

CHAPTER

23

Phosphoinositides

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OUTLINE

Introduction	442	<i>The metabolism of inositol phosphates leads to regeneration of free inositol</i>	449
The Inositol Lipids	443	<i>Highly phosphorylated forms of myo-inositol are present in cells</i>	449
<i>The three quantitatively major phosphoinositides are structurally and metabolically related</i>	443		
<i>The quantitatively minor 3'-phosphoinositides are synthesized by phosphatidylinositol 3-kinase</i>	444	Diacylglycerol	449
Box: Charcot-Marie-Tooth Disease	445	<i>Protein kinase C is activated by the second messenger diacylglycerol</i>	449
<i>Phosphoinositides are dephosphorylated by phosphatases</i>	445	Phosphoinositides and Cell Regulation	451
<i>Phosphoinositides are cleaved by a family of phosphoinositide-specific phospholipase C (PLC) isozymes</i>	446	<i>Inositol lipids can serve as mediators of other cell functions, independent of their role as precursors of second messengers</i>	451
The Inositol Phosphates	448	References	453
<i>D-myoinositol 1,4,5-trisphosphate [$I(1,4,5)P_3$] is a second messenger that liberates Ca^{2+} from the endoplasmic reticulum via intracellular receptors</i>	448		

INTRODUCTION

A large variety of ligands, including neurotransmitters, neuromodulators and hormones, exert their physiological action via an intracellular second-messenger system in which the activated receptor-ligand complex stimulates the turnover of inositol-containing phospholipids, resulting in the formation of the second messengers inositol trisphosphate and diacylglycerol. The original demonstration that phosphoinositide turnover was under neurohumoral control dates back to the early 1950s from the pioneering studies of the Hokins (Hokin & Hokin, 1955). Although it was recognized for many years that alterations in Ca^{2+} homeostasis

and protein phosphorylation accompanied these changes in inositol lipid turnover, it was not until some 30 years later that inositol 1,4,5-trisphosphate and diacylglycerol were identified as second messenger molecules capable of mobilizing intracellular Ca^{2+} and activating protein kinase C, respectively. For a historical perspective, the reader is referred to a review by Irvine (Irvine, 2003). In this chapter, the biochemical and cellular bases of this ubiquitous pathway, as well as its pharmacological significance and physiological importance, are examined in the context of the nervous system. To better understand the events leading to the enhanced inositol lipid turnover, it is necessary first to review the underlying structural chemistry.

THE INOSITOL LIPIDS¹

The three quantitatively major phosphoinositides are structurally and metabolically related

These consist of phosphatidylinositol (PI) and the two polyphosphoinositides, namely phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] (see Fig. 23-1). PI consists of a diacylglycerol (DAG) moiety that is phosphodiesterified to myo-inositol, a 6-carbon polycyclic alcohol (see Fig. 23-1) that has one axial and five equatorial hydroxyls. Myo-inositol (I) is particularly enriched in neural tissues (5–20 mM), where it serves not only as a precursor molecule for inositol lipid synthesis, but also as a physiologically important osmolyte (Fisher et al., 2002). The distinctive configuration of myo-inositol can be more readily understood by making use of Agranoff's "turtle" (Agranoff, 1978), in which the cyclohexane chair conformation is viewed as a turtle with the axial hydroxyl as the head, and hydroxyls in the five equatorial positions serving as the four limbs and tail (Fig. 23-1).

There exists an unusual uniformity in the fatty acid composition of the inositol lipids. All three of the major phosphoinositides are enriched in the 1-stearoyl, 2-arachidonoyl *sn*-glycerol species (~80% in brain). The polyphosphoinositides [PI4P and PI(4,5)P₂] are present in much lower amounts than PI while PI(4,5)P₂ has been shown to be predominantly (although not exclusively) localized to the plasma membrane (Micheva et al., 2001). Because of its high complement of plasma membranes, the brain is one of the most enriched sources of the polyphosphoinositides.

The phosphoinositides are synthesized from the glycerolipid precursor phosphatidic acid (PA) via the formation of the liponucleotide intermediate, cytidine diphosphate diacylglycerol (CDP-DAG; see Fig. 23-2B and Chapter 5), catalyzed by CDP-DAG synthase. Subsequently, PI synthase catalyzes the formation of PI. The latter is then phosphorylated to PI4P in a reaction requiring ATP and catalyzed by PI 4-kinase (PI4K; see Fig. 23-2A). Cloning studies have revealed the presence of four distinct isoforms of PI4K, type II α and β and type III α and β (Sasaki et al., 2009). The type II enzymes contain a split lipid kinase domain and are ubiquitously expressed, although the α subtype is enriched in brain where it may play a role in vesicle trafficking (Guo et al., 2003). Type III PI4K's feature a continuous lipid kinase domain. Whereas the β isoform is ubiquitously expressed, the α isoform is found primarily in brain. In contrast to the type II isoforms, both Type III subtypes are sensitive to the fungal metabolite wortmannin (albeit at relatively high concentrations). The ability of wortmannin to block the sustained agonist-induced turnover of inositol lipids has led to the suggestion that wortmannin-inhibitable isoforms of PI4K are involved in the regulation of hormone-sensitive pools of phosphoinositides (Balla et al., 2008). PI4P is further phosphorylated via PIP kinase to

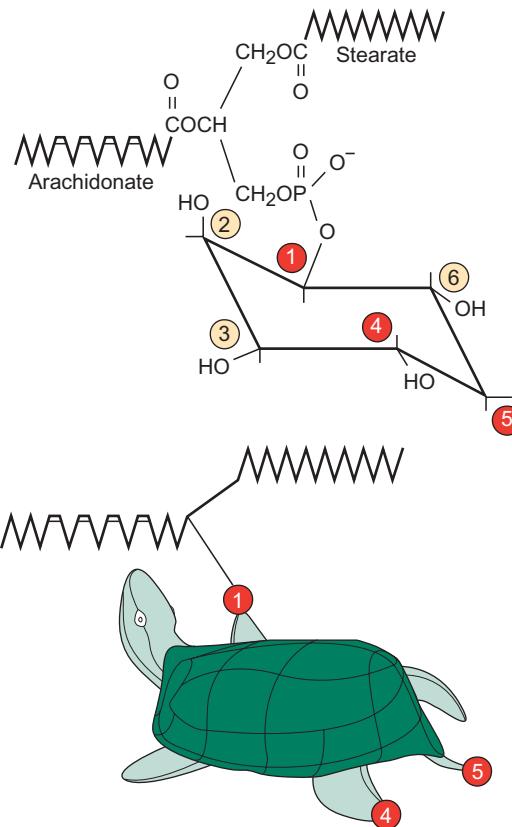


FIGURE 23-1 Stereo chemistry of the quantitatively major inositol lipids. Inositol lipids characteristically contain stearic acid (18:0) and arachidonic acid (20:4 ω 6) esterified to the 1 and 2 position of *sn*-glycerolphosphate, respectively. The phosphate (colored circle) is diesterified to the 1 position of D-myo-inositol. myo-Inositol in its favored chair conformation has five equatorial hydroxyls and one axial hydroxyl. Looking at the chair from above and counting counterclockwise, the axial hydroxyl is then in position 2. As indicated by the drawing, the inositol molecule can be conveniently viewed as a turtle (see Agranoff, 1978) in which the diacylglycerol phosphate moiety is attached to the right front leg (position 1), next to the raised head (the axial hydroxyl in position 2). The other equatorial hydroxyls are represented by the remaining limbs and the tail. Phosphatidylinositol (PI) can be phosphorylated at position 4 (the rear left leg), or the rear left leg as well as the tail (positions 4 and 5 as shown), to yield phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], respectively.

PI(4,5)P₂ (Fig. 23-2A). Three distinct classes of PIP kinase can be identified (Sasaki et al., 2009) and all share a conserved kinase core domain. The type I enzyme (α , β and γ isoforms), preferentially phosphorylates PI(4)P to PI(4,5)P₂ and plays a role in both endo- and exocytosis as well as in actin dynamics. Type II PIP kinase exhibits a preference for PI(5)P over PI(4)P and will phosphorylate at the 4-position of the inositol ring to yield PI(4,5)P₂. Type III PIPK, originally known as PIKfyve because of the presence of an N-terminal "fyve" (or

¹The authors have used the 'Chilton Conference' nomenclature for inositol lipids throughout this chapter, e.g., PI, PI4P, and PI(4,5)P₂ for phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, respectively. Note that the IUB recommended nomenclature for these lipids is PtdIns, PtdIns4P and PtdIns(4,5)P₂ (see Chapter 5).

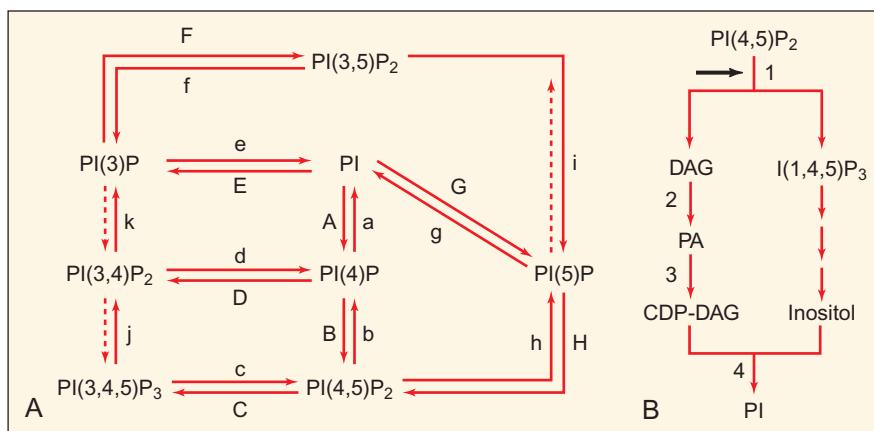


FIGURE 23-2 Pathways of inositol lipid metabolism. **A.** Interrelationship between routes of synthesis and degradation of the “canonical” inositol lipids [PI, PI(4)P and PI(4,5)P₂] and the 3-phosphoinositides (for explanation of abbreviations, see text). Capital letters refer to reactions catalyzed by kinases: A, Type II/III PI4K; B, Type I PIPK; C, Type I PI3K; D, Type I/II PI3K; E, Type I/II PI3K; F, Type III PIPK; G, Type III PIPK; H, Type II PIPK. Lower case letters refer to reactions catalyzed by phosphatases: a, SAC1; b, SAC2, INPP5E, Synaptojanin, OCRL; c, PTEN; d, MTMRs; e, SAC1, MTMRs; f, SAC3; g, PLIP; h, TMEM55; i, INPP5s, SHIP1/2, SAC2, OCRL, synaptojanins; k, INPP4. Broken arrows indicate that the reactions have yet to be confirmed. **B.** Regenerative cycle of phosphoinositide resynthesis following receptor-ligand-mediated breakdown of PI(4,5)P₂ (arrow) and generation of the second messengers DAG and I(1,4,5)P₃. Numbers refer to the following enzymes: 1, phosphoinositide-specific phospholipase C; 2, DAG kinase; 3, CDP-DAG synthase; 4, PI synthase.

FYVE) domain, phosphorylates PI and PI(3)P (see below) to yield PI5P and PI(3,5)P₂, respectively. This further adds to the complexity of phosphoinositide metabolism. Since PI(4,5)P₂ represents only a minor fraction of the total inositol lipid pool, its resynthesis would be a prerequisite for both acute and sustained signaling. Despite the obvious physiological importance, mechanisms that regulate the resynthesis of PI(4,5)P₂ have not been well defined. However, some evidence has been presented in favor of the regulation of PI4-kinase and PIP-kinase activities by a diverse array of agents, including PA, GTP and protein kinase C (Fisher, 1995).

The quantitatively minor 3'-phosphoinositides are synthesized by phosphatidylinositol 3-kinase

These lipids [principally, PI3P, PI(3,4)P₂, PI(3,5)P₂ and PI(3,4,5)P₃], documented by Cantley and colleagues in the mid-1980s, are present in both neural and non-neuronal tissues at concentrations well below those found for either PI4P or PI(4,5)P₂ (Cantley, 2002). In resting cells, the concentration of PI3P exceeds, by an order of magnitude, that of the other 3-phosphoinositides. However, whereas the concentration of PI3P is relatively stable in cells, the mass of PI(3,4)P₂ and PI(3,4,5)P₃ can increase dramatically upon receptor activation (Cantley, 2002). The metabolic interrelationships of the 3-phosphoinositides are shown in Fig. 23-2A. The key enzyme involved in the synthesis of the 3-phosphoinositides is PI 3-kinase (PI3K). Three major classes of PI3K have been described, based upon lipid substrate specificity and regulation. Class I PI3Ks are able to phosphorylate PI, PI4P and PI(4,5)P₂ *in vitro* to form PI3P, PI(3,4)P₂ and PI(3,4,5)P₃, respectively, although their preferred substrate *in vivo* is PI(4,5)P₂. The catalytic subunits of this class of PI3K form heterodimeric complexes with adaptor or regulatory proteins that link

the enzyme to upstream signaling events. Class I A catalytic subunits (p110 α , β , δ) interact with PI3K regulatory proteins (p85) that contain two SH2 domains. The presence of the latter enables the enzyme to more readily associate with phosphorylated tyrosine residues present on upstream proteins, such as receptors with intrinsic tyrosine kinase activity or, alternatively, non-receptor tyrosine kinases, such as Src or JAK kinases, thereby linking PI3K to tyrosine kinase signaling pathways (see Chap. 26). Phosphotyrosine binding to the regulatory domain of PI3K enhances both the lipid kinase activity and the translocation of the enzyme from cytosol to plasma membrane. Class I B catalytic subunits (p110 γ) bind to either p101, p84 or p87 regulatory proteins and are stimulated by $\beta\gamma$ subunits derived from G proteins. Both Type A and B catalytic subunits of Class I PI3K interact with a small GTP-binding protein, Ras, although the significance of this interaction remains uncertain. Class I PI3Ks can be potently inhibited by either wortmannin, a fungal metabolite, or by LY294002, a quercetin analog. Both inhibitors bind to the catalytic subunit of PI3K and inhibit kinase activity. Class II and Class III PI3Ks are monomeric proteins that exhibit a strong preference for PI as a substrate.

In contrast to their more highly expressed counterparts, the 3-phosphoinositides do not serve as substrates for phosphoinositide-specific phospholipase C (PLC), the enzyme activated in stimulated phosphoinositide turnover. This observation indicates that the 3-phosphoinositides themselves, rather than their breakdown products, are likely to be the intracellular mediators of biological activity. PI3P, PI(3,4)P₂ and PI(3,4,5)P₃ can bind to specific domains within proteins, thereby facilitating protein-protein interactions. As will be detailed below (see Phosphoinositides and Cell Regulation), roles for 3-phosphoinositides in the regulation of membrane trafficking, cell survival, maintenance of the cytoskeleton and autophagy have been proposed.

CHARCOT-MARIE-TOOTH DISEASE

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Charcot-Marie-Tooth disease (CMT) is a genetically heterogeneous group of inherited disorders characterized by severe peripheral neuropathy, affecting myelinated motor and sensory axons and leading to distal muscle weakness and atrophy. Among the recessive demyelinating neuropathies (CMT4), three have been found to result from defects in enzymes of phosphoinositide metabolism. CMT4B-1 and -2 exhibit mutations in members of the myotubularin family of PI 3-phosphatases, MTMR2 and MTMR13, respectively, whereas CMT4J exhibits mutations in SAC3 (homolog of yeast FIG4), a PI(3,5)P₂ 5-phosphatase (Scherer et al., 2008). A unique aspect of the MTM family is that nearly half of its members are catalytically inactive. Several of the latter have been found to play important roles in phosphoinositide homeostasis, likely by virtue of their interaction with active MTMs (Robinson et al., 2006). MTMR2, which is catalytically competent, does not display full activity unless it is complexed with the inactive MTMR13. Thus CMT4B-1 and -2 share similar pathology, with myelin outfolding in the nodal/paranodal region. Targeted alteration of the genes for MTMR2 or MTMR13 in mouse Schwann cells reproduces the demyelination found in CMT, in contrast to neuronal ablation of MTMR2, which resulted in no detectable phenotype (Scherer et al., 2008). MTMs are localized to endosomal compartments, underscoring their role in control of PI3P/PI(3,5)P₂ signaling which is important for trafficking through the endosomal system (Nicot et al., 2008). Further indication that PI3P/PI(3,5)P₂ play a crucial role in myelinating Schwann cells is provided by the discovery that CMT4J is caused by mutations in the 5-phosphatase, SAC3. The

latter forms a complex with PIKfyve (a PI3P-5kinase), leading to its activation and production of PI(3,5)P₂ (Liu et al., 2010). Thus, paradoxically, loss of SAC3 (which converts PI(3,5)P₂ to PI3P) leads to a decrease in PI(3,5)P₂. Inactivation of SAC3 in mice produces the “pale tremor mouse,” characterized by both peripheral neuropathy and degeneration of the central nervous system. Although the importance of PI3P and PI(3,5)P₂ homeostasis has been clearly demonstrated in CMT, disorders of autophagy and a wide array of other disease states, the manner in which perturbations in this process contributes to the pathology is poorly understood, although a defect in endosomal function seems likely (McCrea & De Camilli, 2009). Compromised turnover of PI3P/PI(3,5)P₂ and other phosphoinositides is likely to be implicated in additional disorders as more disease genes are identified.

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Phosphoinositides are dephosphorylated by phosphatases

Whereas PI(4,5)P₂ is the only phosphoinositide that undergoes phosphodiesteratic cleavage by PLC, as detailed in the following section, most phosphoinositides are subject to the action of members of various families of phosphatases that dephosphorylate at the D3, D4 or D5 position (Fig. 23-2A; for review, see Sasaki et al., 2009). Phosphoinositide 3-phosphatases, exemplified by myotubularin and PTEN (phosphatase and tensin homolog), share a phosphatase domain containing the CX5R motif. The multimember myotubularin (MTM) family hydrolyzes at the D3 position of PI3P and/or PI(3,5)P₂. Mutations in some MTMs have been implicated in human myopathy and neuropathy (see Box 23-1). PTEN is a tumor suppressor that dephosphorylates both lipids and protein phosphotyrosine residues. Action of PTEN against its preferred substrate, PI(3,4,5)P₃, regulates cell proliferation due to the consequent reduction in Akt activation (see Cell death and survival, below). In addition, a member of the SAC (suppressor of actin) family of phosphatases (SAC1) will cleave at the D3 position of PI3P as well as at the D4 position of PI4P. Two types of inositol polyphosphatase 4-phosphatases (INPP4s) have been identified in mammals: INPP4A and -4B are active on PI(3,4)P₂, while TMTM55A and -55B are active on PI(4,5)P₂.

Recent evidence points to a role for INPP4A (but not INPP4B) in the suppression of NMDA receptor-mediated excitotoxic cell death in neurons (Sasaki et al., 2010; see Ch. 17).

The inositol polyphosphate 5-phosphatases (INPP5s) are characterized by two critical consensus sequences and consist of four classes. Type I hydrolyzes only the water-soluble inositol phosphates, while Types II–IV hydrolyze both lipids and inositol phosphates. Members of the Type II 5-phosphatase family include synaptjanins and OCRL1, a gene that is disrupted in the human X-chromosome-linked developmental disorder called oculocerebrorenal syndrome of Lowe. The wild-type OCRL gene product is reportedly enriched in Golgi or early endosomes and primarily hydrolyzes PI(4,5)P₂, PI(3,4,5)P₃, I(1,4,5)P₃ and I(1,3,4,5)P₄. Lowe’s syndrome results in mental retardation, congenital cataracts and renal tubular acidosis. There are two synaptjanin isozymes: both synaptjanin 1 and synaptjanin 2 are ubiquitously expressed, but the former is enriched in neurons and plays a role in synaptic vesicle recycling. Both synaptjanins are capable of hydrolyzing PI(4,5)P₂ and PI(3,4,5)P₃, as well as I(1,4,5)P₃ and I(1,3,4,5)P₄. By virtue of their N-terminal sac domains, the synaptjanins are also able to act as D3 and D4 phosphatases. Two members of the type III INPP5s have been identified: SHIP1 and SHIP2 (Src Homology Contains Inositol Phosphatase), as well as shorter splice variants of SHIP1. These enzymes contain

an N-terminal SH2 domain and a C-terminal proline-rich domain. The preferred substrate of both SHIP1 and SHIP2 is PI(3,4,5)P₃, but SHIP1 will also hydrolyze I(1,3,4,5)P₄. The only type IV enzyme is INPP5E (also known as pharbin), which has very high affinity for PI(3,4,5)P₃ and is highly expressed in brain, testis and heart. Other enzymes, which lack the defining two consensus motifs of the INPP5's, can also act as phosphoinositide 5-phosphatases. These include PLIP (PTEN-like lipid phosphatase), a dual activity protein tyrosine phosphatase that prefers PI5P as a substrate, and the SAC family phosphatases. Whereas SAC1 preferentially hydrolyzes PI3P and PI4P, SAC2 acts on the D5 position of PI(4,5)P₂ and PI(3,4,5)P₃, and SAC3 targets the D5 position of PI(3,5)P₂.

Phosphoinositides are cleaved by a family of phosphoinositide-specific phospholipase C (PLC) isozymes

PLC catalyzes the phosphodiesteratic breakdown of PI(4,5)P₂, a reaction activated by a large number of pharmacologically distinct CNS receptors (see Table 23-1 and Fisher et al., 1992). The breakdown of PI(4,5)P₂ generates two second messenger molecules, namely diacylglycerol, which is released into the plane of the plasma membrane where it can then activate protein kinase C, and inositol 1,4,5-trisphosphate [I(1,4,5)P₃], which enters the cytosol and mobilizes the release of intracellular Ca²⁺ from ER consequent on the activation of I(1,4,5)P₃ receptors (see below). Further metabolism of these second messengers leads to a regenerative cycle of phosphoinositide resynthesis (Fig. 23-2B). It should be noted that the process whereby PI(4,5)P₂ is cleaved by PLC and subsequently resynthesized is metabolically expensive. Thus, for each mol of PI(4,5)P₂ hydrolyzed and resynthesized, 3 mols of ATP and 1 mol of CTP are consumed. ATP is consumed at the DAG kinase, PI4-kinase and PIP-kinase steps, whereas CTP is utilized by CDP-DAG synthase. Consequently, it is likely that,

under physiological conditions, PI(4,5)P₂ breakdown is kept to a minimum.

Five isoforms of PLC (β , γ , δ , ϵ , η ; Fig. 23-3) have been identified in the brain, with the nomenclature based upon the chronology of their discovery. (The α isoform was subsequently identified as a proteolytic fragment of PLC δ ; for review, see Suh et al., 2008.) Because the lower eukaryotes such as yeast and slime molds possess only the δ isoform, it has been suggested that the other isoforms evolved from an archetypal PLC δ . One additional isoform which is found only in sperm, has been described – PLC ζ . A number of isozymes

TABLE 23-1 Examples of Ligand-Activated Phosphoinositide Hydrolysis in Neural Tissues

Muscarinic	Neuropeptide Y
Adrenergic	Neurotensin
Histaminergic	Gastrin-releasing peptide
Serotonergic	Bombesin
Glutamatergic	Substance P
Endothelin	Oxytocin
Purinergic	Eledoisin
Thromboxane	Neurokinin
Prostaglandin	Vasointestinal peptide
Bradykinin	Angiotensin
Vasopressin	Gonadotropin-releasing hormone
Nerve growth factor	Platelet-activating factor
Cholecystokinin	Thyrotropin-releasing hormone

For the identities of the individual receptor subtypes that couple to phosphoinositide hydrolysis, the reader is referred to the individual chapters on neurotransmitters in this volume.

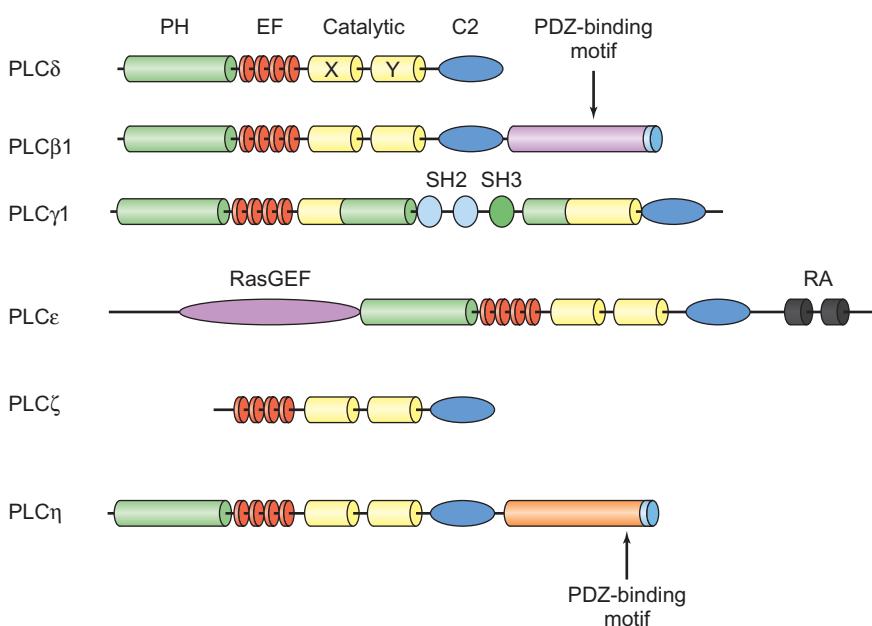


FIGURE 23-3 Linear representation of the six classes of PLC isoforms that can be distinguished by the presence of different domains. Abbreviations: EF, EF-hand domain; PH, pleckstrin homology domain; RA, Ras-binding domain; RasGEF, guanine-nucleotide exchange factor for Ras; SH, Src homology domain; X and Y, catalytic domain. (Adapted from Cockcroft, 2006 with permission.)

of each subtype of PLC have been identified such that at least 13 distinct isozymes are thought to exist. Isozymes of PLC require the presence of Ca^{2+} for full activation and will hydrolyze the inositol lipids with a pronounced selectively for PI(4,5)P₂ over PI *in vivo*. Despite the similarity of function of each isozyme, only two regions of amino acid homology exist (X and Y), one of 150 and a second of 120 amino acid residues that are 54 and 42 percent identical among the isozymes, but are differentially localized within each enzyme (Fig. 23-3). The X and Y domains form the catalytic core of the enzyme. The linker that separates these conserved domains has recently been shown to be autoinhibitory, thereby accounting for the low basal activity of PLC (Hicks et al., 2008). This inhibition is relieved upon binding of the enzyme to membranes. For most of the isoforms, relatively few amino acids separate the X and Y entities, whereas a much larger separation is observed for the PLC γ isoform, wherein the region between X and Y contains amino acid sequences that are found in non-receptor tyrosine kinases (SH2 and SH3 domains). All five isoforms possess pleckstrin homology (PH) domains. The latter are thought to enable the enzyme to become tethered to the plasma membrane via an interaction with PI(4,5)P₂. In addition, all PLC isoforms possess an E-F hand domain, which is located between PH and X domains, and a C2 domain, which is located close to the Y domain. The γ and δ forms of PLC can be distinguished from PLC β by the virtual absence of a 400–500 amino acid consensus sequence present in the C-terminal region of PLC β . Consistent with the absence of putative trans-membrane spanning domains, most PLC activity is localized to the cytoplasm, although a significant amount of activity is associated with membrane fractions.

Various mechanisms of receptor activation of PLC have been identified, most prominently, those involving either receptors with intrinsic tyrosine kinase activity (see Ch. 26) or receptors coupled to G proteins, (see Fig. 23-4 and Ch. 21). Agonist

occupancy of receptors that possess intrinsic tyrosine kinase activation (e.g., platelet derived growth factor; mechanism I) can result in receptor dimerization and autophosphorylation of the cytoplasmic tails on tyrosine residues, thereby recruiting proteins (such as PLC γ) that possess SH2 domains. In this manner, the cytosolic effector enzyme PLC γ is brought into close proximity to the plasma membrane, the site of its substrate, PI(4,5)P₂, and of its activation by phosphorylation. The interaction of a ligand with a GPCR (e.g., muscarinic cholinergic) results in the activation and dissociation of G $_{\text{q}}$, a pertussis toxin-insensitive G protein, to liberate α_{q} . The latter then may activate PLC β_1 and/or PLC β_3 (mechanism II). Alternatively, the ligand-receptor interaction results in the activation and dissociation of a pertussis toxin-sensitive G protein (G $_{\text{i}}$ or G $_{\text{o}}$) that in turn liberates α_{i} or α_{o} and $\beta\gamma$ subunits (mechanism III). In this case, it is the $\beta\gamma$ subunits, not the α subunit, which can then activate PLC β_3 (or in the case of hematopoietic cells, PLC β_2). Different regions of the PLC molecule appear to be required for α_{q} or $\beta\gamma$ activation. Thus, whereas cleavage of the carboxyl terminal portion of PLC β_1 renders it refractory to α_{q} regulation, the amino terminal region of the molecule is required for $\beta\gamma$ activation. The discovery of PLC ϵ revealed yet another mechanism whereby the enzyme can be activated. PLC ϵ possesses two Ras-binding (RA) domains in its carboxyl terminal region and a CDC-25 domain at its N-terminus (see Fig. 23-3) and thus can be activated by various members of the Ras superfamily of small GTPases. $\beta\gamma$ subunits derived from G $_{\text{i}}$ are also able to activate PLC ϵ , and thus this highly versatile enzyme may serve as a “nexus” for both heterotrimeric and small G protein signaling pathways (Wing et al., 2003). In addition, it appears that cyclic AMP can indirectly activate PLC ϵ , via the cyclic nucleotide’s ability to activate EPAC (a guanine nucleotide exchange factor, GEF), which in turn will activate Rap2B. The latter (like Ras and Rap1A) can then bind to the Ras domain and activate PLC ϵ . Both PLC β_2 and PLC γ_2

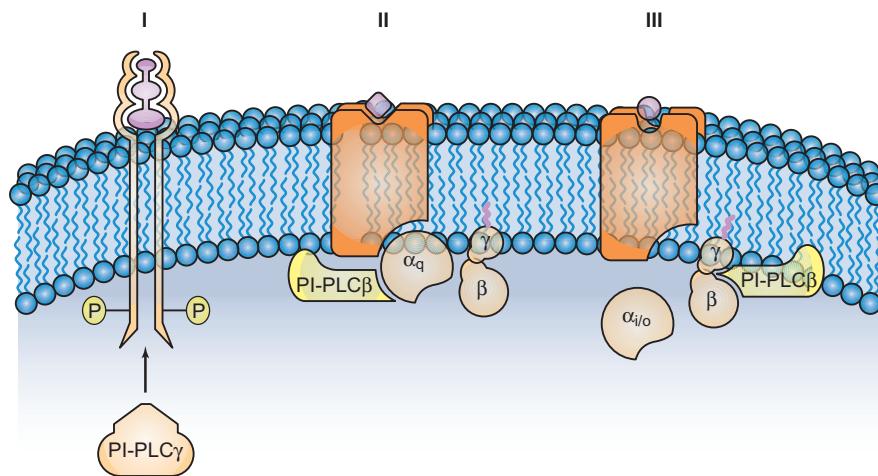


FIGURE 23-4 Three distinct mechanisms for the activation of phosphoinositide-specific phospholipase C (PLC). In mechanism I, the ligand activates a receptor which possesses an intrinsic tyrosine kinase. Dimerization and autophosphorylation of the receptor leads to recruitment of PLC γ to the plasma membrane and its activation by phosphorylation. In mechanism II, the ligand-receptor interaction results in the dissociation of a pertussis toxin-insensitive G protein (G $_{\text{q}}$), to liberate α_{q} , which then activates either PLC β_1 or PLC β_3 . In mechanism III, the ligand-receptor interaction results in the dissociation of a pertussis toxin-sensitive G protein (e.g., G $_{\text{i}}$ or G $_{\text{o}}$) which in turn liberates $\alpha_{\text{i/o}}$ and $\beta\gamma$ subunits. The latter can then activate either PLC β_2 or PLC β_3 . Note that although $\beta\gamma$ subunits derived from G $_{\text{q}}$ could also theoretically activate this pathway, this possibility is less likely, given the abundance of G $_{\text{i}}/G_{\text{o}}$ relative to G $_{\text{q}}$.

may also be activated by Rac, a member of the Ras superfamily of GTPases (Harden et al., 2009). An additional isoform, PLC η , appears to be neuron specific and highly Ca^{2+} sensitive, although little is presently known about its mechanism of activation. (Cockcroft, 2006).

THE INOSITOL PHOSPHATES

D-myo-inositol 1,4,5-trisphosphate [$\text{i}(1,4,5)\text{P}_3$] is a second messenger that liberates Ca^{2+} from the endoplasmic reticulum via intracellular receptors

The unique ability of $\text{I}(1,4,5)\text{P}_3$ to mobilize intracellular Ca^{2+} was first demonstrated by Berridge and colleagues in the early 1980s (Streb et al., 1983). When directly injected into cells, or added to permeabilized cells or membrane fractions, $\text{I}(1,4,5)\text{P}_3$ elicits increased release of Ca^{2+} from a store that has been associated with the endoplasmic reticulum. That specific receptor sites mediate the action of $\text{I}(1,4,5)\text{P}_3$ was first inferred from the presence of binding sites in membrane fractions obtained from selected brain regions, notably the cerebellum, the brain region most enriched in these receptors. The purified receptor is a glycoprotein with a MW of 260–313 kD and is highly selective for $\text{I}(1,4,5)\text{P}_3$ (for reviews, see Mikoshiba, 1993, 2007). The IP_3 -receptor (IP_3 -R) consists of three distinct

regions (Fig. 23-5). A ligand-binding domain is located at the amino terminal region of the molecule. A regulatory domain contains potential phosphorylation sites for protein kinase A and protein kinase C, an immunophilin binding site, a Ca^{2+} -sensor binding site and two ATP-binding sites that regulate the affinity with which IP_3 binds to the receptor. There is also a six-transmembrane-spanning channel domain through which Ca^{2+} is released. The region to the N-terminal side of the IP_3 binding domain serves as a suppressor that negatively modulates IP_3 affinity. The native IP_3 -R exists as a homotetramer with both amino- and carboxyl-termini facing the cytoplasm. Upon binding of an IP_3 molecule to each of the four subunits, a conformational change in the receptor is presumed to occur which results in the opening of the intrinsic Ca^{2+} channel. A critical feature of IP_3 -Rs is that their opening is regulated by cytosolic Ca^{2+} , the latter acting as a "co-agonist" at the IP_3 -R. Thus, paradoxically, the ability of $\text{I}(1,4,5)\text{P}_3$ to mobilize intracellular Ca^{2+} is itself regulated by Ca^{2+} , with nM concentrations of Ca^{2+} being a prerequisite for Ca^{2+} channel opening. This sensitivity to Ca^{2+} allows IP_3 -Rs to act as Ca^{2+} -induced Ca^{2+} release channels that promote the amplification of smaller triggering events. Activation of PLC-linked receptors frequently results in oscillations of the intracellular Ca^{2+} signal. The relationship between IP_3 production and signal oscillations is discussed in Chapter 26. At least three distinct forms of the IP_3 -R have been identified, and these share an overall amino acid homology of 60–80%. Type I predominates

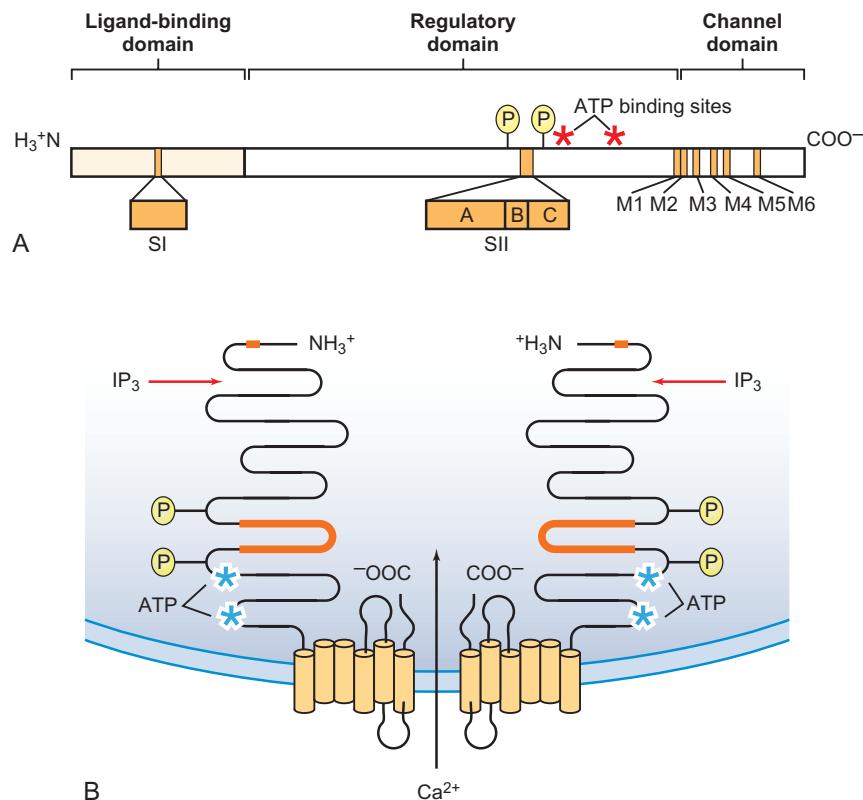


FIGURE 23-5 A. Linear representation of the IP_3 receptor showing the three functional domains. P, phosphorylation sites; SI and SII isoforms produced by alternative RNA splicing; M1-M6, membrane-spanning regions. B. Model of IP_3 receptor showing transmembrane topology. One half of the homotetrameric structure is shown. *, ATP-binding sites. From Mikoshiba, 1993, with permission.

in the cerebellum and has been most extensively studied. It is the largest of the three forms of the receptor and, unlike Type II and III receptors, the gene possesses a 120-nucleotide insert. Type II IP₃-Rs are found mainly in non-neuronal tissues, whereas Type III receptors occur in both neural and non-neuronal tissues. In response to chronic activation, IP₃-Rs are degraded via the ubiquitin-proteasome pathway (Taylor et al., 1999). Evidence that IP₃-Rs have numerous binding partners suggests that they may function like a scaffolding protein as part of a macromolecular signaling complex (Mikoshiba, 2007). IP₃-Rs are also subject to regulation by protein kinases and phosphatases (Vanderhyden et al., 2008), and functional abnormalities in these Ca²⁺ release channels have been implicated in certain neurological disorders (Foskett, 2010).

The metabolism of inositol phosphates leads to regeneration of free inositol

I(1,4,5)P₃ can be metabolized either by a 5-phosphatase (a membrane-bound enzyme) to yield inositol 1,4-bisphosphate [I(1,4)P₂] or by a cytosolic 3-kinase to form inositol 1,3,4,5-tetrakisphosphate [I(1,3,4,5)P₄] (for review, see Irvine & Schell, 2001). Both activities may be regarded as “off signals,” terminating the action of I(1,4,5)P₃. The I(1,4)P₂ that results from 5-phosphatase action is dephosphorylated further by an inositol bisphosphatase to inositol 4-monophosphate [I(4)P] and then by the action of inositol monophosphatase to

free inositol. It has been suggested that I(1,3,4,5)P₄ possesses a second-messenger function in facilitating the entry of Ca²⁺ into cells. It also serves as a substrate for the same 5-phosphatase that acts on I(1,4,5)P₃, with the resultant formation, in this instance, of inositol 1,3,4-trisphosphate [I(1,3,4)P₃]. Unlike the 1,4,5-trisphosphate isomer, I(1,3,4)P₃ is ineffective at mobilizing intracellular Ca²⁺. I(1,3,4)P₃ can be further metabolized to inositol 1,3-bisphosphate [I(1,3)P₂] or inositol 3,4-bisphosphate [I(3,4)P₂]. These compounds are then dephosphorylated by 4- or 3-phosphatases to yield inositol 3- or 1-monophosphates, respectively (Fig. 23-6).

A single enzyme, inositol monophosphatase, leads to loss of the remaining phosphate and the regeneration of free inositol. The enzyme is inhibited by Li⁺ in an uncompetitive manner; i.e., the degree of inhibition is a function of substrate concentration. It has been suggested that the uncompetitive inhibition of inositol monophosphatase by Li⁺ mediates its therapeutic action in affective disorders (Berridge et al., 1989; Agranoff & Fisher, 2001). The inositol depletion hypothesis proposes that monophosphatase inhibition *in vivo* lowers inositol levels in cells that are most actively producing inositol monophosphate, i.e., have maximally activated their phosphoinositide-linked receptors. While the inositol depletion hypothesis appears attractive in many respects (Deraniah & Greenberg, 2009), its validity remains to be demonstrated. A number of other biochemical sites of Li⁺ action have been reported (Jope, 1999; Phiel & Klein, 2001), and it may be that multiple actions of Li⁺ are necessary for its clinical effectiveness.

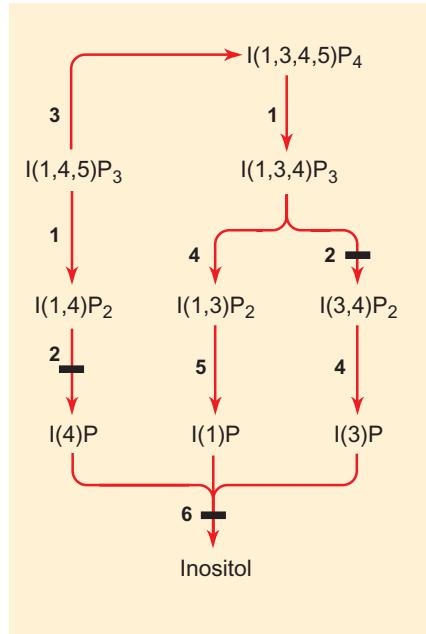


FIGURE 23-6 Pathways of I(1,4,5)P₃ metabolism. Only the quantitatively major established pathways are depicted. Li⁺ is known to block the dephosphorylation reactions indicated by the black bars. For details of the metabolic pathways of the higher inositol phosphates (i.e., IP₅, IP₆ and the diphosphorylated inositol phosphates) see Shears, 2009. Numbers refer to the following enzymes: 1, inositol polyphosphate 5-phosphatase (I); 2, inositol polyphosphate 1-phosphatase; 3, I(1,4,5)P₃ 3-kinase; 4, inositol polyphosphate 4-phosphatase; 5, inositol polyphosphate 3-phosphatase; 6, inositol monophosphatase.

Highly phosphorylated forms of myo-inositol are present in cells

Phytic acid (IP₆) is the most abundant species (Fisher et al., 2002). IP₆ had long been known as the principal phosphate storage molecule in plants, and is ubiquitously expressed in animal cells at concentrations of 10–60 μM. Biosynthesis of IP₆ occurs by a complex series of reactions (Shears, 2004, 2009) consisting of two possible routes for the conversion of I(1,4,5)P₃ to I(1,3,4,5,6)P₅, followed by the action of IP₅ kinase to form IP₆. An additional layer of complexity was introduced upon the discovery of the existence of diphosphorylated inositol phosphates, which contain energy-rich pyrophosphoryl (PP) residues (Shears, 2009). These include 5PP-IP₄, 5PP-IP₅, (IP₇) and 1/3,5(PP)₂-IP₄ (IP₈). Multiple physiological roles for the highly phosphorylated inositol species have been proposed. These include effects on endocytosis, mRNA transport and telomere length. The diphosphoinositol polyphosphates serve as phosphate donors in non-enzymatic protein phosphorylation and may play a role in the maintenance of the bioenergetic status of the cell (Shears, 2009).

Protein kinase C is activated by the second messenger diacylglycerol

This unique family of protein kinases, which represents ~2% of the human kinome (Rosse, et al., 2010) was originally

identified in the late 1970s by Nishizuka and colleagues (reviewed in [Nishizuka, 1995](#); [Battaini & Mochly-Rosen, 2007](#)). All protein kinase C (PKC) isoforms contain a highly conserved catalytic domain separated by a short “hinge” region from a variable regulatory domain (Fig. 23-7). The latter, which contains a “pseudosubstrate” sequence, folds over the catalytic domain to render it inactive in the resting state. PKC isoforms can be distinguished by the presence or absence of motifs in the regulatory region that dictate the cofactor requirements for activation. The conserved region 1 (C1) contains two cysteine-rich repeats and represents the site at which DAG and phospholipid binding regulates the enzyme. The classical C2 region has an anionic phospholipid-binding site and a Ca^{2+} binding region that confers the Ca^{2+} sensitivity of the “conventional/classical” isoforms of PKC (cPKC: α , β and γ). The “novel” isoforms (nPKC: δ , ϵ , η and σ) possess a C2 region that lacks the conventional calcium coordinating residues and these isoforms are accordingly Ca^{2+} -insensitive. An additional variation in PKC structures is observed for the ζ and ι/λ isoforms, which possess only one cysteine-rich repeat in the C1 region. These isoforms, termed “atypical” (aPKC), are not regulated by DAG or Ca^{2+} but are activated by protein–protein interactions via their PB1 (Phox/Bem1) domain ([Rosse et al., 2010](#)).

All isoforms of PKC are predominantly localized to the cytosol and, upon activation, undergo translocation to either plasma or nuclear membranes. However, newly synthesized PKCs are localized to the plasma membrane and are in an “open” conformation in which the autoinhibitory pseudosubstrate sequence is removed from the substrate-binding domain. The “maturation” of PKC isoforms is effected by

phosphoinositide-dependent kinase-I (PDK-I), which phosphorylates a conserved threonine residue in the activation loop of the catalytic (C4) domain ([Newton, 2003](#)). This in turn permits the autophosphorylation of C-terminus threonine and serine residues in PKC, a step that is a prerequisite for catalytic activity. The phosphorylated enzyme is then released into the cytosol, where it is maintained in an inactive conformation by the bound pseudosubstrate.

Although many cellular proteins are potential targets for phosphorylation by PKC, there is remarkably little overlap among the substrates for the different PKC isoforms, a specificity that appears to depend on regulation of their intracellular localization. Targeting of PKC isozymes to subcellular loci appears to occur via interaction of the enzyme with localized intracellular binding proteins that have been termed C-KIPs (PKC-interacting proteins, reviewed in [Poole et al., 2004](#)). Such proteins may or may not be substrates for PKC. An example of the latter category would be RACK 1 (Receptors for Activated C Kinase). It is thought that RACKs interact only with activated PKCs and target translocated PKCs.

The phorbol esters are useful for studying the function of PKC since they mimic the stimulatory effects of DAG on the enzyme. These tumor-promoting plant products and their synthetic derivatives are able to penetrate intact cells. Many inferences regarding the intracellular actions of PKC are based on results of studies on whole-cell preparations with the phorbol esters. These substances, like DAG, may produce feedback inhibition of signal transduction at a number of metabolic levels. Results of experiments using phorbol esters in whole cells are thus often complex and must be interpreted cautiously.

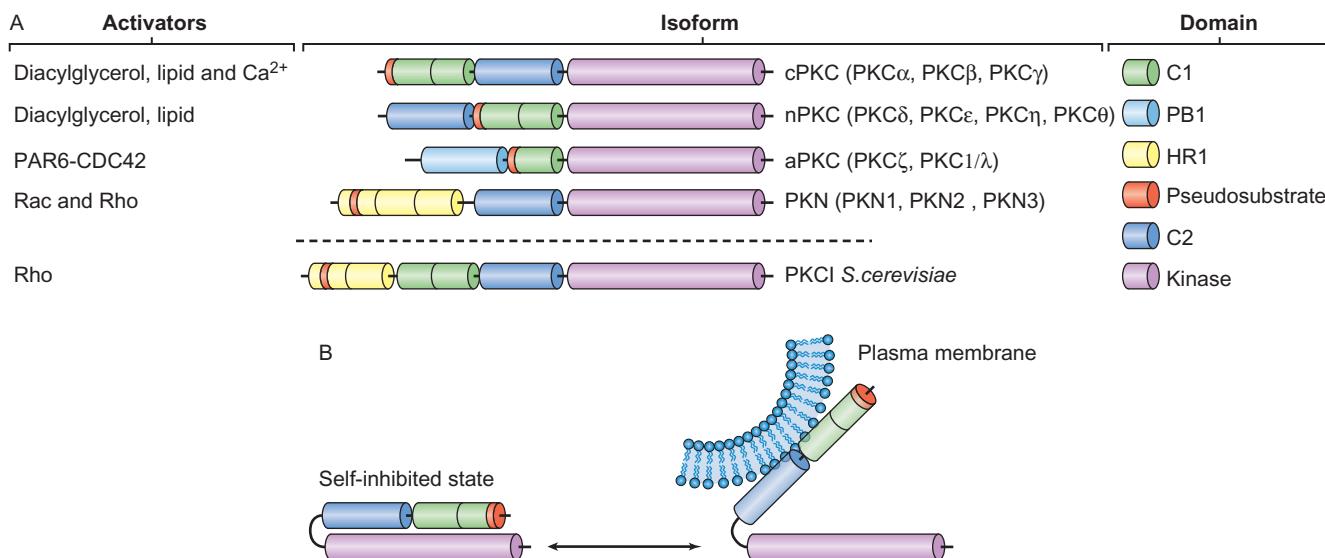


FIGURE 23-7 A. A schematic of the domain structure of the four subgroups of the mammalian protein kinase C (PKC) family members is shown above the *Saccharomyces cerevisiae* PKC1. These are the classical isoforms (cPKC), novel isoforms (nPKC), atypical isoforms (aPKC) and the PKC-related kinases (known as PKN). B. A cPKC is shown in its self-inhibited state, with the pseudosubstrate site binding to the substrate-binding pocket in the kinase domain. When the regulatory domain is recruited to plasma membranes through Ca^{2+} , phospholipid and diacylglycerol, autoinhibition is relieved and the catalytic domain of cPKC is free to exert its action on target substrates. Conserved region 1 (C1) can confer binding of diacylglycerol and phospholipids, and C2 confers binding to phospholipids and Ca^{2+} . Phox/Bem domain 1 (PB1) acts as a dimerization domain in various proteins. Homology region 1 (HR1) confers small-GTPase binding properties to PKCs. From [Rosse et al., 2010](#), with permission.

Notwithstanding this consideration, based upon experiments with phorbol esters there is now ample evidence to implicate DAG-sensitive isoforms of PKC in a number of complex processes, including long-term potentiation (see Chapter 56).

Since $I(1,4,5)P_3$ production leads to increased intracellular Ca^{2+} , which in turn can stimulate PKC action, the two messengers DAG and $I(1,4,5)P_3$ often act in concert. DAG can, however, arise in cells from sources other than the phosphoinositides. For example, DAG can be generated from the action of a phosphatidylcholine (PC)-specific phospholipase C or from the transfer of phosphorylcholine from PC to ceramide in the synthesis of sphingomyelin (see Chapter 5). As a second messenger, DAG is rapidly metabolized under normal conditions, and the predominant route is via phosphorylation to PA, a reaction catalyzed by DAG kinase, of which multiple forms have been identified and characterized. All possess a C-terminal catalytic domain and two or three cysteine-rich repeat sequences. The α , β and γ forms possess E-F hand domains and are thus likely to be regulated by changes in the concentration of cytosolic Ca^{2+} . Once DAG is phosphorylated to PA, this in turn can be converted into PI via CDP-DAG (see Fig. 23-2B).

PHOSPHOINOSITIDES AND CELL REGULATION

Inositol lipids can serve as mediators of other cell functions, independent of their role as precursors of second messengers

Although studies of the phosphoinositides have centered on the role played by these lipids in transmembrane signalling events, in particular the generation of second messengers linked to Ca^{2+} homeostasis and protein phosphorylation, it is now abundantly clear that inositol lipids are important mediators of a diverse array of cell functions. This can be attributed to the fact that the phosphoinositides, in particular PI(4,5)P₂ and PI(3,4,5)P₃, can specifically interact with protein-targeting modules. Many proteins have evolved lipid-binding domains that exhibit some degree of specificity for different classes of phosphoinositides (Fig. 23-8; reviewed in Kutateladze, 2010). Through interaction of these high-affinity lipid domains, phosphoinositides can recruit specific proteins and regulate multiple cell functions both in the CNS and in non-neuronal cells. Roles for phosphoinositides in membrane trafficking,

cell death/survival and regulation of ion channels are discussed below.

Membrane trafficking

A direct role for the quantitatively major phosphoinositides, i.e., PI4P and PI(4,5)P₂, in the exocytosis of neurosecretory granules was first proposed by Holz and colleagues (Eberhard et al., 1990), who observed that the ATP requirement for Ca^{2+} -regulated exocytosis in chromaffin cells could be attributed to the generation of PI(4,5)P₂. Subsequent studies revealed that PI4-kinase, PI4P 5-kinase and the PI-transfer protein appear to be prerequisites for exocytosis. The requirement for PIP₂ in these exocytotic events reflects the ability of the lipid (which is localized in the plasma membrane) to interact with a variety of proteins, including Ca^{2+} -dependent activator protein for secretion (CAPS) and synaptotagmin, a putative Ca^{2+} sensor for exocytosis. The latter protein is located in the membrane of the secretory organelle and interacts with PI(4,5)P₂ via its C2 domains. The synaptotagmin-PI(4,5)P₂ interactions are proposed to facilitate the close apposition of the vesicle and target membranes (Bai et al., 2004). PI(4,5)P₂ also regulates the clathrin-dependent endocytosis of synaptic vesicles by binding to key endocytic proteins, including the clathrin adaptor AP-180, dynamin and epsin (via PH or ENTH/ANTH domains; see Fig. 23-8). The interaction of PI(4,5)P₂ [or PI(3,4,5)P₃] with these cytosolic proteins facilitates their recruitment to the plasma membrane and, in the case of dynamin, also stimulates the GTPase activity (Osborne et al., 2006). The 3-phosphoinositides also appear to play a role in late endocytic events, since inhibition of PI3K with wortmannin prevents the post-endosomal sorting of platelet-derived growth factor receptor (PDGF-R). Moreover, mutations of the PDGF-R that prevent its association with PI3K result in receptors that fail to undergo trafficking from the Golgi to lysosomes. Through its interaction with the FYVE domain (see Fig. 23-8) of the clathrin adapter protein-2 (AP-2), PI3P has also been suggested to play a role in the assembly of clathrin-coated pits at the plasma membrane. The continuing cycle of the endocytic process requires the rapid dissociation of clathrin adaptor proteins, epsin and dynamin which is accomplished by the action of the 5-phosphatase, synaptojanin 1 on PI(4,5)P₂ and PI(3,4,5)P₃ (Di Paulo & De Camilli, 2006). Remodeling of the actin cytoskeleton also occurs in parallel with membrane rearrangements during endocytosis thus providing another site for a contribution of phosphoinositides to membrane trafficking.

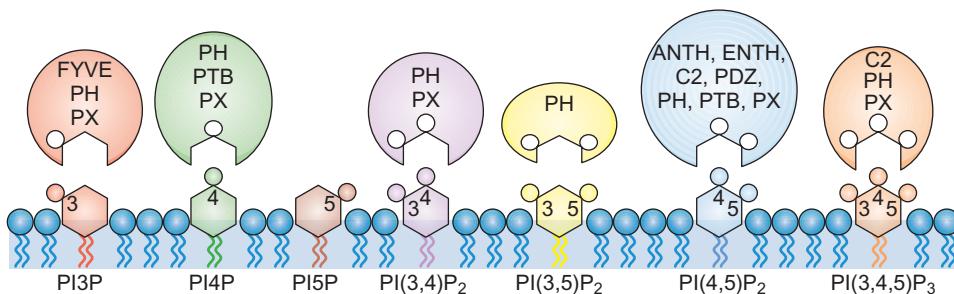


FIGURE 23-8 Phosphoinositide binding domains and preferred lipid binding partners. Adapted from Kutateladze, 2010, with permission.

Regulation of the actin cytoskeleton has best been characterized for PI(4,5)P₂, which interacts directly with actin-binding proteins. PI(4,5)P₂ typically activates proteins that promote actin filament assembly and inhibit those which promote disassembly. Among the latter are gelsolin and profilin. Gelsolin, which can sever actin filaments, also prevents actin assembly via its barbed end capping activity, whereas profilin sequesters actin monomers. Interaction of PI(4,5)P₂ disrupts the binding of both of these proteins to actin, leading to a stimulation of actin filament formation. PI(4,5)P₂ also activates N-WASP (Wiskott-Aldrich syndrome protein), a regulator of Arp2/3 (actin-activated protein complex 2/3), resulting in stimulation of actin polymerization. Rho family GTPases such as RhoA, Rac1 and Cdc42 play important roles in actin polymerization and exemplify proteins, which can both be regulated by phosphoinositides and serve directly or indirectly as regulators of phosphoinositide metabolizing enzymes (Saarikangas et al., 2010).

PI(4,5)P₂ also plays a role in the formation of membrane adhesion complexes — sites where the actin cytoskeleton is linked to the extracellular matrix (focal adhesions) or to cell-cell contacts through interactions with integrins or cell adhesion molecules (CAMs). Many proteins in these adhesion complexes bind PI(4,5)P₂ and by doing so, have increased binding to each other. For example, when vinculin binds to PI(4,5)P₂, there is a conformational change in the molecule which then allows its binding to a number of cytoskeletal and adhesion proteins such as talin, actin, paxillin and Arp 2/3 complex, thereby allowing newly synthesized actin filaments to be linked to integrins. Similarly, members of the ERM family of proteins (ezrin, radixin and moesin) display enhanced binding to CAMs in the presence of PI(4,5)P₂ after the interaction of the lipid with the FERM domain (Fig. 23-8).

Cell growth and cell survival

The 3-phosphoinositides play a key role in the control of cell death (apoptosis) or survival, via their ability to regulate the activity of a kinase that is pivotal in these processes, namely, PKB (Protein kinase B; Akt), a serine-threonine kinase [reviewed in Duronio, 2008; see also Ch. 25]. After PI3K activation, PI(3,4,5)P₃ [or PI(3,4)P₂] recruits PKB to the plasma membrane via its PH domain. Phosphoinositide-dependent kinase-1 (PDK-1), which also possesses a PH domain with high affinity for PI(3,4,5)P₃, is targeted to the same region of the membrane and phosphorylates ³⁰⁸threonine on PKB. Additional phosphorylation of PKB on ⁴⁷³serine by a PDK-2 kinase allows for full activation of PKB, which is then released from the membrane and is able to phosphorylate multiple target proteins. Among these are numerous proteins involved in the apoptosis pathway, including Bcl-2 (B-cell lymphoma 2) family members, activators of NF- κ B (nuclear factor- κ type B), and GSK-3 (glycogen synthase kinase-3), as well as components of the protein translational machinery. The ability of 3-phosphoinositides to stimulate cell proliferation/survival via activation of PKB is countered by the 3-phosphatase PTEN, which hydrolyzes PI(3,4)P₂ and PI(3,4,5)P₃. Cells in which PTEN activity is reduced have increased tumorigenic properties, since PKB inhibits apoptosis and promotes cell survival. Conversely, PTEN activity programs the fate of the cell toward apoptosis. Mutations of PTEN have been

shown to occur in a wide range of tumor types, but with a particularly high frequency in glioblastomas.

In contrast to the pro-apoptotic properties of PTEN, another 3-phosphoinositide phosphatase, INPP4A (which specifically dephosphorylates PI(3,4)P₂ to PI3P), has been found to have a pro-survival function (Sasaki et al., 2010). Mice subjected to targeted disruption of the gene encoding INPP4A exhibited involuntary movement disorders and displayed striatal lesions upon histological examination which were typical of those induced by excessive activation of N-methyl-D-aspartate glutamate receptors. This neurodegeneration could be rescued by expression of recombinant INPP4A.

An additional cellular process in which 3-phosphoinositides have been shown to be required is that of autophagy, a tightly regulated catabolic process involving lysosomal degradation of the cell's own components (see Ch. 37). Although autophagy can be a mechanism of non-apoptotic cell death following exposure to various cytotoxic agents, it mainly serves as a fundamental intracellular trafficking pathway which functions to degrade deleterious cellular components or to supply nutrients during conditions of cellular starvation. PI(3)P plays a role in recruitment of specific protein components to the autophagosome. There is also evidence that PI(3)P is involved in the inactivation of TOR (target of rapamycin), a process that leads to autophagy (Burman & Ktistakis, 2010). Since deleterious consequences to the cell would ensue if autophagy were prolonged, appropriate termination of the PI(3)P signal is also crucial. Experimental depletion of the PI(3)P phosphatases Jumpy and MTMR3 leads to an increased number/size of autophagic vacuoles, indicating an important role for this pathway in regulating the duration of the autophagy process (Vergne & Deretic, 2010). PI(3)P is also a substrate for PIP5-kinase and PI(3,5)P₂ has been implicated in autophagosome maturation. Spontaneous mouse mutants exhibiting severe neurodegeneration were found to have defects in two of the major components of the PI(3,5)P₂ regulatory complex (Ferguson et al., 2010). Such studies underscore the growing appreciation that defects in regulation of autophagy may underlie numerous neurodegenerative disorders.

Regulation of ion channel activity

A large number of ion channels have been found to be dependent upon PI(4,5)P₂ for activity since it was first demonstrated that Kir (inward rectifier potassium) channels display no activity in its absence (Hilgemann & Ball, 1996; for recent review, see Suh & Hille, 2010; Ch. 4). PI(4,5)P₂ facilitates the opening of some transient receptor potential channels, voltage-gated K⁺ channels, P/Q- and N-type Ca²⁺ channels, ryanodine-sensitive Ca²⁺ release channels and P2X receptors (ATP-gated cation channels). Other channels, e.g., cyclic-nucleotide ion gated channels in rods and I(1,4,5)P₃-gated calcium release channel, are reportedly inhibited by PI(4,5)P₂, while voltage-gated Na⁺ channels appear to be completely unaffected. Little is known of the molecular mechanism whereby PI(4,5)P₂ regulates ion channel activity, except that the lipid is presumed to bind directly to cationic sites on the channels, and that these are distinct from the phosphoinositide binding domains listed in Fig. 23-8. The physiological significance of phosphoinositide dependence is also unclear. It may be that the latter prevents the channels from becoming

active until inserted in the plasma membrane and/or provides a means for receptor-mediated regulation of channel activity via PLC-catalyzed depletion of PI(4,5)P₂ (Hilgemann et al., 2001; Suh & Hille, 2010). The physiological relevance of this PI(4,5)P₂ requirement is supported by the recent recognition that alterations in the affinity of channel proteins for PI(4,5)P₂ may underlie various diseases characterized as channelopathies (Logothetis et al., 2010).

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