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Avia Rosenhouse-Dantsker *Editor*

Cholesterol and PI(4,5)P2 in Vital Biological Functions

From Coexistence to Crosstalk

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Preface

Cholesterol is an essential component of the plasma membrane. Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), although a minor phospholipid, is the most abundant membrane phosphoinositide. Although initially presumed to serve only as membrane components, these two lipids perform a broad range of cellular functions. For example, cholesterol is the precursor of crucial substances, and $PI(4,5)P_2$ is critical in membrane trafficking. Furthermore, cholesterol and $PI(4,5)P_2$ regulate signaling processes and protein activity. While studies of these lipids have traditionally focused on the individual effect of each lipid, a growing number of studies demonstrate that cholesterol and $PI(4,5)P_2$ can act together to modulate protein function and biological processes. Also, cholesterol and $PI(4,5)P_2$ may cross-regulate each other's levels. Yet, there is a lack of an overview of cellular functions and molecular mechanisms in which lipid functions extend from parallel independent existence to allosterism, synergism, and crosstalk. This book seeks to fill this void.

The first part of the book introduces the fundamental facets of cholesterol and $PI(4,5)P_2$. Starting from the structure, synthesis, and regulation of these two lipids, the first chapter in this part continues to describe the primary functions and signaling processes in which cholesterol and $PI(4,5)P_2$ are involved. In line with the focus of the book, the chapter also provides examples demonstrating that these two lipids can act via common mechanisms to alter protein function through direct binding. To conclude the introduction to cholesterol and $PI(4,5)P_2$, the second chapter in this part discusses how cholesterol and $PI(4,5)P_2$ affect the biophysical properties of membranes and underscores the potential implications of these effects for biological activity and membrane lipid-protein interactions.

The second part is on the mutual influence of cholesterol and $PI(4,5)P_2$ on their levels. It begins with a chapter on the cross-regulation of plasma cholesterol and $PI(4,5)P_2$. The chapter discusses molecular processes by which $PI(4,5)P_2$ may modulate low- or high-density lipoprotein cholesterol and describes the regulation of $PI(4,5)P_2$ by cholesterol. The part continues with a chapter on cholesterol and phosphoinositides in the biology of cilia, underscoring the roles of these lipids in signal transduction via cilia and the building of cilia. The part ends with a chapter focused

on alterations in cholesterol and phosphoinositide levels in the Niemann-Pick C1 disease, an intracellular cholesterol trafficking disorder.

The third part of the book is on the co-regulation of protein function by cholesterol and $PI(4,5)P_2$. The first six chapters in this part focus on plasma membrane ion channels. These chapters describe intriguing findings concerning the modulation of ion channels by cholesterol and $PI(4,5)P_2$ and discuss the ensuing mechanistic implications. The ion channels reviewed are inwardly rectifying potassium (Kir) channels, the voltage-gated potassium Kv7.2/3 channels, BK channels – the large conductance calcium-activated potassium channels, thermo-transient receptor potential (TRP) channels, the calcium-activated chloride channels TMEM16A and TMEM16B, and the principal components of the calcium release-activated calcium (CRAC) channels – the STIM1 and Orai1 proteins. The part concludes with two chapters on lipid transport proteins. One chapter discusses the relationship between cholesterol, phosphoinositides, and lipid transfer proteins, especially oxysterol-binding protein-related proteins (ORPs). The second chapter describes the crosstalk between cholesterol, $PI(4,5)P_2$, and ABC transporters and its implication for inflammation and cardiovascular disease.

The fourth part delves into two other cellular processes at the intersection of cholesterol and $PI(4,5)P_2$ involvement. One chapter reviews unconventional protein secretion pathways involving engrailed-2 (En2) homeoprotein and fibroblast growth factor 2 (FGF2). The second chapter discusses how calcium homeostasis and annexins affect the sensing and transfer of cholesterol and phosphoinositides across membrane contact sites.

Collectively, the chapters in this book portray the emerging relationship between cholesterol and $PI(4,5)P_2$ in a broad array of biological systems and processes. As the reader will discover, many questions remain open. Furthermore, the intricate relationship between these and other lipids has yet to be interrogated. Undoubtedly, the roles of lipids in biological processes and the complex relationships between these lipids will remain an active area of research for many years to come.

The editor is deeply thankful to all the authors who made this project come to life through their enthusiasm, excitement, and dedication. The editor would also like to thank the anonymous book proposal reviewers for their invaluable suggestions at the very beginning of this project. Last but not least, the editor is grateful to her collaborators and colleagues for providing a nurturing environment for the completion of this collection of substantial contributions to the emerging field of lipid interplay in biological processes.

Chicago, IL, USA Avia Rosenhouse-Dantsker

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About the Editor

Avia Rosenhouse-Dantsker, DSc, is a clinical associate professor at the University of Illinois Chicago. Her research background ranges from quantum theory and computational biology to biophysical chemistry, molecular biology, and electrophysiology. Her current research employs experimental and computational approaches to elucidate structural and functional mechanisms of protein modulation with a special emphasis on the co-modulation of ion channels by lipids (e.g., cholesterol, phosphoinositides, and others).

Part I
Introduction to Cholesterol and PI(4,5)P_2

$PI(4,5)P_2$ and Cholesterol: Synthesis, Regulation, and Functions

Avia Rosenhouse-Dantsker, Dimitris Gazgalis, and Diomedes E. Logothetis

Abstract Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) is the most abundant membrane phosphoinositide and cholesterol is an essential component of the plasma membrane (PM). Both lipids play key roles in a variety of cellular functions including as signaling molecules and major regulators of protein function. This chapter provides an overview of these two important lipids. Starting from a brief description of their structure, synthesis, and regulation, the chapter continues to describe the primary functions and signaling processes in which $PI(4,5)P_2$ and cholesterol are involved. While $PI(4,5)P_2$ and cholesterol can act independently, they often act in concert or affect each other's impact. The chapters in this volume on "Cholesterol and $PI(4,5)P_2$ in Vital Biological Functions: From Coexistence to Crosstalk" focus on the emerging relationship between cholesterol and $PI(4,5)P_2$ in a variety of biological systems and processes. In this chapter, the next section provides examples from the ion channel field demonstrating that $PI(4,5)P_2$ and cholesterol can act via common mechanisms. The chapter ends with a discussion of future directions.

Keywords Phosphatidylinositol 4,5-bisphosphate · Cholesterol · Lipid homeostasis · Lipid modulation of protein function · Cellular signaling · Lipid regulation

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1 Introduction

In recent years, it has become evident that lipids are not merely passive entities, e.g., as membrane components, but also play numerous active roles including in signaling processes and the modulation of protein function. Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and cholesterol are examples of such lipids, and this chapter will review their structure, synthesis, regulation, and functions. As the chapter covers a wide array of research areas, unintended omissions are inevitable, and we apologize for missing references or topics. The discussion of each lipid highlights their unique aspects and our differential state of understanding of these central lipids. For example, whereas the cholesterol biosynthesis pathway is complex and intricate, involving over 30 reactions, the synthesis of $PI(4,5)P_2$ is straightforward and generally achieved via phosphorylation or dephosphorylation of other phosphoinositides. Another example is our understanding of the interactions between each of these lipids and proteins. For many years, cholesterol was thought to affect protein function indirectly, e.g., by altering membrane properties. While the possibility that cholesterol binds directly to proteins was proposed in the mid-1970s, investigations of the mechanisms of cholesterol modulation of protein function and the roles of this essential lipid in signaling pathways gained a central stage in research efforts only about three decades later. In contrast, the active roles of $PI(4,5)P_2$ in protein function and signaling processes were appreciated several decades earlier, and the underlying molecular mechanisms began to unravel already in the 1990s. Thus, the current level of mechanistic understanding of $PI(4,5)P_2$ functions is more detailed than that of cholesterol. Hence, the chapter design is not symmetrical, but rather aims to depict the current understanding of $PI(4,5)P_2$ and cholesterol. Furthermore, beyond their diverse array of functions, a growing number of studies published in the past decade have revealed that $PI(4,5)P_2$ and cholesterol often function in parallel or in concert. Thus, while the focus of this chapter is to introduce each of these lipids individually, the chapter will end with a section underscoring their ability to modulate protein function via common mechanisms using an example from the ion channel field.

2 Phosphatidylinositol Phosphates (PIPs)

2.1 PIPs: Overview, Structure, Synthesis, and Membrane Distribution

Phosphatidylinositol phosphates (PIPs, the acronym used in this review) or phosphoinositides (also referred to in the literature as PPIs) are phosphorylated inositol phospholipids found in cellular membranes, where they serve critical roles in the physiology of eukaryotic cells, such as in actin dynamics, membrane trafficking, signal transduction, and regulation of transmembrane protein activity [1–9].

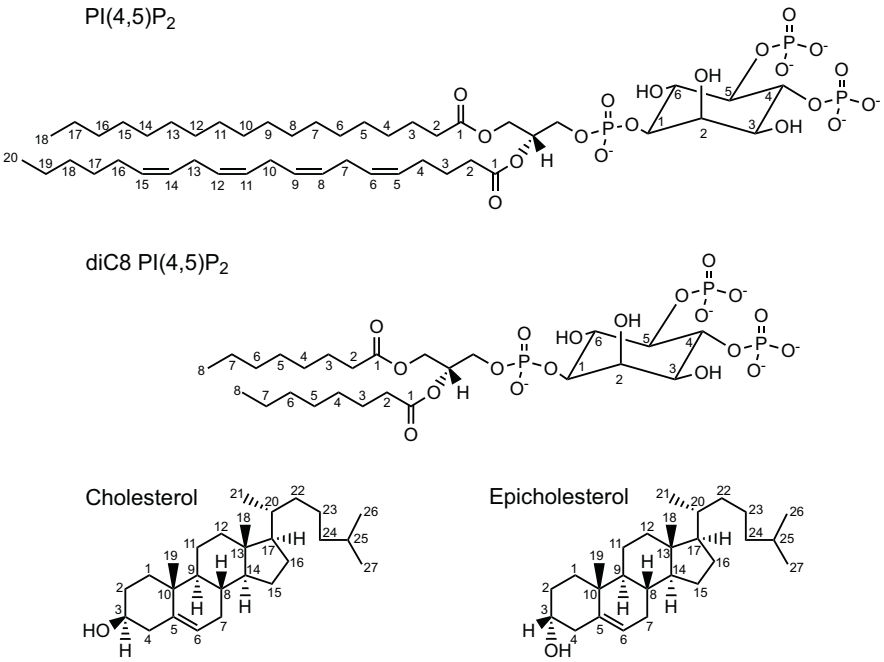


Fig. 1 Structures of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2), diC8-P(4,5)P_2, cholesterol, and epicholesterol drawn using ChemDraw Version 20.1.0.112 (PerkinElmer Informatics)

Although PIPs in the plasma membrane (PM) are only a minor component (~1%) of the total cellular phospholipid, they have been recognized as "master regulators," integrating cytosolic and membrane cellular functions. The most abundant PIP of the plasma membrane, phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P_2 or PIP_2) (Fig. 1), is unique in that it possesses a high negative charge density ranging from –3e to –5e (depending on pH and the counterions present), yielding a net lipid charge of –4e. Not only do these negative charges regulate electrostatic interactions of PIP_2 with basic domains of numerous proteins but also electrostatic interactions with neighboring lipids contribute to this phospholipid's lateral distribution. PIP_2 can form small clusters or large stable but interconvertible aggregates that are dependent on divalent (Ca^{2+}, Mg^{2+}, and Zn^{2+}) and trivalent (Fe^{3+} and Al^{3+}) ions [10, 11].

2.1.1 Headgroup

The PIP headgroup consists of a polyol myo-inositol on which phosphates can be added or removed to carbons 3′, 4′, and/or 5′ of the inositol ring by specific lipid kinases and phosphatases, respectively [12–15]. Phosphatidylinositol (PI) is phosphorylated by specific PI kinases at the 3′ (by PI3KII or PI3KIII), 4′ (by PI4K), or

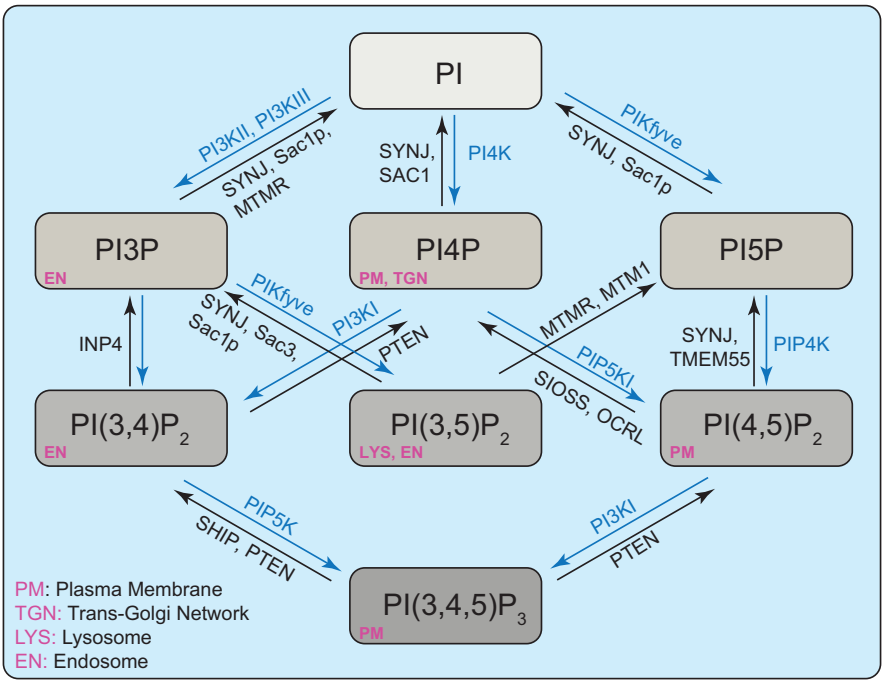


Fig. 2 Mono-, di-, and tri-phosphorylated forms of phosphatidyl inositides. By the action of lipid kinases and phosphatases, phosphatidylinositol (PI) is interconverted to three distinct isoforms of PIPs and PIP_2s, as shown. The predominant presence of specific phosphatidylinositol phosphates (PIPs) in distinct organellar membranes is also indicated. *MTM* myotubularin; *OCRL* Lowe oculo-cerebrorenal syndrome protein; *PTEN* phosphatase and tensin homolog; *SAC* suppressor of actin; *SYNJ* synaptojanin; *SHIP* SH2 domain-containing phosphatidylinositol 5-phosphatase; *SIOSS* a group of phosphatases (SYNJ 1/2, inositol polyphosphate 5-phosphatases (INPP5) B/J/E, OCRL1, SAC2, skeletal muscle and kidney enriched inositol polyphosphate phosphatase (SKIP))

5′ (by PIKfyve) positions to yield mono-phosphorylated PIPs (Fig. 2). The reverse reactions are catalyzed by phosphatases acting at the 3′ (by synaptojanin (SYNJ), Sac1P, or MTMR), 4′ (by SYNJ or SAC1), or 5′ (by SYNJ or Sac1p) positions to dephosphorylate the mono-phosphorylated PIPs back to PI.

Phosphatidylinositol phosphate (PIP) kinases recognize the mono-phosphorylated species and phosphorylate them to yield the di-phosphorylated PIPs. Thus, PI3P can be phosphorylated to $PI(3,5)P_2$ (by PIKfyve) or to $PI(3,4)P_2$ (by an unidentified PIP kinase), PI4P can be phosphorylated to $PI(3,4)P_2$ (by PI3KI) or to $PI(4,5)P_2$ (by PIP5KI), and PIP5 to $PI(4,5)P_2$ (by PIP4K), while its phosphorylation to $PI(3,5)P_2$ has not been reported. The reverse reactions from the di- to the mono-phosphorylated PIPs are catalyzed by phosphatases to make PI3P from $PI(3,4)P_2$ (by INP4) or from $PI(3,5)P_2$ (by SYNJ, Sac3, or Sac1p), to make PI4P from $PI(3,4)P_2$ (by PTEN) or from $PI(4,5)P_2$ (by SIOSS or OCRL), or to make PI5P from $PI(3,5)P_2$ (by MTMR or MTM1) or from $PI(4,5)P_2$ (by SYNJ or TMEM55).

Finally, some PIP kinases also use PIP_2 species as substrates to phosphorylate them to the tri-phosphorylated PI(3,4,5)P_3 from PI(3,4)P_2 (by PIP5K) or from PI(4,5)P_2 (by PI3KI). Dephosphorylation of PIP_3 to PI(3,4)P_2 (by SHIP or PTEN) or to PI(4,5)P_2 (by PTEN) regulates the duration of the presence of PIP_3.

2.1.2 Acyl Chains

Phospholipids possess two fatty acid chains, esterified to a glycerol backbone, that through a phosphate attach to the polar headgroup at position 1. In PIPs the two acyl chains are asymmetric, consisting of a saturated stearic acid and an unsaturated (four double bonds) arachidonic acid (Fig. 1).

2.1.3 Membrane Distribution

PIPs are distributed differentially in membranes of different cellular compartments. They reside exclusively in the inner leaflet of the bilayer. The PM contains predominantly PI4P, PI(4,5)P_2, and PI(3,4,5)P_3. PI(4,5)P_2 is the most abundant PIP of the PM [16] (Fig. 2). The Golgi membrane is most abundant in PI4P and PI3P [1, 17, 18]. Endosomes and multivesicular bodies are enriched in PI3P [19, 20], while endosomes also show higher PI(3,5)P_2 levels, as do multivesicular bodies and lysosomes [21]. PI(3,4)P_2 levels are also enriched in early endosomal membranes. One can think of the seven PIPs as different zip codes identifying the membranes of specific compartments within the cell that cellular proteins will be sent to or retrieved from by virtue of their interactions with the particular PIP(s) present.

2.2 Functions of PIPs

One can assess the importance of any biological process of interest when its dysregulation leads to disease. Defects in some of the kinases and phosphatases depicted in Fig. 2 have been greatly implicated in many types of cancers, in diseases of the visual system, myopathies, kidney diseases, asthma, human immunodeficiency virus-1 (HIV-1), and neuropathologies including mental retardation, Down syndrome, Alzheimer's disease, and bipolar disorder. Here, we will proceed with a brief discussion of the role of PIPs, and PIP_2 in particular, on: (i) a number of diverse processes (such as endo- and exocytosis, actin dynamics, focal adhesion dynamics, intracellular trafficking, membrane curvature, and lipid transfer); (ii) specific signaling transmembrane proteins such as G-protein-coupled receptors (GPCRs) and a more extensive discussion on ion channel gating, aligned with the focus of this volume; and (iii) cellular signaling at large. For a greater discussion of the function/physiology and pathophysiology of PIPs on topics that are covered only briefly here, recent more extensive reviews are available (e.g., [22, 23]).

2.2.1 PIP_2-Dependent Processes

2.2.1.1 Endocytosis/Exocytosis

In our discussions below, a common feature to all functions orchestrated by PIP_2 will be its property to interact specifically with many different proteins and control the formation and spatiotemporal organization of the groups of proteins involved in a particular process. With respect to endo- and exocytosis in the example of the presynaptic terminus of neuronal membranes, PIPs, and specifically PIP_2, control clathrin-mediated endocytosis by coordinating the biogenesis of clathrin-coated vesicles at multiple steps [24]. Recruitment of coat and fission factors to the membrane depends on the association of the adaptor proteins AP-2 and AP-180 with PIP_2 [25–27], whereas fission of clathrin-coated pits involves a PIP_2-dependent recruitment and activation of the GTPase dynamin I, which polymerizes into rings around the neck of the pits [28, 29]. Under conditions of intense neuronal activity, clathrin-mediated endocytosis is largely replaced by activity-dependent bulk endocytosis, which also depends on PIP_2 [30].

Priming of synaptic vesicles for exocytosis also depends on PIP_2, either indirectly, via its second messenger diacylglycerol (DAG), or directly through synaptotagmin [31, 32]. At the calcium concentrations required for transmitter release, synaptotagmin-I binds PIP_2 and PIP_3 via a poly-lysine region in its C2 domain, facilitating PM penetration and exocytosis [31, 33].

2.2.1.2 Intracellular Trafficking

As we considered earlier in the "Membrane Distribution" section, PIPs are spatially localized in different compartments in intracellular organelles. The case of clathrin-mediated endocytosis provides a good example of the utility of the different PIPs in best serving the process they support. $PI(4,5)P_2$ predominates over other PIPs in the PM and it is required for the nucleation of the endocytic clathrin-coated pits [34]. Yet, the obvious question of how conversion occurs from $PI(4,5)P_2$ to PI3P, which dominates the various stages of endosomes, needed to be elucidated. It has been shown that a specific PI3K-mediated $PI(4,5)P_2$ conversion to $PI(3,4)P_2$ was required for clathrin-coated pit maturation by recruiting the Bin-Amphiphysin-Rvs (BAR) domain protein SNX9 at a late stage preceding dynamin-mediated fission [35]. This conversion mechanism that regulated clathrin-coated pit maturation and constriction may be preparing endocytic vesicles for fusion with PI3P-containing endosomes.

2.2.1.3 Actin Dynamics

Actin cytoskeleton remodeling and regulation of actin filament assembly and organization are fundamental to many cellular activities, including cellular architecture, adhesion strength, force generation, intracellular transport, motility, and migration

[28, 36–38]. Actin dynamics are also critical for docking priming and mobilization of synaptic vesicles at the presynaptic terminus [39], as well as for dendritic spine morphogenesis [40]. Actin polymerization is a dynamic process regulated by a variety of actin-binding proteins involving the attachment of globular monomers (G-actin) at the barbed (+) end and dissociation at the pointy end defining the length of the polymerized fibrous actin form (F-actin). PIP_2 regulates the function of many actin-binding proteins, such as inactivating proteins that inhibit actin polymerization (e.g., gelsolin, villin, cofilin, and profilin), while activating proteins that promote filamentous assembly (e.g., vinculin, talin, α-actinin, and ezrin) [41, 42]. Thus, for example, excessive epidermal growth factor (EGF) receptor activity leads to hydrolysis of PIP_2 by phospholipase-Cγ, activating the local membrane pool of cofilin in carcinoma cells [43] causing inhibition of formation of F-actin and an excess of G-actin.

2.2.1.4 Focal Adhesion Dynamics

Focal adhesions are macromolecular assemblies that transmit mechanical force and regulatory signals between the extracellular matrix (ECM) and the interior of a cell. PIP_2 binds to many focal adhesion proteins and regulates the signals between the ECM and the cell interior. A good example is talin, the actin-binding protein that is activated by PIP_2 to promote filamentous assembly. Talin also plays a crucial role in activating integrins [44, 45]. In its inactive form, talin's C-terminal rod domain binds to the N-terminal head domain. Disruption of this head-to-tail interaction by PIP_2 activates talin to interact with the cytosolic domain of β3-integrin on one end and actin on the other, thus coupling the ECM to the actin cytoskeleton.

2.2.1.5 Membrane Curvature

PIP_2 interacts with membrane-curvature-sensing proteins called BAR domain transmembrane proteins that regulate membrane shape transitions during endocytosis and membrane trafficking [46, 47], an example of which we encountered in Sect. 2.2.1.2 in clathrin-coated pit maturation with the recruitment of the BAR domain protein SNX9. PIP_2 binds to both sides of BAR proteins forming a membrane protrusion. Amphiphysin1 (BIN1) for example has been shown to induce curvature in PIP_2-containing membranes, while in PIP_2-lacking membranes curvature sensing and generation is abrogated [2]. Genetic mutations that interfere with such interactions lead to the group of congenital centronuclear myopathies (showing severe hypotonia and hypoxia) characterized by a centralized rather than a peripheral muscle cell nucleus [48, 49].

2.2.1.6 Lipid Transfer

In vitro evidence suggests that lipid transfer proteins use PIPs to couple the energy of ATP hydrolysis to the transport of lipid cargoes against a concentration gradient at membrane contact sites. Trans-Golgi network cholesterol and PM phosphatidylserine are examples, where members of the oxysterol-binding protein (OSBP) family and PI4P are involved. Although OSBPs and other lipid transfer proteins can certainly facilitate lipid exchange reactions in vitro, in vivo evidence is lacking and thus their true utility is actively debated in the field [23].

2.2.2 Regulation of Signaling Transmembrane Proteins

2.2.2.1 G-Protein-Coupled Receptors (GPCRs)

GPCR signaling involves competing signal transducers (either G-proteins or β-arrestin) binding to the receptor that in the presence of agonists catalyzes downstream signaling through each of the transducers. G-protein activation involves nucleotide exchange (GDP for GTP) on the Gα subunit of the heterotrimeric Gαβγ to cause Gα-GTP dissociation from Gβγ and downstream signaling. β-arrestin activation involves GPCR phosphorylation by kinases called G-protein receptor kinases (GRKs) that increase receptor affinity to β-arrestin [50]. GPCRs can signal as monomers but ample evidence has suggested that they can also exist in oligomeric states [51], the functional significance of which is not fully appreciated, as structural guidance of how such GPCR oligomers could accomplish downstream signaling is currently lacking.

Phospholipids allosterically modulate the activation of G-protein-coupled receptors (GPCRs) [52], which may play a role in controlling their oligomeric state [53]. Regarding G-protein signaling, the adenosine 2A receptor (A2AR), for example, is stabilized in its active state and its selectivity for G-protein coupling is enhanced by PIP_2 [54]. On the other hand, for β-arrestin signaling, the dissociation of signaling competent β-arrestin may be dependent on PIPs [55]. Moreover, it has been shown that the presence of PIP_2 promotes complex formation between the β2-adrenergic receptor and GRK5 [56] and β-arrestin-mediated endocytosis of GPCRs is proposed to require arrestin binding to PIPs [57]. This preliminary picture of the PIP_2 dependence of GPCR-stimulated G-protein/β-arrestin signaling will certainly be evolving as additional evidence is generated and allows us to put the pieces together in incorporating the important element of PIP contributions to GPCR signaling.

Small GTPases are cytosolic G-proteins, homologous to the Gα subunits of heterotrimeric membrane-associated G-proteins. As GTPases, they are able to hydrolyze GTP to GDP. GTP hydrolysis is accelerated by GTPase activating proteins (GAPs), while GTP exchange is catalyzed by guanine nucleotide exchange factors (GEFs). PIPs regulate GTPase activity by mediating their recruitment as well as that of GEFs and GAPs to membranes [20]. Ras proteins, the best studied small GTPases that have been shown to interact tightly with PIP_2 and operate in the inner leaflet of

the PM, are found to be mutated in many cancer types [58, 59]. It has not been reported whether Gα subunits of heterotrimeric G-proteins also directly interact with PIP_2.

2.2.2.2 Ion Channels

Ion channels are oligomeric transmembrane proteins that provide a hydrophilic pathway to ion flow down an electrochemical gradient across cellular membranes. Three types of stimuli regulate one or more constrictions of the ion pathway, referred to as gates, to open and allow ion flow or to close and stop ion flow, a process coined as "gating." The three types of stimuli use molecular sensors in the protein structure that are allosterically coupled to the gate(s): a voltage sensor for voltage-gated channels, binding sites for extracellular or intracellular chemicals for ligand-gated channels, and mechanical sensors for mechanosensitive channels [60, 61]. The PIP_2 phosphates localized at the inner leaflet of the bilayer are proximal to the channel gates and have been shown to exert critical allosteric control of gating of most ion channels, including voltage-gated K^+ and Ca^{2+} channels, inwardly rectifying K^+ and two-pore domain K^+ channels, transient receptor potential (TRP) channels, Ca^{2+}-activated K^+ and Cl^- channels, Ca^{2+} release and receptor-operated channels, and renal epithelial Na^+ channels (reviewed by [62]). Many channel activators have been shown to strengthen interactions with PIP_2, while allosteric inhibitors (i.e., inhibitors that are not pore blockers) weaken interactions with PIP_2. Channel activators or inhibitors can be physiological chemical modulators, post-translational modifications, drugs, etc.

Perhaps the best studied ion channel in terms of how PIP_2 regulates its gating is the G-protein-gated inwardly rectifying potassium (GIRK or Kir3) channel. We will use this channel as an example to illustrate the critical role of PIP_2 in channel gating. Kir3 channel clones have been available for almost three decades [63–66]. They code for four mammalian isoforms, the ubiquitously expressed Kir3.1, the neuronal Kir3.2 and Kir3.3, and the predominantly peripherally expressed Kir3.4, such as in supraventricular cardiac cells. Kir3.1 and Kir3.3 are not thought to be expressed as homomers, while Kir3.2 and Kir3.4 are expressed both as low-activity homotetramers and high-activity heterotetramers (e.g., Kir3.1/Kir3.2, Kir3.1/Kir3.4, Kir3.1/Kir3.3) (recently reviewed by [67]). They are intracellular ligand-gated channels, where the Gβγ dimer of pertussis toxin-sensitive G-proteins serves as the ligand to activate them following GPCR activation [68]. Na^+ ions serve as another intracellular ligand that gates Kir3.2 and Kir3.4 (both as homotetramers and their heterotetramers with Kir3.1), independently of G-proteins [69]. PIP_2 is absolutely required for channel gating by Gβγ or Na^+, even though it is unable to gate the channel by itself [70–74]. In fact, Gβγ, Na^+, and PIP_2 all synergize together to yield the maximum activity that is much higher than Gβγ/PIP_2 or Na^+/PIP_2 alone [75].

In the last decade, crystal structures of a truncated but functional Kir3.2 alone or together with its gating partners have been solved by the MacKinnon group [76, 77]. Figure 3a shows a side view of two opposite subunits of the homotetrameric Kir3.2

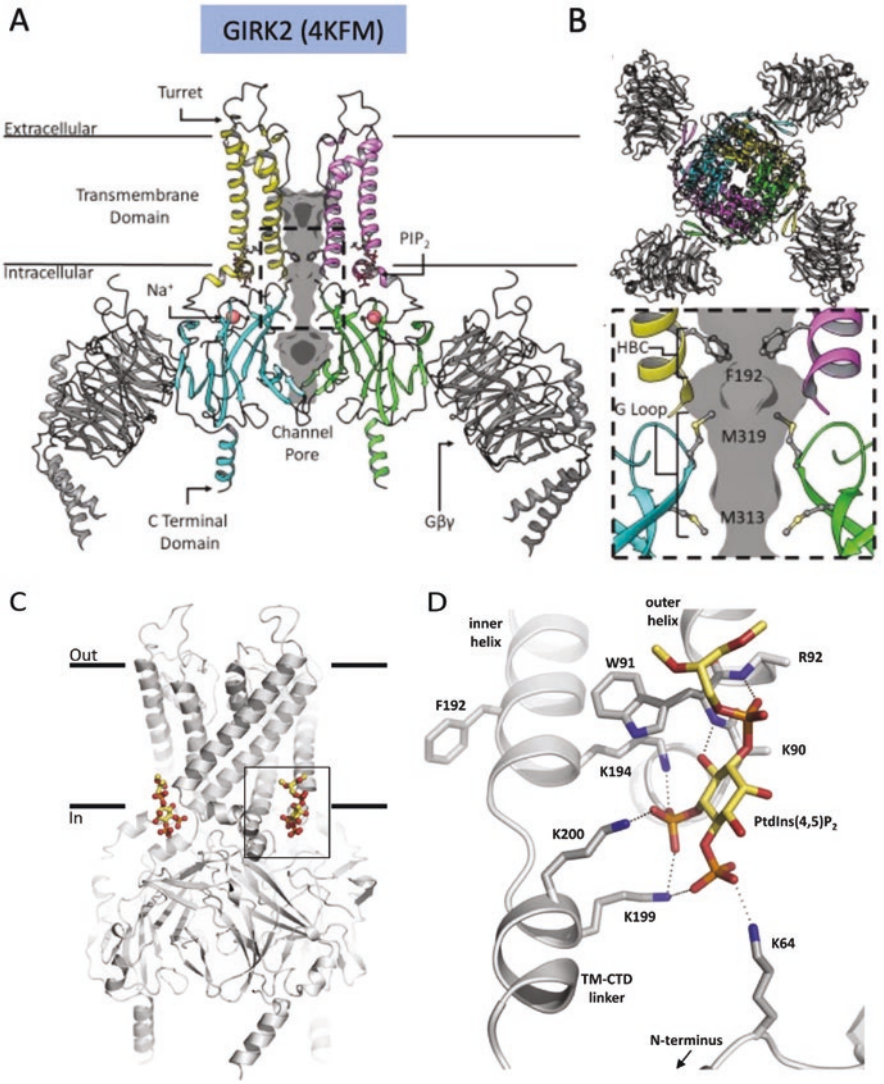
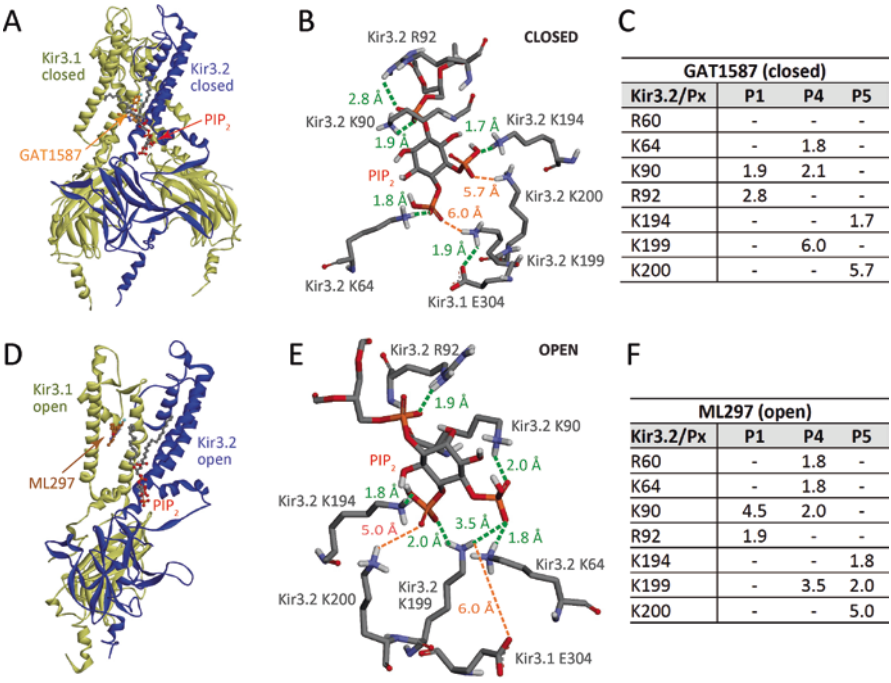


Fig. 3 Kir3 channel architecture and binding to PIP_2. (**a**) Side view of Kir3.2 homotetramers (4KFM) in a pre-open state in the presence of Gβγ, Na^+ (pink spheres), and PIP_2 (sticks). The thick black lines define the boundaries of the phospholipid bilayer (**b**) The top-down view of the Kir3.2-Gβγ complex (top) and the two gates (bottom): the helix bundle crossing or HBC (F192) and the G-loop (M313/M319) (Adapted from Gazgalis et al. 2022 [81]) (**c**) PIP_2 molecules are colored yellow, orange, and red with the whole channel (gray) on the left, and (**d**) a zoomed view on the right. The black box highlights the region of the zoomed view on the right, where the main coordinating residues are shown as sticks. P1 of PIP_2 is coordinated by K90 and R92, P4 by K64 and K199, and P5 by K199, K200, and K194. The important HBC gate residue F192 is also shown for reference. (Adapted from Whorton and MacKinnon 2011 [76])

channel (front and back opposite subunits have been removed to allow a clear view of the permeation pathway shown in gray). Two gates can be readily discerned, one at the level of the interface of the inner membrane leaflet with the cytosol, where the helices cross (Fig. 3c) called the helix bundle crossing (HBC) gate, and a second cytosolic gate, coined as the G-loop gate (like a girdle at the waist of the channel) comprised by two methionines, M313 (Ile in Kir3.1) and M319, defining the width of the girdle (zoomed on top and side views in Fig. 3b). The positions of each of the three gating molecules, PIP_2, Gβγ, and Na^+, are revealed in the structure. Gβγ interacts with the Kir3.2 channel on its cytosolic side in a cleft formed by the DE and LM loops of adjacent subunits [77]. This site of Gβγ interaction was found independently to be positioned similarly in a Kir3.1 model that was validated experimentally [78]. Na^+ ions were found to be coordinated by the CD loop, consistent with previous experimental and modeling studies [74, 76, 79]. PIP_2 was found to be coordinated by four distinct regions (Fig. 3d): (i) two basic residues in the transmembrane to cytoplasmic domain (TM-CTD) linker (also referred to as the B-loop), K200 coordinating phosphate 5 (P5) and K199 coordinating both P5 and P4 of PIP_2; (ii) an N-terminal basic residue K64 coordinating P4 of PIP_2; (iii) K194, in the transmembrane domain 2 (TM2 or inner helix), which lines up part of the permeation pathway, one helical turn away from F192 that comprises the HBC gate coordinating P5 of PIP_2; and (iv) the basic residues K90 and R92 in the highly conserved KWR motif in the TM1 (or outer helix) of inwardly rectifying K^+ channels coordinating P1 of PIP_2. The 4KFM structure that contained all gating molecules (PIP_2, Gβγ, and Na^+) was captured in a pre-open state with only two of the four subunits having their gates open [77].

Li and colleagues proceeded to run microsecond-long molecular dynamics (MD) atomistic simulations of Kir3.2 in a lipid environment and with an electrical gradient across the membrane. They observed gating transitions from the pre-open to the open state that displayed the synergy between the gating components that had been described 20 years earlier [75, 80]. The dynamic simulations revealed that Na^+ ions controlled the G-loop gate through an anti-clockwise rotation, whereas Gβγ stabilized the HBC gate through a rocking outward movement of the cytosolic domains. These movements enhanced the interactions of the channel with PIP_2 to stabilize the channel in an open conductive state. Gazgalis and colleagues extended the work of Li et al. [80] to study the gating transition from closed to open [81]. Instead of the physiological gating molecules (Gβγ and Na^+), they used the potent urea-based activator ML297 [82] that binds the Kir3.1 subunit and ran MD simulations on Kir3.1/Kir3.2 models to assess gating by urea compounds. In addition, they took advantage of a published molecular switch chemical change that turns ML297 into an inhibitor (which they called GAT1587) [83]. Starting from the 4KFM pre-open structure, ML297 could gate the channel to the open conductive state, while GAT1587 could gate the channel to a closed non-conductive state.

Figure 4 shows the distances of seven key basic residues of the Kir3.2 subunit from the three phosphates of the PIP_2 headgroup (P1, P4, and P5) in the closed/inhibited (GAT1587) versus open/conducting (ML297) states. The most striking finding from this comparison is the position of the Kir3.2(K199) residue that forms



GAT1587 (closed)			
Kir3.2/Px	P1	P4	P5
R60	-	-	-
K64	-	1.8	-
K90	1.9	2.1	-
R92	2.8	-	-
K194	-	-	1.7
K199	-	6.0	-
K200	-	-	5.7

ML297 (open)			
Kir3.2/Px	P1	P4	P5
R60	-	1.8	-
K64	-	1.8	-
K90	4.5	2.0	-
R92	1.9	-	-
K194	-	-	1.8
K199	-	3.5	2.0
K200	-	-	5.0

Fig. 4 Kir3.1/Kir3.2 snapshots in the closed or open conformations stabilized by similar urea-based compounds. (**a**) Side view of Kir3.1/Kir3.2 heterotetramer (the back blue Kir3.2 subunit was removed for clarity) stabilized in the closed conformation by GAT1587 after hundreds of nanoseconds of MD simulations. (**b**) Zoomed distances mainly of Kir3.2 from the PIP_2 phosphates as well as of the Kir3.2(K199) with the G-loop Kir3.1(E304) residue. (**c**) Summary of the interactions of PIP_2 with the Kir3.2 subunit of a Kir3.1/Kir3.2 heteromer in the closed state. (**d**) Side view of two adjacent subunits of the heterotetrameric Kir3.1/Kir3.2 channel stabilized in the open conformation by ML297 after hundreds of nanoseconds of MD simulations. (**e**) Zoomed distances mainly of Kir3.2 from the PIP_2 phosphates as well as of the Kir3.2(K199) with the G-loop Kir3.1(E304) residue. (**f**) Summary of the interactions of PIP_2 with the Kir3.2 subunit of a Kir3.1/Kir3.2 heteromer in the open state

a salt bridge with the G-loop gate residue Kir3.1(E304) in the closed state (1.9 Å distance), while in the open state it critically coordinates the P5 of the PIP_2 to stabilize the HBC gate in an open conducting state. Thus, Kir3.2(K199) in the closed state is found to block the G-loop gate but in the open state the G-loop gate block is reversed and now Kir3.2(K199) stabilizes the HBC gate open by switching its salt bridge partner with the P5 of PIP_2. This example of Kir3 channel gating via PIP_2 highlights the power of computational models in predicting stable conformational states in the presence of the appropriate ligands. Its predictions could also be pursued experimentally with atomic resolution structures to further validate the models.

2.2.3 Cellular Signaling

2.2.3.1 Receptor-Stimulated Phospholipases C

Activation of phospholipases C (PLCs) cleaves PIP_2 at the glycerol side of the phosphodiester bond, generating diacylglycerol (DAG, the two acyl chains) and inositol tris-phosphate (IP_3, the inositol headgroup with phosphates at P1, P4, and P5) [84–86]. IP_3 releases Ca^{2+} from intracellular stores. There are 13 PLCs that have been identified belonging to 6 families ($\beta_{1\text{-}4}$, $\gamma_{1,2}$, $\delta_{1,3,4}$, ε, ζ, and $\eta_{1,2}$). G-protein activation involves either the Gαq or the Gβγ dimer of Gαi/o pertussis toxin-sensitive proteins. Tyrosine kinase receptors (RTKs) recruit PLCγ to activated (trans-phosphorylated) RTKs via SH2 domain–phosphotyrosine interactions, where they are further activated by RTK-mediated phosphorylation. PLCs and their distinct functions have been recently reviewed [87].

2.3 Methods to Monitor Membrane PIP Levels

Methods to monitor membrane PIP_2 processes, especially as it pertains to ion channel research, have been reviewed [88]. There are three main approaches at different levels that have been utilized extensively.

At the *cell biology* level, visualizing with fluorescent proteins tagging protein domains interacting distinctly with different PIPs has been used for this purpose. There are numerous protein domains that interact specifically with PIPs, and do so even as independent domains when they are excised from the protein they are normally a part of. When tagged by fluorescent proteins, these domains can track through fluorescence methods the PIPs they bind. The PLCδ-PH domain is one of many such examples (reviewed in [89]).

At the *functional* level, the versatility of the patch-clamp technique offers the ability to assess apparent affinities of ion channel proteins for different PIPs in either the excised inside-out patch or in the whole-cell modes of the technique. In inside-out patches from cells expressing the PIP-interacting channel protein, water-soluble (di-C8) synthetic PIPs are used to construct concentration–response relationships. More recently, in whole-cell experiments expressing two interacting component systems, one is tethered to the PM (e.g., through a lipid anchor) while the second changes conformation with a stimulus, such as a chemical (e.g., rapamycin) or light, to interact with the membrane-bound component. This allows for membrane recruitment of the cytosolic component upon stimulation. A plant-derived N-terminal region of CIB1 (CIBN) (tethered to the membrane) and a photosensitive CRY2 component tagged with different PIP_2 phosphatases (the 4′ Sac, the 5′ OCRL, or the 4′ and 5′ pseudojanin) have been used effectively [90, 91]. This approach of dephosphorylating specifically either the 4′ or 5′ or both phosphates of PIP_2 can discern their relative importance in the control of gating a particular PIP_2-interacting protein. Moreover, the speed of the blue light-activated mechanism

allows for sensitive kinetic studies. This system has been used to assess how post-translational modifications, like phosphorylation, or drugs strengthen or weaken channel–PIP_2 interactions by slowing or accelerating the kinetics and decreasing or increasing the extent of current inhibition respectively upon PIP_2 dephosphorylation [62, 91, 92].

Finally, at the *purified protein–lipid* level, several sensitive methods such as isothermal calorimetry, centrifugation assays, and surface plasmon resonance (SPR) have been used [93]. Perhaps standing out among them is the SPR approach, which immobilizes either a biotinylated PIP at the biosensor chip of the Biacore instrument or the purified PIP-interacting protein of interest. Different concentrations of the non-immobilized interacting partner are run through the instrument yielding sensitive refractive index (and thus mass concentration of protein or lipid) measurements. The advantage of this approach is that one obtains direct binding and unbinding values based on the affinity of the protein for the lipid. Using this approach, direct estimates of the K_d for diC8-PIP_2 for the truncated Kir3.2 used in the structural studies can be obtained in the order of tens of nanomolar concentrations, which compare to three orders of magnitude lower than the apparent affinities obtained in concentration–response studies performed in inside-out patch recordings [94].

3 Cholesterol

3.1 Cholesterol: Overview and Structure

Cholesterol is an essential lipid that plays critical roles in maintaining cell viability and growth in mammalian cells. Constituting between 20% and 50% of membrane lipids [95–100], cholesterol supports the integrity and fluidity of cellular membranes. In addition, cholesterol serves as a precursor for the synthesis of vital substances, regulates signaling processes, and alters the function of membrane proteins [99–103]. Considering the involvement of cholesterol in this variegated list of functions, it is not surprising that both an excess and a deficiency in cholesterol levels are pathophysiological [96, 104–112]. Specifically, disrupted cholesterol metabolism can result in genetic diseases (e.g., Niemann-Pick type C disease, Schnyder corneal dystrophy, Smith-Lemli-Opitz syndrome, Familial hypercholesterolemia, Tangier disease, and Sitosterolemia) [113–120]. Additionally, alterations in cholesterol levels have been associated with acquired cardiovascular diseases, neurodegenerative disorders (e.g., Alzheimer's disease), severe physical malformations, and different types of cancer [106–112, 120–126]. The sections below discuss the diverse physiological roles of this important lipid.

From a structural point of view, cholesterol is a 27-carbon isoprenoid composed of a rigid fused tetracyclic ring system with a hydroxyl group on one end (ring 1) and a flexible 8-carbon alkyl group on the other end (ring 4) (Fig. 1). The structural elements of cholesterol along with its hydrophobic nature are key to its essential

roles. (i) These features allow cholesterol to stabilize membrane integrity by increasing lipid packing and membrane thickness [127]. Specifically, the fused ring structure of this hydrophobic molecule enhances the rigidity of phospholipid membrane bilayers. Within the membrane, the orientation of the cholesterol molecule is often influenced by the preference of its hydroxyl group to be at an interface with an aqueous environment. (ii) The tetracyclic ring structure of this lipid constitutes an essential starting point for the synthesis of steroid hormones and bile acids. (iii) The ring structure of cholesterol often participates in stacking interactions with aromatic protein residues while the hydrophobic nature of the cholesterol molecule is key to its abundant interactions with hydrophobic residues. Together, the interactions of cholesterol with hydrophobic and aromatic residues often define the binding sites of cholesterol in proteins. As such, the structural features of cholesterol underlie not only its roles in membrane integrity and as a metabolic precursor, but also its ability to affect protein function and signaling processes. Crucially, the latter forms the basis for cholesterol-driven effects on physiology and pathology.

3.2 Biosynthesis, Regulation, and Metabolism of Cholesterol

The regulation of cellular cholesterol content is tightly controlled by an elaborate feedback mechanism encompassing cholesterol biosynthesis, uptake, storage, transfer, and excretion pathways [120, 128–131] (Figs. 5 and 6).

The de novo cholesterol biosynthesis pathway, also called the mevalonate pathway, is the primary source of cholesterol in humans, yielding ~10 mg cholesterol per day per kg body weight. Hence, a 70 kg human would synthesize a total of ~700 mg cholesterol per day [130, 132, 133]. Almost all organs require cholesterol for synthesizing new membranes and as precursors for other compounds (e.g., steroids, bile acids, oxysterols) [132, 134–136]. Therefore, cholesterol synthesis occurs in different tissues, with the liver, intestines, and skin making the primary contributions. Within the cells, cholesterol synthesis occurs primarily in the membranes of the endoplasmic reticulum (ER) [98, 137]. Starting from acetyl-coenzyme A (CoA), cholesterol is synthesized in a series of about 30 reactions tightly regulated by a fleet of over 20 enzymes (Fig. 5) [138]. With lanosterol being the first sterol intermediate on route to the synthesis of cholesterol, the intracellular biosynthesis pathway is commonly divided into pre- and post-lanosterol pathways [138]. In the pre-lanosterol pathway, six isoprene units formed from acetyl-CoA are combined to form the isoprenoid hydrocarbon squalene. Once formed, squalene is oxidized to squalene 2,3-epoxide, which then undergoes cyclization to form lanosterol. Conversion of lanosterol to cholesterol proceeds via about 20 reactions involving the removal of three methyl groups (at C-4, C-14) in addition to the reduction of one double bond (at C-24) and the migration of another (at C-8). While some of the reactions must occur in a specific order, the double bond reduction can occur at different points along the pathway, giving rise to two alternative post-lanosterol pathways [128, 139, 140].

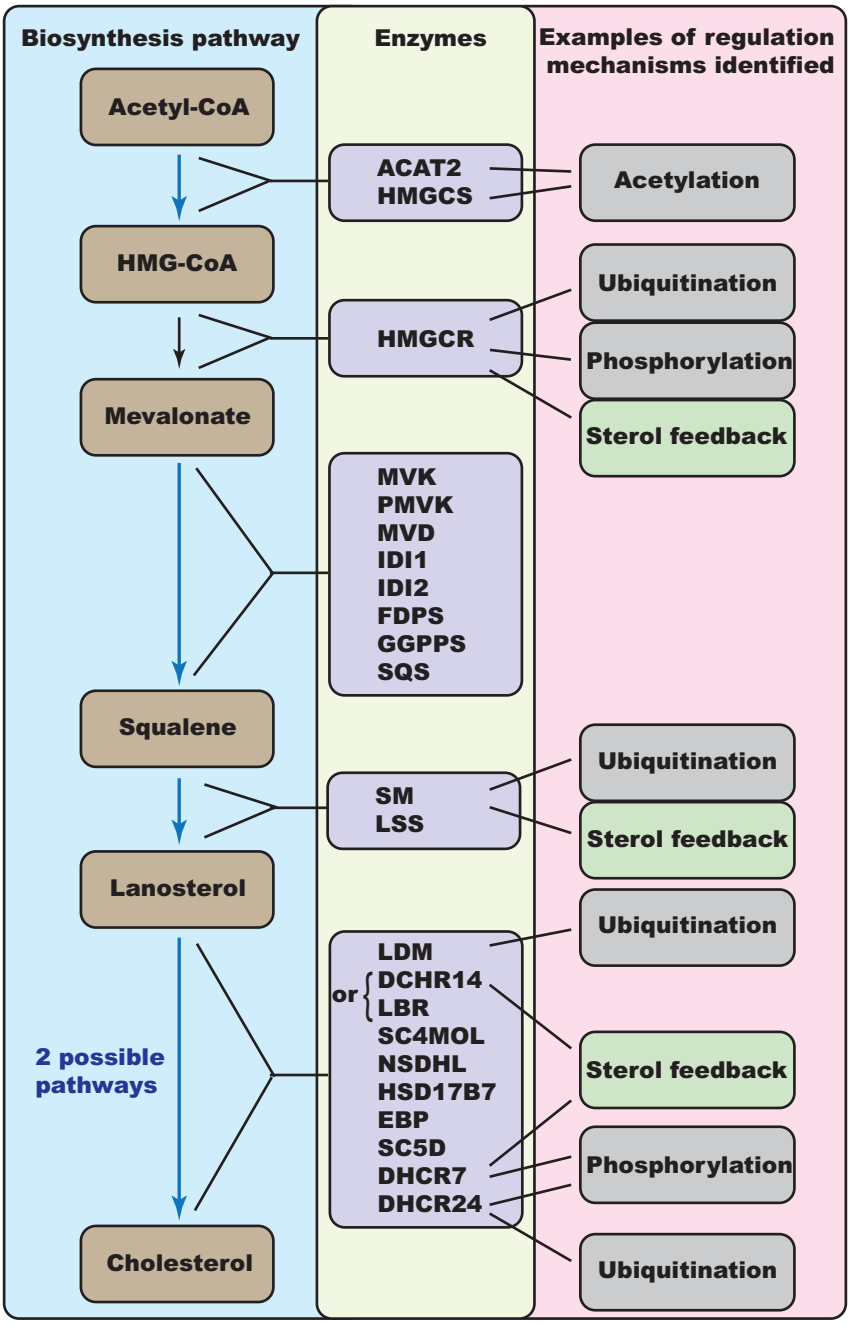


Fig. 5 Simplified mevalonate pathway showing key intermediates along the cholesterol biosynthesis pathway (left column), enzymes involved in cholesterol synthesis (center column), and examples of post-translational and sterol feedback regulation mechanisms (right column)

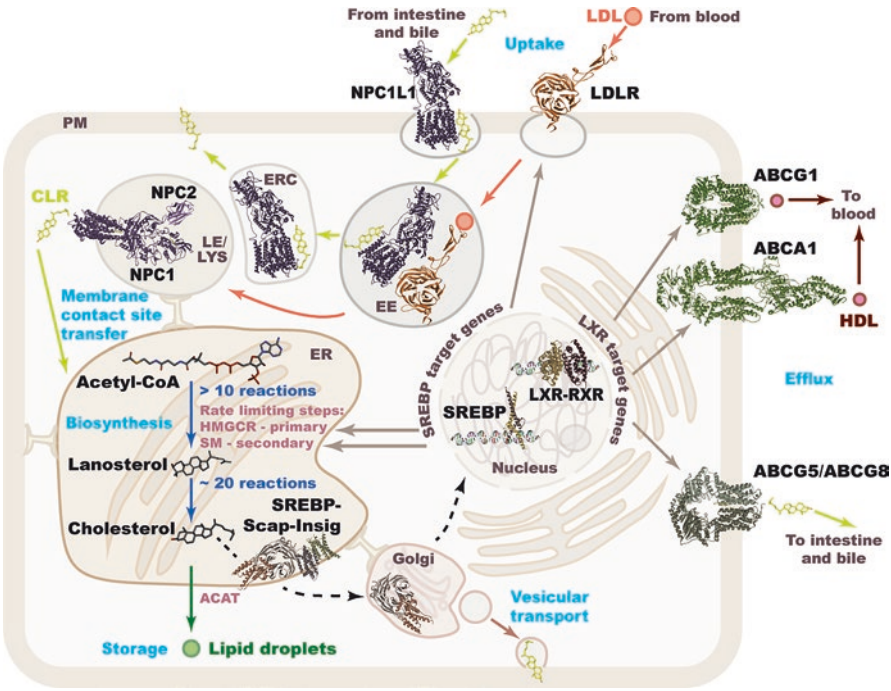


Fig. 6 Overview of major pathways in cholesterol homeostasis: cholesterol biosynthesis, uptake, efflux, vesicular transport, membrane contact site transfer, and storage. The following structures from the Protein Data Bank (PDB) were used to represent different molecular entities in the figure: PDB ID 5xjy for ABCA1, PDB ID 7r8d for ABCG1, PDB ID 7r8b for the ABCG5/ABCG8 heteromer, PDB ID 3p5b for LDLR, PDB ID 6v3f for NPC1L1, PDB ID 6w5v for NPC1 and NPC2, PDB ID 5grs for the SREBP–Scap complex, PDB ID 6m49 for the Scap–Insig transmembrane domain, PDB ID 1am9 for SREBP interacting with DNA, and PDB ID 1uhi for the RXR LXR complex. The following structures were obtained from PubChem: CID 444493 for acetyl-CoA (2D conformer), CID 246983 for lanosterol (3D conformer), and CID 5997 for cholesterol (3D conformer)

Two rate-limiting steps have been identified in the pre-lanosterol pathway (Fig. 6). The primary rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate catalyzed by HMG-CoA reductase (HMGCR) [141]. A secondary rate-limiting step controlling the synthesis of cholesterol downstream of HMGCR involves the synthesis of squalene 2,3-epoxide from squalene catalyzed by squalene monooxygenase (SM) [142, 143]. While these two rate-limiting steps have received the most attention, other steps have also been investigated in recent years. These studies have revealed that other enzymes also contribute to the tight regulation of cholesterol biosynthesis (Fig. 5) (reviewed by [138]). Therefore, and in view of the sequential nature of the cholesterol synthesis pathway, it is not surprising that multiple human disorders involving enzyme defects have been described.

Most of the genes encoding the enzymes catalyzing each of the cholesterol biosynthesis steps are regulated by the sterol regulatory element-binding protein 2 (SREBP) [130, 144]. In the ER, this transcription factor is bound to the SREBP cleavage activating protein (Scap), which acts as a sterol sensor (Fig. 6) [120, 130, 145–149]. When sterol levels are high, the SREBP–Scap complex interacts with the insulin-induced gene (Insig); a sterol bound between the Scap and Insig proteins stabilizes this interaction. The interaction between Scap and the sterol alters Scap's conformation, retaining the SREBP–Scap complex in the ER. When cholesterol levels are low, Insig dissociates from the SREBP–Scap complex, and Scap escorts SREBP to the Golgi (Fig. 6). In the Golgi, SREBP undergoes two sequential proteolytic cleavages that release its N-terminus. The N-terminus of SREBP is then translocated to the nucleus, where it binds to the sterol regulatory element (SRE) in the promoters of genes encoding for enzymes catalyzing steps along the mevalonate pathway, thereby upregulating their transcription (Fig. 6).

A counter mechanism aimed at rapidly reducing cholesterol levels involves the proteolytic degradation of HMGCR and SM in the presence of excess levels of sterols. To mark these enzymes for degradation, they are ubiquitinated by E3 ligases (Fig. 5) [150–153]. Interestingly, the degradation of HMGCR is stimulated by excess levels of oxysterols and metabolic intermediates of the cholesterol biosynthesis pathway, whereas the degradation of SM is stimulated by excess levels of cholesterol [138]. Beyond HMGCR and SM, other enzymes (e.g., DCHR7, DCHR14) are also regulated via post-translational ubiquitination that leads to their degradation in response to sterol excess (Fig. 5). Notably, ubiquitination is the dominant type of post-translational modification of enzymes along the cholesterol biosynthesis pathway. However, enzymes along this pathway can be subjected to other post-translational modifications, such as phosphorylation or acetylation (for examples, see Fig. 5) [154–156]. For example, HMGCR is inhibited following phosphorylation and reactivated following dephosphorylation. In contrast, DCHR7 is activated by phosphorylation [157, 158], indicating phosphorylation may have a differential effect on the activity of different enzymes along the cholesterol biosynthesis pathway.

The synthesis of cholesterol via the biosynthesis pathway is supplemented by a receptor-mediated lipoprotein uptake mechanism involving clathrin-mediated endocytosis (Fig. 6). Delivery of dietary cholesterol from the blood is regulated by members of the low-density lipoprotein (LDL) receptor (LDLR) family at the basal surface of enterocytes or hepatocytes [159–161]. After reaching the late endosome (LE)/lysosome (Lys), the LDL-derived cholesterol is transported to the PM to fulfill its roles (Fig. 6). The transport of cholesterol from the LE/Lys is coordinated by the NPC1 and NPC2 proteins [162–167]. When cholesterol levels in the PM are too high, the LDL-derived cholesterol is transported to the ER to downregulate the biosynthesis pathway by blocking the activation of SREBP through the inhibition of the Scap/SREBP complex, thereby contributing to the feedback mechanism regulating cellular cholesterol levels (Fig. 6).

Dietary input of cholesterol can yield ~300 mg cholesterol per day [130], which would reach most organs except for the brain where all the cholesterol is synthesized de novo, as lipoproteins cannot cross the blood-brain barrier [168–171]. Similar to the genes encoding the enzymes along the cholesterol biosynthesis pathway, the gene encoding LDLR is regulated by SREBP, albeit via a more rapid mechanism. Specifically, LDLR has one SRE whereas upregulation of the genes of several of the enzymes along the cholesterol biosynthesis pathway requires the cooperativity of two SREs. As the intricate cholesterol biosynthesis pathway is an energetically expensive process, this ensures that it is not induced when cholesterol can be obtained through dietary intake.

Another protein that plays an essential role in dietary cholesterol uptake is the cholesterol absorption protein Niemann-Pick C1-Like 1 (NPC1L1), which is abundantly expressed on the apical membrane of enterocytes in the small intestine [172–175]. Additionally, NPC1L1 is also expressed in the canalicular membrane of hepatocytes where it is thought to contribute to cholesterol reabsorption and enterohepatic recycling of sterols (Fig. 6).

Conversely, reduction of excess cholesterol levels is achieved through efflux pumps, such as the ATP-binding cassette transporters ATP-binding cassette subfamily A member 1 (ABCA1), ATP-binding cassette subfamily G member 1 (ABCG1), and ABCG5/ABCG8. ABCA1 and ABCG1 export cholesterol to the blood and the ABCG5/ABCG8 heterodimer exports cholesterol to the intestinal lumen and bile ducts (Fig. 6) [176–189]. Excess cholesterol can also be esterified by acyl coenzyme A: cholesterol acyltransferase (ACAT) [190]. The resulting cholesteryl esters are then stored in lipid droplets or incorporated into plasma lipoproteins for secretion (Fig. 6).

Within the cells, cholesterol can be transferred between different membrane domains (e.g., lipid rafts, caveolae) via vesicular transport [191]. Additionally, cholesterol can be transported between different cellular organelles (e.g., between the ER and the PM, between the ER and the Golgi or Lys) at membrane contact sites (Fig. 6) [192–194]. Such non-vesicular transport is facilitated by lipid transport proteins, such as steroidogenic acute regulatory protein (StAR)-related lipid transfer (StART) proteins and members of the OSBP-related protein (ORP) family [120, 195–210]. For example, this mechanism is used by ORP1L to transfer cholesterol between the ER and the LE/Lys at membrane contact sites of these organelles [211–217].

Although many details in the mechanisms and pathways underlying the regulation of cellular cholesterol content have been uncovered (see for example, the reviews [98, 120, 130, 131, 138, 191, 218–221]), future research will undoubtedly reveal further information regarding these complex processes and the numerous proteins and enzymes involved in them.