3-P (Section 5.2.2), it is likely that the class III enzymes have important signaling roles.

5.1.3. PTEN and other phosphoinositide phosphatases

Inositol phosphatases remove phosphates from the inositol ring and many of these enzymes can use both soluble inositol phosphates and lipid phosphoinositides as substrates. The focus in this section is on the lipid phosphatases that hydrolyze phosphate from the D3, D4, or D5 position of the phosphoinositides. The overall function of these phosphatases is to down-regulate phosphoinositide-dependent signaling reactions.

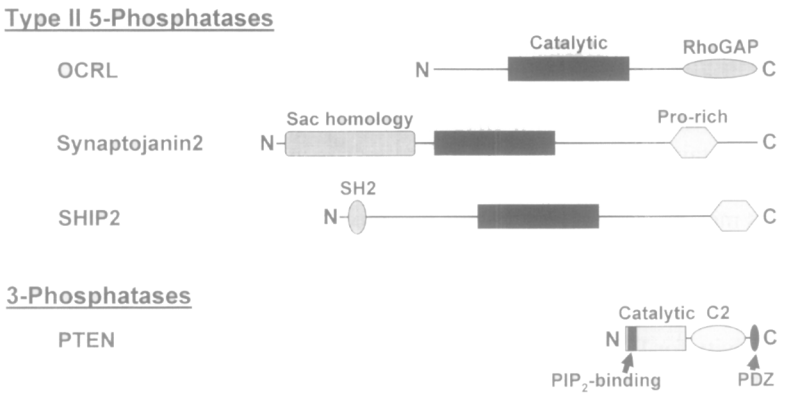

Fig. 12. Domain structures of representative phosphoinositide phosphatases. Shown are selected members of the Type II 5-phosphatases and the 3-phosphatases. Domains include: catalytic (different for the 5- and the 3-phosphatases); Rho-GTPase-activating protein (RhoGAP) domain; Sac homology domain, a region with lipid phosphatase activity; polyproline (Pro-rich) region; Src homology 2 (SH2) domain; C2 domain; a phosphatidylinositol-4,5-bisphosphate binding motif (PIP_2-binding) and a PDZ motif.

Mutations in genes encoding some of these enzymes are responsible for certain dystrophies and myopathies and also are common in various tumors. These phosphatases are classified into three families, based on selectivity for the different positions on the inositol ring: the 5-phosphatases, the 3-phosphatases, and the 4-phosphatases [25]. Domain structures of representatives from the 5- and 3-phosphatase families are shown in Fig. 12.

The largest family is the 5-phosphatases, which is divided into two major groups, based on substrate preference and structural differences [6,25]. They all share a homologous phosphatase domain, defined by two signature motifs involved in catalysis and divalent metal ion binding (Y. Tsujishita, 2001). Type I enzymes hydrolyze only water-soluble substrates, as discussed in Section 2.1.2. Type II enzymes prefer PI-4,5-P_2 and/or PI-3,4,5-P_3 as substrates. Although they share the 5-phosphatase catalytic domain, Type II members differ with respect to other regions. One subclass is typified by OCRL-1 that, when mutated, causes Lowe syndrome or oculocerebrorenal dystrophy. Characteristics of the disease include cataracts, mental retardation and the development of renal failure. OCRL-1 contains a C-terminal Rho-GTPase-activating protein domain of unknown function, which could be involved in localization of the enzyme.

A second Type II subclass consists of the synaptojanins, which participate in synaptic vesicle trafficking. These proteins have a C-terminal polyproline region, presumably mediating interactions with specific SH3 domain-containing proteins. Synaptojanins also contain an N-terminal Sac homology domain, which possesses a separate lipid phosphatase activity (W.E. Hughes, 2000). The Sac domain shows selectivity for monophosphoinositides or phosphoinositides without adjacent phosphates (i.e. PI-3,5-P_2). Thus, synaptojanins have two phosphatase domains, which allows hydrolysis of PI-4,5-P_2 to PI.

A third Type II sub-class consists of SHIP1 and SHIP2, which show specificity for 3-phosphate-containing substrates (e.g. PI-3,4,5-P_3). These enzymes contain a SH2

domain at the N-terminus, which is responsible for their acronym (*SH2*-containing *i*nositol *p*hosphatase). SHIP1 is restricted to hematopoietic cells, while SHIP2 is more widely distributed. The SHIPs also can be phosphorylated on C-terminal region Tyr residues, which allows interaction with SH2 domain-containing proteins (e.g. Shc). SHIPs additionally contain one or more polyproline motifs in the C-terminal half of the protein, which mediates binding to SH3 domain-containing proteins. The SH2 domains and the other motifs mediate assembly of SHIPs into growth factor and cytokine receptor signaling complexes, where the enzymes can hydrolyze newly formed PI-3,4,5-P_3. This limits the duration of PI-3,4,5-P_3-mediated signaling.

The fourth Type II subclass of 5-phosphatases (not shown in Fig. 12), like the SHIPs, shows selectivity for PI-3,4,5-P_3. These enzymes lack SH2 domains, but contain numerous N-terminal polyproline motifs, as well as an immunoreceptor tyrosine-based activation motif, usually found only in receptor molecules (M.V. Kisseleva, 2000). Binding partners for these motifs, which presumably regulate localization and function of the enzyme, are not identified.

The 3-phosphatases are a novel family of proteins, which contain a protein tyrosine phosphatase active site motif [26]. PTEN/MMAC1/TEP1 (acronyms for *p*hosphatase and *ten*sin homolog, *m*utated in *m*ultiple *a*dvanced *c*ancers, and *T*GFβ-regulated and *e*pithelial cell-enriched *p*hosphatase) was the first member identified and it was originally thought to be a dual specificity protein tyrosine kinase involved in tumor suppression. Mutations in the PTEN gene cause tumorigenesis and contribute to specific syndromes with increased risk for breast and thyroid cancers. The known functions of PTEN are to switch off cell proliferation and to permit programmed cell death (apoptosis), which clearly are tumor suppressive activities. Maehama and Dixon (Maehama, 1998) first reported that PTEN had lipid phosphatase activity, hydrolyzing the D3 phosphate from PI-3,4,5-P_3 with high efficiency. Hydrolysis of PI-3,4-5-P_3 seems to be the primary function of the enzyme in vivo, based on studies using mutations that eliminate lipid, but not protein, phosphatase activity. Regulation of PTEN function is most likely by membrane translocation, mediated by a N-terminal PI-4,5-P_2 binding domain, a C2 domain in the C-terminal region, and a C-terminal PDZ-binding site. Both the PI-4,5-P_2 binding domain and the C2 domain mediate binding to membrane phospholipids. Interaction with PDZ proteins, which are scaffolding proteins, may aid in assembly of PTEN with signaling complexes.

A second group of enzymes, the myotubularins, also hydrolyze the D3 phosphate with preference for PI-3-P [26]. Mutations in two myotubularin family members, MTM1 and MTMR2, cause two human diseases: X-linked myotubular myopathy and Type 4B Charcot–Marie–Tooth syndrome, respectively. The mutations usually affect catalytic activity, suggesting the requirement for phosphatase activity in the normal functioning of the proteins. However, the physiological functions controlled by the myotubularins are not understood. These proteins contain several regulatory domains, including a PDZ-binding motif. Some family members also contain lipid-binding domains (i.e. PH or FYVE domains), which may help to localize the enzyme near its substrate.

The 4-phosphatases are also involved in regulation of 3-phosphoinositide signaling. These enzymes hydrolyze the D4 phosphate of PI-3,4-P_2 to yield PI-3-P [6]. This action will limit the duration of receptor-mediated increases in the levels of PI-3,4-P_2 (see

Section 5.2.1). The 4-phosphatases are Mg^{2+}-independent and may be down-regulated by receptor-triggered proteolysis. Little is known of their physiological functions.

5.2. Cellular targets

Phosphoinositides exert their effects by binding to specific domains in target proteins. Lipid binding may cause a conformational change in the target protein, thereby altering enzyme activity, or it may affect the localization of the target protein and access to enzymatic substrates. Multiple domains and motifs have been shown to bind phosphoinositides in vitro [25,27]. PH domains generally show specificity for PI-4,5-P_2, PI-3,4-P_2 or PI-3,4,5-P_3. PI-4,5-P_2 also binds to the ENTH (acronym for epsin N-terminal homology) domain and to certain Lys/Arg-rich motifs in proteins. Some SH2 and PTB domains bind PI-3,4,5-P_3 in vitro, but the relevance of this interaction in vivo is unclear. FYVE domains [named after the first four proteins shown to contain it: *F*ab1p, *Y*OTB, *V*ac1p, and *e*arly endosome antigen 1 (EEA1)] and certain PX domains selectively bind PI-3-P. Other PX domains show greater specificity for other lipids, as discussed in Section 5.2.2.

5.2.1. Domains that bind polyphosphoinositides

PH domains contain about 120 amino acids and were first identified as targets for PI-4,5-P_2. These domains are found in over 100 proteins, including protein kinases, phospholipases, and regulatory proteins for small GTPases (exchange factors and GTPase-activating proteins). PH domains are divided into four groups, based on their selectivity for binding various phosphoinositides [27]. Group I, typified by PH domains in Bruton's tyrosine kinase, exchange factors for the small GTPase Arf (ARNO, GRP1), and GTPase-activating proteins for Arfs (centaurin α) and Ras (GAP1), show high affinity binding for PI-3,4,5-P_3. Group II PH domains selectively bind PI-4,5-P_2 and are found in PLCδ, oxysterol-binding protein, β-adrenergic receptor kinase, and β-spectrin. Group III PH domains are found in the protein kinases Akt/protein kinase B (PKB) and phosphoinositide-dependent kinase 1 (PDK1) and bind PI-3,4-P_2 and PI-3,4,5-P_3 with equal affinities. Group IV PH domains bind phosphoinositides with relatively low affinity, typified by the PH domain in the endocytic GTPase dynamin.

The Group III PH domain-containing proteins are important cellular targets for PI-3,4,5-P_3 and PI-3,4-P_2, because of their central roles in the regulation of cell survival and proliferation. Akt/PKB is a 57 kDa Ser/Thr protein kinase identified as the cellular homolog of the viral oncoprotein v-Akt. It was also shown to have high homology to protein kinases A and C (hence the name PKB). Akt/PKB is activated by the binding of PI-3,4,5-P_3 or PI-3,4-P_2, which localizes the enzyme to the membrane and induces conformational changes allowing phosphorylation of a critical Thr residue by PDK1. PDK1 is a 63 kDa Ser/Thr kinase, which binds to membranes containing polyphosphoinositides, placing the enzyme in close proximity to Akt/PKB. After interaction with another protein kinase (PKC-related kinase 1), PDK1 is then able to phosphorylate a C-terminal Ser residue in Akt/PKB, rendering the enzyme fully active. Active Akt/PKB phosphorylates a variety of substrates involved in the regulation of apoptosis, cell metabolism, and gene expression. The overall effect of

activated Akt/PKB is to promote cell survival, increase cell size, and enhance cell proliferation.

PI-4,5-P_2 has additional binding sites in proteins, i.e. stretches of Lys/Arg-rich sequences, found primarily in cytoskeletal proteins and in PTEN, and the ENTH domain, a region of about 140 residues found in several proteins involved with endocytosis [27]. Cytoskeletal protein targets include the ezrin/radixin/moesin family of proteins and vinculin, which help to cross-link actin to the plasma membrane, and proteins that regulate actin polymerization, such as gelsolin, cofilin, and profilin (M.J. Bottomley, 1998). ENTH domain-containing proteins include epsin and other proteins involved in assembly of the clathrin lattice. PI-4,5-P_2 also accumulates in the early phagosome, where it recruits PLC. Thus, PI-4,5-P_2 plays major roles in the regulation of the cytoskeleton, endocytosis, and phagocytosis (T. Nebl, 2000; A. Simonsen, 2001).

5.2.2. Domains that bind phosphatidylinositol-3-phosphate

A role for phosphoinositides in endosomal trafficking was first discovered in yeast, where PI-3-P was shown to be the major phosphoinositide involved. Recently, a specific binding domain for PI-3-P, the FYVE domain, was discovered (H. Stenmark, 1996). This domain consists of about 60–80 residues and coordinates two Zn^{2+} atoms [27]. Binding of PI-3-P involves initial contact between a hydrophobic loop of the domain with the membrane, followed by interaction with the headgroup of PI-3-P. Use of the isolated domain coupled to green fluorescent protein demonstrated that PI-3-P is selectively enriched in early endosomes. Many FYVE domain-containing proteins target to endosomes, where they regulate endocytosis, endosomal fusion, and vacuolar transport. About 60 proteins are known to contain FYVE domains, including early endosomal antigen 1, which is essential for endosomal fusion, and PI-3-P-metabolizing enzymes (some of the myotubularin-related 3-phosphatases and the PI 5-kinase PIKfyve).

Another binding target for PI-3-P is the PX domain, first identified as a conserved motif of about 120 residues in the N-terminal regions of the NADPH oxidase proteins $p47^{phox}$ and $p40^{phox}$. It is present in over 70 human and yeast proteins, and PX domain-containing proteins are also found in plants, flies, and worms [27]. The proteins have diverse functions, although a large group includes the sorting nexins, which are involved in targeting of proteins to lysosomes. Recent studies established that several PX domains bind selectively to PI-3-P, including those in several sorting nexins, the yeast SNARE protein Vam7p, and the NADPH oxidase component $p40^{phox}$. However, PX domains from several other proteins ($p47^{phox}$, class II PI 3-kinase, cytokine-independent survival kinase) preferentially bind polyphosphoinositides (PI-4,5-P_2, PI-3,4-P_2, PI-3,4,5-P_3). The PX domain of $p47^{phox}$ also binds PA with higher affinity than the polyphosphoinositides (L.C. McPhail, unpublished results). The lipid-binding specificity for numerous other PX domains is not known. The diversity in lipid-binding and in the types of proteins containing PX domains suggest that PX domains have multiple functions in lipid-signaling pathways.

6. *Lysophosphatidic acid and other lysophospholipids*

For many years it was believed that lysophospholipids were simply intermediates in the metabolism of glycerolipids. However, it has now been clearly established that LPA, LPC and the sphingolysolipids sphingosylphosphorylcholine and sphingosine-1-phosphate act as extracellular signaling molecules [28]. These lipids bind to a family of related cell-surface heptahelical receptors and are implicated in tumorigenesis, angiogenesis, immunity, atherosclerosis, and neuronal survival. Because sphingolipids are discussed in a separate chapter, the focus here will be on LPA and lysophosphatidylcholine.

6.1. Sources of lysophospholipids

A number of cell types have been shown to produce lysophospholipids, including platelets, macrophages, other leukocytes, some epithelial cells, and some tumor cells [29]. The regulation of the synthesis and metabolism of extracellular lysophospholipids is not well understood. Phosphatidylcholine is likely to be the major source for both LPC and LPA and several pathways are possible. The action of a phospholipase A directly yields LPC. LPA can be formed by the sequential actions of PLD and a phospholipase A or of a phospholipase A and a lyso-PLD. The nature of the phospholipases A_2 or A_1 involved may vary, depending on cellular source and stimulus, and is not clearly delineated. Although lyso-PLD activity has been detected in various cell types, the cDNA encoding the enzyme has not been cloned. It is not known how lysophospholipids exit the cell, once they are synthesized. Albumin is probably the main LPA-binding protein in plasma, although plasma gelsolin also binds LPA with higher affinity than albumin. LPC is present in oxidized low density lipoproteins. Metabolism of lysophospholipids is by reacylation and/or, for LPA, dephosphorylation. The enzyme LPA acyltransferase can convert LPA to PA. Lipid phosphate phosphatase-1 is positioned in the plasma membrane of cells such that it can dephosphorylate extracellular LPA to form monoacylglycerol.

6.2. Lysophospholipids are extracellular signaling molecules

LPA induces a number of cellular responses, including proliferation, decreased apoptosis, platelet aggregation, smooth muscle contraction, chemotaxis, and tumor cell invasion. LPC has inflammatory effects in the body, including up-regulation of endothelial cell adhesion molecules and growth factors, and activation of macrophages. Both LPA and LPC may be elevated in certain pathophysiological conditions, such as ovarian cancer and artherosclerosis. LPA and LPC are ligands for members of a family of heptahelical receptors, formerly known as the EDG receptors and now termed the LP receptors [28]. Family members number 12 so far and can be divided into three distinct groups. The first group consists of three receptors (termed LPA_{1-3}) showing high selectivity for LPA. The second group (5 receptors, termed $S1P_{1-5}$) preferentially binds sphingosine-1-phosphate. The third group (SPC_1, LPC_1, PSY, GPR4) is more divergent and shows greater specificity for LPC and sphingosylphosphorylcholine. As

a group, the LP receptors couple to several G proteins, including G_i, G_q, and $G_{12/13}$. Thus, the lysophospholipids exert their effects on cell functions by triggering a variety of G protein-linked second messenger pathways, including activation of PLC, PLD, phosphoinositide 3-kinases, PKC, MAP kinases, and small GTPases. LPA_1 receptor (also called EDG-2) null mice have a complex phenotype, with 50% neonatal lethality and neuronal and other abnormalities (J.J. Contos, 2000). This suggests a critical role for LPA_1 in multiple aspects of development. LPC_1 null mice show T cell abnormalities and develop a late-onset autoimmune disease, similar to systemic lupus erythematosus (L.Q. Li, 2001). Creation of null mice deficient in other receptors in the LP family is needed to help elucidate the spectrum of biological functions of these lysophospholipids.

7. Future directions

Much remains to be learned about glycerolipid signaling. It is becoming clear that the signaling intermediates and protein targets involved in the regulation of specific cell functions assemble and disassemble in dynamic complexes at specific intracellular locations. Movement of proteins in and out of these complexes is likely orchestrated by the transient presence of specific signaling lipids, along with the cytoskeleton. It will be a challenge to design tools to visualize these processes at high resolution within living cells without disrupting the signaling pathways or the functions being regulated. The engineering of fluorescently tagged protein domains that recognize specific lipids is providing some insight [20]. With the dawn of the genomic age, it should soon be possible to identify all of the enzymes involved in the synthesis and metabolism of the signaling lipids, based on homologies with known proteins. Similarly, additional targets for the lipids will be found. However, discerning the biological functions of the numerous enzymes and target proteins involved in lipid signaling will continue to require extensive research using biochemical, cell biological, and molecular tools. Such a global and integrated approach has been established for understanding G protein-dependent cell signaling (The Alliance for Cellular Signaling, http://cellularsignaling.org/), which, of course, encompasses glycerolipid signaling.

Abbreviations

DG	Diacylglycerol
Ins-1,4,5-P_3	Inositol-1,4,5-trisphosphate
$InsP_6$	Inositol hexakisphosphate
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
PA	Phosphatidic acid
PC	Phosphatidylcholine
PH	Pleckstrin homology
PKB	Protein kinase B
PKC	protein kinase C

PLC	Phospholipase C
PLD	Phospholipase D
PI	Phosphatidylinositol
PIP	Phosphatidylinositol monophosphate
PI-4,5-P_2	Phosphatidylinositol-4,5-bisphosphate
PX	Phox homology
SH2	Src homology 2
SH3	Src homology 3

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