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Biochimica et Biophysica Acta 1768 (2007) 836–852

Biochimica et Biophysica Acta BBA

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Review

Lipid–protein interactions in GPCR-associated signaling

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Received 25 July 2006; received in revised form 5 September 2006; accepted 6 September 2006
Available online 16 September 2006

Abstract

Signal transduction via G-protein-coupled receptors (GPCRs) is a fundamental pathway through which the functions of an individual cell can be integrated within the demands of a multicellular organism. Since this family of receptors first discovered, the proteins that constitute this signaling cascade and their interactions with one another have been studied intensely. In parallel, the pivotal role of lipids in the correct and efficient propagation of extracellular signals has attracted ever increasing attention. This is not surprising given that most of the signal transduction machinery is membrane-associated and therefore lipid-related. Hence, lipid–protein interactions exert a considerable influence on the activity of these proteins. This review focuses on the post-translational lipid modifications of GPCRs and G proteins (palmitoylation, myristoylation, and isoprenylation) and their significance for membrane binding, trafficking and signaling. Moreover, we address how the particular biophysical properties of different membrane structures may regulate the localization of these proteins and the potential functional consequences of this phenomenon in signal transduction. Finally, the interactions that occur between membrane lipids and GPCR effector enzymes such as PLC and PKC are also considered.

Keywords: Signal transduction; Membrane structure; G-protein-coupled receptor; G protein; Palmitoylation; Myristoylation; Isoprenylation; Farnesyl; Geranylgeraniol; Phospholipase C; Protein kinase C

Contents

Abbreviations: Arg, arginin; cPH, C-terminal half of pleckstrin homology; Cys, cysteine; DAG, diacylglycerol; DHA, docosahexaenoic acid; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; H_{II}, hexagonal II (inverted hexagonal); His, histidin; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; l_o, liquid-ordered; LH/hCG receptor, luteinizing hormone/human chorionic gonadotropin receptor; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphoinositide; PH, pleckstrin homology; PKC, protein kinase C; PLC, phospholipase C; RACK, receptor for activated C kinase; Ser, serine; Trp, tryptophan; TRPC3, transient receptor potential 3

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doi:10.1016/j.bbamem.2006.09.001

1. Introduction

Biological membranes are vital components of living organisms, providing a diffusion barrier that separates cells from the extracellular environment or which compartmentalizes specialized organelles within the cell. The lipid bilayer of membranes hinders the free diffusion of ions, a prerequisite to create electrochemical potentials that can be used for the synthesis of ATP or for active transport. Beside this general purpose of biological membranes, the interaction of lipids and membrane proteins impinges on basic biochemical processes between the separated areas on both sides of the membrane, such as respiration, photosynthesis, protein and solute transport, motility, and signal transduction. The importance of these proteins is underlined by the fact, that the proportion of putative membrane proteins predicted from the sequenced human genome is around 30% [1]. In order to maintain the diffusion barrier and to keep it electrochemically sealed, a close interaction of membrane proteins with the lipid bilayer is obligatory. This is especially important given that many membrane proteins undergo conformational changes that affect their transmembrane regions and that may regulate their activity, as seen for G-protein-coupled receptors (GPCRs) and G proteins [2–4]. The lateral and rotational mobility of membrane lipids supports the sealing function while allowing a structural rearrangement of membrane proteins, because they can adhere to the surface of integral membrane proteins and flexibly adjust to a changing microenvironment. In this context, different membrane proteins are selective for certain phospholipids [5] and likewise particular phospholipids are essential for the activity of certain membrane proteins [6]. However, in most cases the basis underlying the specific lipid requirements of membrane components remains unclear.

One special kind of lipid–protein interaction that participates in G-protein-associated signaling involves well-known co- or post-translational lipid modifications of the majority of signaling proteins (myristoylation, palmitoylation and isoprenylation). These modifications are particularly prevalent in integral or peripheral membrane proteins, such as GPCRs, G proteins, G-protein-coupled effector enzymes and receptor kinases [7–10]. Protein lipidation is the first important lipid–protein interaction that occurs in GPCR-related signaling, and it includes the covalent binding of a hydrophobic lipid molecule to the signaling protein. The other important kind of lipid–protein interaction is the influence of plasma membrane lipids and their membrane organization on signaling proteins, and consequently on signal transduction. In this context, the lipid–protein interaction is mediated by the chemical nature of the lipids and the resulting biophysical properties of the membrane in which they are inserted. By these means, membrane lipids are not only involved in the trafficking of newly synthesized proteins to the plasma membrane and their lateral distribution within it, but they also modulate their enzymatic activity. The chemical properties of lipids can also lead to the segregation of specific membrane regions into microdomains, a phenomenon that also seems to be implicated in signal transduction, membrane transport, and protein sorting [11,12]. However, the fact that the influence between membrane lipids and proteins is not unilateral and that can occur in both directions is often overlooked. This means that integral and peripheral proteins are able to perturb their immediate lipid environment (e.g., to facilitate or inhibit the chain and translational ordering of adjacent phospholipids), thereby modifying plasma membrane properties and organization [13,14]. Taken together, the variety of interactions between membrane lipids and proteins reflects the complexity of their relationship and the multitude of possible regulatory processes involved in GPCR-associated signal transduction.

2. Lipid modifications of GPCRs

GPCRs form by far the largest family of cell surface proteins involved in signaling across biological membranes. Indeed, within the human genome approximately 950 genes encode proteins of this superfamily [15]. Hence, the wide diversity of the possible agonists and the factors that may modulate signaling, coupled with the widespread distribution of these receptors enables GPCRs to be employed in the physiological regulation of virtually all biological activities.

2.1. Fatty acylation of GPCRs

GPCRs are integral plasma membrane proteins that contain seven membrane-spanning domains, connected by three extracellular and intracellular loops. Therefore, in contrast to peripheral proteins that depend on fatty acylation for tight membrane attachment (e.g., G proteins, see Section 3), GPCRs do not need any kind of co- or post-translational lipid modification to ensure proper membrane binding. Nevertheless, GPCRs are also subject to lipid modifications. While the most frequent lipid modifications of proteins are generally N-myristoylation, isoprenylation and thio(S)-acylation, as a rule

the lipidation of GPCRs is limited to thio-acylation, through the attachment of palmitate to one or more cysteine residues via a thioester bond. The exact site is typically a cysteine residue in the cytoplasmic tail of the protein, positioned 10 to 14 amino acids downstream of the last transmembrane domain (i.e., close to the carboxy terminus of the receptor [16]). Rhodopsin was the first GPCR to be identified as a target for palmitoylation [7], and it was suggested that the attached palmitate might be incorporated into the plasma membrane bilayer, thereby creating an additional intracellular loop. This was later corroborated by the use of fluorescent fatty acid analogues [17]. As a consequence, palmitoylation has a profound effect on the local conformation of this domain, and it possibly controls the interaction of GPCRs with specific regulatory proteins. When the palmitoylation of the β_2-adrenergic receptor was characterized, the palmitoylated cysteine was again found in the carboxy terminal tail of the protein at an analogous position to that in rhodopsin [18]. Moreover, cysteine residues at similar locations were found in about 80% of all GPCRs (Table 1), indicating that palmitoylation is a general characteristic of this type of receptor. However, GPCR palmitoylation may not be restricted to the carboxy terminal of the receptor. Indeed, when all cysteines in the carboxyl tail of the rat μ-opioid receptor were mutated there was no effect on palmitate incorporation [19], suggesting that palmitoylation sites exist outside of this receptor domain. Further evidence of other palmitoylation sites, even in receptors that are palmitoylated in their carboxy terminal tail, came from studies of the V1a vasopressin receptor [20]. In this case, mutations of the cysteines in the carboxyl tail did not completely eliminate palmitoylation, suggesting the presence of palmitoylated cysteines in the intracellular loops that connect the transmembrane spans.

Beside palmitoylation, the only other lipid modification of GPCRs identified is the isoprenylation of the prostacyclin receptor. The prostacyclin receptor from a number of species contains an identical conserved putative isoprenylation CaaX motif in the carboxy terminal tail that corresponds to the sequence CSLC. Indeed, it was shown that the human prostacyclin receptor is C-15 farnesylated on Cys383 in its carboxy terminal tail and that it is also palmitoylated on Cys308 and 311 [21,22]. Dual lipidation via palmitoylation and isoprenylation, is not unique to the prostacyclin receptor as it occurs in a variety of different proteins (e.g., RhoB, Ha-Ras and N-Ras: [23,24]). Palmitoylation of isoprenylated proteins generally takes place at membranes whereby isoprenylation precedes palmitoylation [25]. In general, a second lipid modification may serve to further augment the hydrophobicity of the protein and consequently increase its membrane affinity, converting the affected proteins into permanent residents of the membrane. Nevertheless, since palmitoylation occurs after isoprenylation, the first signal may also be required to permit an initial interaction with membranes thereby facilitating palmitoylation by a membrane-bound palmitoyltransferase [26,27], a process that else would be unable to occur.

2.2. Role of lipid modifications in GPCR trafficking

In contrast to other lipid modifications, palmitoylation is a very dynamic modification. The acyl-thioester bond attaching the 16-carbon saturated palmitate moiety to the specific cysteine residues of its target protein is chemically labile and it has been shown to be rapidly turned over [28–30]. This rapid turnover suggests that palmitoylation/depalmitoylation may exert a regulatory influence on protein function. However, when considering possible functional roles of palmitoylation, it is important to differentiate between constitutive and dynamic palmitoylation. In this context, constitutive palmitoylation refers to the first protein acylation that occurs during or shortly

Table 1
Palmitoylation dependence for G protein coupling

	Cysteines	Reference	Selected sites of modification [a]
GPCRs with palmitoylation-dependent G protein coupling			
Bovine rhodopsin	322, 323	[201]	QFRNCMVTTL**CC**GKNPLGDDEA
Human β_2-adrenergic receptor	341	[18]	DFRIAFQELL**C**LRRSSLKAYG
Human M2 muscarinic acetylcholine receptor	457	[202]	KKTFKHLLM**C**HYKNIGATR
Human somatostatin receptor, type 5	320	[203]	DNFRQSFQKVL**C**RLKGSGAKDADA
Human endothelin receptor, subtype A, ET_A	383, 385–388	[204] [205]	KFKNCFQS**C**L**CCCC**YSKSLMTSV
Human endothelin receptor, subtype B, ET_B	402, 403, 405 [+ Cys 400]	[53] [205]	RFKNCFKSCL**CC**W**C**QSFEEKQSLE RFKNCFKS**C**L**CC**W**C**QSFEEKQSLE [b]
Human prostacyclin receptor, IP	308, 311 383	[22] [21]	AVFQRLKLWV**CC**L**C**LGPAHGDSQTP SAVGTSSKAEASVA**C**SLC [c]
GPCRs w/o palmitoylation-dependent G protein coupling			
Porcine α_{2A}-adrenergic receptor	442	[52]	HDFRRAFKKIL**C**RGDRKRIV
Rat LH/hCG receptor	621, 622	[206]	DFLLLLSRFG**CC**KRRAELYRRK
Human dopamine D_1 receptor	347, 351	[207]	RKAFSTLLG**C**YRL**C**PATNNAIETV
Human adenosine A_1 receptor	309	[58]	FLKIWNDHFR**C**QPAPPIDEDL
Human thyrotropin receptor	699	[208]	VFILLSKFGI**C**KRQAQAYRGQ

[a] Bold cysteine residues represent sites for thioester-linked palmitoylation, as identified by mutagenesis.
[b] Palmitoylated cysteine residues were detected by mass spectrometry.
[c] Bold, underlined cysteine residue represents site for thioether-linked isoprenylation as identified by mutagenesis.

after protein synthesis. In contrast, dynamic palmitoylation corresponds to the regulation of the palmitoylation state of the mature protein once it has reached its specific location. It is recommendable to define these differences, since there is strong evidence that GPCR palmitoylation may be implicated in the efficient expression of the functional receptor at the cell surface. Thus, palmitoylation could fulfill two different roles: the processing and targeting of the protein to the correct membrane site and independently, a signaling function in the final receptor. This is consistent with the fact that palmitoylation of membrane proteins has been detected in several cellular locations. Accordingly, palmitoylation of caveolin-1 [31] and of the transferrin receptor [32] appears to take place at the plasma membrane, whereas the palmitoylation of viral polypeptides and glycoproteins seems to occur in the Golgi [33,34]. Other proteins are palmitoylated even earlier in their biosynthetic pathway (i.e., in the endoplasmic reticulum [35]). It is difficult to corroborate whether all of these locations are true sites of palmitoylation or whether the results only reflect the rapid intracellular movement of the corresponding proteins. Nevertheless, it is possible that a single membrane protein could be modified at various sites in the cell during its lifespan. In this context, the δ-opioid receptor has recently been shown to be palmitoylated during or shortly after export from the endoplasmic reticulum [36]. Blockage of this palmitoylation inhibited the cell surface expression of the receptor implying that this lipid modification is crucial for its correct intracellular trafficking. In the same study the δ-opioid receptor was also shown to be palmitoylated at the plasma membrane, and that the palmitoylation turnover in this domain is dependent on receptor activation by agonist. More evidence indicating that other GPCRs are processed in the same way was contributed by several studies demonstrating an intracellular accumulation of the receptor, when its specific palmitoylation sites were mutated. This was the case for bovine opsin [37], the lutropin/choriogonadotropin receptor [38], the H_2 histamine receptor [39], and the chemokine CCR5 receptor [40]. Intracellular receptor retention upon mutating the two specific palmitoylated cysteines (Cys341 and Cys342) was also reported for the vasopressin V2 receptor [41], although palmitoylation of only one of these cysteines was sufficient to completely restore cell surface expression of the receptor [42].

2.3. The role of lipid modifications in GPCR signaling

The fact that palmitic acid turns over more rapidly than the half-life of the protein [43,44] supports the idea that palmitoylation, like phosphorylation, is likely to be a regulatory mechanism in the cell [45]. Accordingly, an increase in palmitate turnover can be provoked in response to extracellular stimuli [46]. Indeed, for a variety of GPCRs, such as the β_2-adrenergic and α_{2A}-adrenergic receptor, the palmitoylation/depalmitoylation cycle is activation-dependent [20,47–51]. However, when considering the effect of GPCR palmitoylation on G protein coupling the situation is somewhat less clear (Table 1). While receptors like the β_2-adrenergic receptor are uncoupled from their corresponding G protein upon mutagenesis of their palmitoylation site [18], the signaling of others like the α_{2A}-adrenergic receptor is not significantly affected by this modification [52]. In some cases, the elimination of specific palmitoylated cysteines seems to exert a different influence on G protein coupling in terms of the type of G protein α subunit (Gα) with which the receptor may associate. For example, the loss of all palmitoylation sites (Cys402, Cys403 and Cys405) from the endothelin receptor subtype B abolishes receptor coupling with $G\alpha_q$ and $G\alpha_i$. When Cys402 alone is restored, the receptor can couple with $G\alpha_q$ but not $G\alpha_i$, the latter clearly requiring the more palmitoylation sites available to interact accurately with the receptor [53]. The reason for the uncoupling of some GPCRs and the loss of downstream signaling could be due to enhanced receptor phosphorylation in the absence of palmitoylation. Indeed, the phosphorylated amino acids of GPCRs are in close vicinity to the sites of palmitoylation. In this context, the unstimulated β_2-adrenergic receptor mutant is already hyperphosphorylated and even in the presence of its agonist isoproterenol, it does not undergo further phosphorylation [54]. Phosphorylation is the first step of receptor desensitization and it initiates receptor uncoupling from the interacting G protein. Consequently, it has been recently shown in transgenic knock-in mice expressing a palmitoylation-deficient rhodopsin that this mutation facilitated receptor phosphorylation leading to an increased shutoff of phototransduction upon stimulation [55]. Thus, receptor palmitoylation is believed to regulate the access to phosphorylation sites in the receptor, thereby inhibiting rapid desensitization. Phosphorylation of GPCRs usually initiates their internalization, an event that terminates in protein degradation (down-regulation) or receptor recycling to the cell surface (resensitization). Accordingly, phosphorylation, desensitization and internalization were enhanced in a palmitoylation-deficient mutant of the 5-HT_{4a} hydroxytryptamine and the luteinizing hormone receptor, further supporting the relevance of receptor palmitoylation as a regulator of GPCR-mediated signal transduction [56,57].

Nevertheless, great care must be taken when interpreting results from experiments carried out with proteins containing mutated cysteine residues. The free sulfhydryl (-SH) group of the specific unpalmitoylated cysteine residue may also be responsible for other important interactions apart from possible modification by lipid attachment. Free sulfhydryl groups can form hydrogen or disulfide bonds that may be vital to maintain specific protein configurations in the absence of the palmitate residue. The mutation of the cysteine in these motifs would also affect these structural considerations. In this context, the drastically different half-lives of the wild-type and cysteine-mutated human A1 adenosine receptor [58] may be attributed either to the loss of palmitoylation as such or to the enhanced degradation due to the misfolded tertiary protein structure of the unpalmitoylated protein.

3. Lipid modifications of heterotrimeric G proteins

Heterotrimeric G proteins (αβγ), like many signaling proteins, bind tightly, and often reversibly, to cellular membranes. Covalent lipid modifications mediate membrane binding of these peripheral membrane proteins by serving as

hydrophobic anchors. G protein α subunits (Gα) are modified at their N-termini by the fatty acids myristate and/or palmitate, while γ subunits of βγ dimers (Gβγ) are modified by farnesyl or geranylgeranyl isoprenoids at their C-termini (Table 2).

3.1. Fatty acylation of Gα subunits

Myristoylation occurs exclusively on Gα of the α_i family [59,60]. As is the case with all myristoylated proteins, an N-myristoyltransferase catalyzes the amide bond attachment of the 14-carbon saturated fatty acid myristate to the extreme N-terminus of a protein [61]. This modification occurs co-translationally and requires a glycine at the extreme N-terminus of a substrate protein; thus, a prerequisite is removal of the initiating methionine by a methionyl amino peptidase. Not all extreme N-terminal glycines, however, are myristoylated, since the N-myristoyltransferase displays a strong preference for specific amino acids following the glycine. For example, α_s is not myristoylated even though it possesses a glycine at position 2. Most evidence indicates that myristoylation is an irreversible modification. Interestingly, α_t, also known as transducin, is found as a mixture in which the protein is modified by myristate or one of three less hydrophobic fatty acids; however, this diversity of modification at the N-terminal glycine appears to be retina specific [62,63].

All mammalian Gα, with the exception of α_t and α_{gust} undergo palmitoylation [59,60]. The 16-carbon palmitate is linked post-translationally via a thioester bond to one or more cysteines within the first 20 N-terminal amino acids of Gα. α_i Family members thus contain a tandem myristoylation plus palmitoylation motif, while other Gα are only palmitoylated. No clear recognition motif has been identified for palmitoylation, although the presence of an initial membrane targeting signal, for example myristoylation in the case of α_i or interaction with Gβγ in the case of non-myristoylated Gα, often serves as a prerequisite. Although a palmitoyltransferase has not yet been clearly identified for Gα, bona fide palmitoyltransferases have very recently been identified for several proteins, including yeast and mammalian Ras [64]. These identified palmitoyltransferases are members of a large family of proteins, and thus it appears likely that a relevant palmitoyltransferase will soon be identified for Gα.

Table 2
Lipid modifications of heterotrimeric G proteins

Gα	Lipid modification	Selected sites of modification [a]
α_s, α_{olf}	Palmitoylation and N-palmitoylation [b]	α_s M**G****C**LGNSKT-
α_q, α_{11}, α_{14}, α_{16}	Palmitoylation (2 or more sites)	α_q MTLESIMA**CC**LSEEA-α_{16} MARSLRWR**CC**PW**C**L- [c]
α_{i1}, α_{i2}, α_{i3}, α_o, α_z	Myristoylation and palmitoylation	α_{i1} M**G****C**TLSAED-
α_{t1}, α_{t2}, α_{gust}	Myristoylation	α_{i1} M**G**AGASAEE-
α_{12}, α_{13}	Palmitoylation (α_{12}=1 site; α_{13}=2 sites)	α_{12} MSGVVRTLSR**C**LLPAE- α_{13} MADFLPSRSVLSV**C**FPG**C**V-
Gγ	**Lipid modification [d]**	**Selected sites of modification [e]**
γ_1, γ_9, γ_{11}	Farnesylation	γ_1-KNPFKELKGG**C**VIS
γ_2, γ_3, γ_4, γ_5, γ_7, γ_8, γ_{10}, γ_{12}, γ_{13}	Geranylgeranylation	γ_2-ENPFREKKFF**C**AIL

[a] Shown are selected extreme N-termini of Gα. Underlined glycine residues represent sites for N-myristoylation or N-palmitoylation (for α_s) after removal of the initiating methionine. Bold cysteine residues represent sites for thioester-linked palmitoylation, as identified by mutagenesis and palmitate labeling studies.

[b] N-terminal palmitoylation has not been tested for α_{olf}.

[c] Bold cysteine residues represent probable sites for palmitoylation of α_{16}; however, mutagenesis of these cysteines has not yet been reported.

[d] Although all mammalian Gγ are modified by a single isoprenyl group, the *S. cerevisiae* Gγ, Ste18, is modified by both farnesylation and palmitoylation at the C-terminus.

[e] Shown are selected extreme C-termini of Gγ. Bold cysteine residues represent sites for isoprenylation. Underlined residues indicate the three amino acids removed by a CaaX protease.

Although palmitoylation generally occurs on cysteine residues, a recent report demonstrated that a purified preparation of α_s contained palmitate covalently attached to the N-terminal glycine through an amide bond [65]. This raises the intriguing possibility that α_s, at least under certain conditions, might be modified by dual palmitoylation, one through an amide linkage on glycine 2 and one through a thioester linkage on cysteine 3. This would be somewhat analogous to the dual myristoylation and palmitoylation of the α_i family of subunits. However, no enzyme activity responsible for such amide linked palmitoylation has been identified, and the functional significance of N-terminal palmitoylation of α_s has not yet been addressed.

One particularly important aspect of palmitoylation is that it is a reversible modification, and indeed regulation of palmitoylation has been demonstrated for Gα [66]. G-protein-coupled receptor (GPCR)-mediated activation of Gα causes an increased rate of turnover of the attached palmitate, as measured through pulse-chase labeling studies using radiolabeled palmitate. This has been most clearly demonstrated for α_s, but activation-induced palmitate turnover has also been shown for several other Gα, suggesting that regulation of the state of palmitoylation of Gα by GPCR-mediated activation is a general phenomenon. For α_s, it has been demonstrated that activation causes a reversible translocation from the plasma membrane into the cell's cytoplasm [67–70]. This correlation between depalmitoylation and redistribution of α_s suggests a causative relationship, although this mechanism still remains to be confirmed. Changes in palmitoylation can clearly affect the subcellular localization and trafficking of α_s, or other Gα, but understanding the functional significance of such regulated changes in localization is a current challenge.

3.2. Isoprenylation of Gγ subunits

All 12 mammalian G protein γ subunits are isoprenylated [71], meaning thioether attachment of either the 20 carbon geranylgeranyl or 15 carbon farnesyl moiety to a C-terminal cysteine in a CaaX motif, in which the cysteine is followed by two aliphatic amino acids and the X amino acid that specifies recognition by either well-characterized geranylgeranyl or farnesyl transferases. Most γ subunits are geranylgeranylated, but γ1 (retina specific), γ9, and γ11 are modified by farnesylation. After irreversible isoprenylation, most

isoprenylated proteins undergo the additional obligate modifications of proteolysis of the C-terminal three -aaX residues and then carboxy methylation of the new isoprenylcysteine C-terminus [72]. Though recognized for many years, the role of these two subsequent modifications is surprisingly still not clear; however, carboxy methylation does appear to increase membrane binding of farnesylated proteins, most likely by a general increase in hydrophobicity. On the other hand, the greater hydrophobicity of the geranylgeranyl lipid compared to farnesyl makes it a strong membrane anchor that does not seem to require methylation to increase strength of membrane binding.

3.3. Role of lipid modifications in G protein membrane binding, trafficking and signaling

It is clear that a major role for lipid modifications of G proteins is to serve as hydrophobic membrane anchors for attachment of the proteins to the cytoplasmic face of cellular membranes, presumably by direct insertion of the attached lipid into a lipid bilayer. Consistent with this, a number of studies have shown that mutation of the lipid acceptor amino acid (e.g., Gly to Ala for myristoylated Gα, or Cys to Ser for palmitoylated Gα or isoprenylated Gγ) to prevent modification causes the G protein subunit to redistribute from being tightly attached to cellular membranes to being cytoplasmic/soluble, as measured by cell fractionation or immunofluorescence microscopy. Moreover, lipid modification of G proteins is essential for them to carry out their signaling functions. The simplest explanation is that G proteins must be stably anchored at plasma membranes to transmit signals, and thus G proteins that are not lipid modified show signaling defects simply because they cannot get to the proper membrane location. However, additional roles for the various lipid modifications are possible, and recent studies have suggested that myristoylation is critical for maintaining N-terminal structure of α_t [73] and that the isoprenyl group of Gγ can bind to a hydrophobic pocket in Gβ under certain circumstances [74].

Not only do lipid modifications of G proteins allow tight membrane binding of G proteins, they play a critical role in trafficking of G proteins to the plasma membrane. Recent studies on heterotrimeric G proteins [75–77] combined with a number of groundbreaking studies on lipid-modified small GTPases of the Ras family [78] have led to a working model for how G proteins transit to the plasma membrane after synthesis. After isoprenylation of the Gγ subunit of the Gβγ dimer by a cytosolic farnesyl or geranylgeranyl transferase, Gβγ localizes to the cytoplasmic face of the endoplasmic reticulum (ER) where the subsequent processing enzymes, Rce (the -aaX protease) and Icmt (isoprenyl carboxy methyltransferase) are located. Isoprenylated proteins appear to require a second membrane targeting signal, such as palmitoylation, to proceed from the ER to plasma membranes, but Gβγ contains no obvious membrane targeting signals other than isoprenylation. Thus, interaction of Gβγ with a Gα subunit to form a heterotrimer before reaching the plasma membrane would provide palmitoylation (and myristoylation in the case of α_i subunits) *in trans* as an additional membrane targeting signal. Consistent with this proposal of heterotrimer formation before reaching the plasma membrane, mutational studies have demonstrated a reciprocal requirement for Gα and Gβγ interacting with each other for proper plasma membrane localization [76,79,80]. The exact intracellular localization where Gα and Gβγ interact is not clear; one study suggested that α_i and βγ first interact at the Golgi and proposed that this is also where palmitoylation would occur [75]. However, other studies have shown that disruption of the Golgi by treatment with brefeldin A did not affect palmitoylation or plasma membrane localization of several Gα [77,81,82]. Golgi-independent transport thus suggests that Gα and Gβγ may interact at ER membranes. Nonetheless, the location(s) at which Gα undergo post-translational palmitoylation remains undefined. The exact pathway the G proteins take when trafficking from the ER to the plasma membrane also remains unresolved.

4. Membrane lipids and their relevance for GPCR-related signaling

If the only purpose of membranes were to form a molecular barrier and a support for proteins, then two simple lipids such as phosphatidylcholine (PC) and cholesterol would be sufficient to produce a selective and consistent lipid bilayer. However, this rather passive view of the membrane has had to be revised owing to recent discoveries showing that membrane lipids are involved in crucial cell functions. This more active role justifies the presence of the multitude of different lipid classes and species found in biological membranes.

4.1. The modern membrane model

The diverse lipid species that can be encountered in the different cell membranes and the precise regulation of their composition reflects their significant role in cell physiology. For example, the numerous organelles within a cell have discrete membranes, each with a unique lipid composition and some containing specific lipid species [83,84]. Indeed, even within an single membrane, the exoplasmic and cytoplasmic leaflets can display quite different lipid compositions [85–87]. Consequently, membranes are far more organized than was initially believed. Clearly, the image of the membrane as a simple accumulation of bipolar lipid molecules in a liquid crystalline state with total lateral and rotational freedom, and with a random distribution of its molecular components is now outdated. Over and above the heterogeneity in the membrane lipid composition already illustrated, the existence of microdomains adds a further complexity to these structures. Certain areas within a given membrane (e.g., lipid rafts, pre- and postsynaptical membrane areas, basal, apical and lateral regions of epithelial cells, etc.) have a defined lipid composition that diverges significantly from other regions of the same cell membrane. These domains coexist in a single membrane without intermixing their lipid components and as such, they maintain their own special biophysical properties and are enriched in certain membrane proteins [88]. The resulting mosaic structure of the membrane may be stable over long

periods, as in the case of apical, lateral and basal endothelial membranes. Alternatively, they may be more dynamic and volatile as is the case of lipid rafts [89]. Thus, it is clear that such precisely regulated membrane lipid distribution contradicts the classical view of the membrane as a mere support for proteins. As a result, a more complex membrane model has been proposed to encompass these findings, namely the dynamically structured mosaic model, which highlights the non-random distribution of specific membrane lipids and proteins [90].

4.2. Membrane structures

Cellular lipids usually assemble in the form of lipid bilayers, also known as lamellar lipid phases, and to date several subtypes of lamellar structures have been described. The most abundant of these structures is the Lα or liquid crystalline phase, characterized by the high mobility of the fatty acid moieties in the central hydrophobic region of the bilayer. Indeed, this organization essentially represents the fluid membrane state that Singer and Nicolson described in the early seventies [91]. However, under different conditions lamellar bilayers organize into more ordered states, such as the Lβ (gel), Lc (pseudo-crystalline) or Pβ' (rippled) membrane state [92]. A special subtype of the lamellar membrane phase, the l_o (liquid-ordered) phase, has been examined intensely over the last decade since it existence was first predicted [93]. Today it is generally accepted that this phase represents a real plasma membrane microdomain known as the lipid raft, which fulfils an important role in signal transduction [88]. The l_o phase is characterized by the tight packing of the extended acyl chains of lipids, as in the gel phase, while it still maintains a certain degree of lateral mobility [94]. In contrast, membranes with high levels of non-lamellar prone lipids have different physical properties. In these non-lamellar membranes the acyl chains are more loosely packed and the lipids only apply weak lateral pressure on the polar regions of the membrane, the reason why they are also referred to as frustrated (Lε) membranes. This low lateral surface pressure can lead to a splayed configuration of some phospholipids, whereby one of the phospholipid acyl chains extends out of the bilayer while the other remains within the membrane [95]. In vitro, lipid curvature stress dominates the organization of lipids into hexagonal or inverted hexagonal phases (H_I or H_{II}, respectively), cubic phases (discontinous, or micellar, and bicontinuous) and rhombohedral phases [92]. The presence of real non-lamellar structures in vivo is not very frequent and they are typically restricted to membrane conditions with extreme curvature like vesicle fission during endo- and exocytosis [96] or cell division [97]. However, the membrane propensity to adopt these structures is also associated with important cellular functions, especially G-protein-coupled signaling.

4.3. Lipid–protein interactions between membrane lipids, GPCRs and G proteins

We must also bear in mind that the post- or co-translational lipid modifications of GPCRs and G proteins described above only constitute one side of the story. In this case, the nature of the lipid moiety attached dictates the specific requirements of the optimal membrane lipid environment necessary for the correct localization and function of these signaling proteins. It is obvious that the association of both GPCRs and G proteins to the plasma membrane makes them susceptible to their lipid environment, such that lipid–protein interactions may be critical to protein function. However in turn, these proteins are also capable of modulating lipid structure and the organization of the membrane with which they interact.

4.3.1. Membrane fluidity

Membrane fluidity, or the reciprocal value microviscosity, was one of the first properties of membranes shown to influence the activity of important proteins [98]. Since then, many studies have set out to elucidate the relationship between GPCR function and the biophysical properties of membranes in terms of bilayer viscosity. Membrane fluidity depends on the lipid composition of the membrane, especially on the type of fatty acid moieties in the membrane phospholipids and the amount of cholesterol. As a rule of thumb, a high degree of unsaturation in the phospholipid fatty acids increases the fluidity of the membrane [99]. For this reason, alterations in lipid composition provoked by metabolic disorders or nutritional interventions can affect membrane fluidity [100,101]. Indeed, diseases in which GPCR-mediated signaling plays an important role, such as hypertension and Alzheimer disease, have been associated with altered membrane fluidity [102–105]. Decreased membrane fluidity was detected in different cell types of spontaneously hypertensive rats [106] and hypertensive patients [107]. These changes not only restricted rotational mobility, but they also affected lateral diffusion in the membrane of genetically hypertensive animals [108]. Moreover, Gurdal and coworkers found a correlation between low membrane fluidity and impaired coupling of α_s with the β_2-adrenergic receptor, which mediates vasorelaxation and as a result reduction of blood pressure, in aortas of rats [109]. In contrast, another study carried out in crude membrane preparations from rat lung showed the opposite relationship, concluding that the age-related alteration in coupling between the receptor and the G protein is difficult to explain by alterations in membrane fluidity [110]. However, membrane fluidity is a general biophysical parameter that only broadly characterizes membrane properties. It does not distinguish between the properties of distinct membrane areas and as such it is fundamentally based on the traditional Singer-Nicolson model that envisages the plasma membrane as a unique, uniform structure. Therefore, perhaps the measurement of membrane fluidity has lost some of its scientific interest in recent years, although alterations of this parameter obviously indicate (albeit not in detail) changes in the status of membrane lipids.

4.3.2. Interaction of membrane lipids with GPCRs and G proteins

The binding of an agonist activates GPCRs by inducing a conformational change in protein structure that leads to the activation of heterotrimeric G proteins. Subsequently, the Gα-subunit of the heterotrimeric complex exchanges bound GDP

for GTP and it dissociates from the Gβγ dimer, enabling both molecular entities to regulate the activity of their specific effectors. A general feature of this receptor-mediated signaling pathway is that it operates as a signal amplification cascade, the first amplification step occurring at the plasma membrane where a signal propagated from a GPCR activates numerous G proteins. Hence, it would be advantageous if a large number of inactive heterotrimeric G protein molecules were readily available in the proximity of the receptor. Indeed, the results of an early study of signal transduction kinetics in human platelet membranes suggested that about one third of the α_2-adrenergic receptors seem to be coupled to α_i prior to agonist binding [111]. However, while the protein–lipid interactions between G proteins and lamellar membranes have been comprehensively studied [112], much less attention has been paid to the potential interactions with non-lamellar lipid phases. Non-lamellar prone lipids are able to modulate the physical properties of biomembranes by inducing alterations in the intrinsic curvature of the monolayer, in the lateral surface pressure, and in the hydration of membranes [113]. Cell membranes are usually rich in the non-lamellar prone, inverted hexagonal (H_{II}) phase inducing lipid phosphatidylethanolamine (PE), which is mainly distributed on the inner side of the plasma membrane [114] (i.e., the normal site for G protein binding). The relative amount of PE in membranes under physiological conditions is sufficient to provoke the formation of regions with a negative curvature strain in vivo, which might be stabilized or induced by the presence of particular membrane proteins [115]. Indeed, it has recently been shown that the greater the hexagonal-phase propensity, the higher the affinity of heterotrimeric α_i proteins for the membrane [116]. Therefore, PE-rich domains or regions with negative curvature strain could act as a membrane platform for these peripheral proteins, either as a stable microdomain or as a dynamic transitory arrangement. This concept is supported by the loss of G proteins from brain plasma membranes observed after disruption of non-lamellar H_{II} structures by daunomycin [117].

Interestingly, GPCRs seem to display similar preferences to heterotrimeric G proteins. It was proposed that lipids with a negative spontaneous curvature favor elongation of the G-protein-coupled photoreceptor rhodopsin during the activation process. This was explained by the fact that they facilitated the conformational change of rhodopsin to its activated state in model membranes [118]. Moreover, measurements of the interaction of this receptor and its corresponding G protein transducin (α_t) in lipid bilayers demonstrated that PE markedly increased receptor affinity for α_t upon light activation, while the affinity of α_t for dark-adapted rhodopsin remained unchanged [119]. In contrast, in pure lamellar PC bilayers the affinity of α_t for light-activated rhodopsin was substantially lower. The polyunsaturated docosahexaenoic acid (DHA) comprises about 50% of the lipid fraction of retinal rod outer segment membranes where rhodopsin is normally found, and this lipid displays a strong tendency to induce curved structures [120]. Nevertheless, the observation that rhodopsin activation can also be supported by other non-lamellar lipids, such as the hexagonal-prone PE, indicates that it is more likely the physical curvature stress induced by these lipids than a direct lipid-specific reaction between DHA and rhodopsin, which facilitates the conformational change of this GPCR. It is noteworthy that peptides with α-helical structures, such as the transmembrane regions of GPCRs, promote the formation of hexagonal phases under certain conditions in vitro [121]. In fact, a pronounced restructuring of membrane lipids during photoactivation of rhodopsin occurs, which provides strong evidence for the dynamic interaction of this GPCR with the lipid phase of the disc membranes [122]. It may be possible that upon agonist activation the GPCRs themselves initiate the assembly of microdomains with specific hexagonal-prone biophysical characteristics around them and that the presence of non-lamellar-prone membrane lipids is substantial for this conversion of the receptor environment. Thus, hexagonal phase-forming membrane lipids seem to fulfill a dual role in signal transduction, by inducing the co-localization of the first signaling components and by participating in GPCR activation.

In contrast to G protein heterotrimers, the activated monomeric α_i-subunit showed a marked preference for pure lamellar structures [116]. This would provoke the rapid exit of activated α_i-monomers from the receptor environment where the hexagonal-phase propensity is increased. Mobilization of the Gα protein subunit away from the receptor environment may favor its interaction with effector proteins located in other membrane domains (e.g., adenylyl cyclase). In fact, both αi and αs have been found in lipid rafts [123]. Another study confirmed that some G protein subunits did indeed co-localize with adenylyl cyclase in l_o membrane structures, in this case in caveolae. However, most G proteins resided in irregular structures of the plasma membrane, which could not be morphologically identified [124]. When considered in conjunction with the finding that α_t translocates to lipid rafts only upon activation [125], it seems obvious that the opposite binding preferences of activated Gα subunits indeed target them away from the receptor surroundings to membrane sites where they may efficiently interact with their effectors. This leads to a model where two spatially separated membrane areas have contrasting biophysical properties, one harboring the pre-active G heterotrimer and the other, the activated Gα monomer. This idea is further strengthened when considering that a considerable number of signal transduction proteins are located in specialized membrane sites, namely lipid rafts [126,127]. Thus, lipid rafts appear to represent platforms whose specific biophysical properties enhance the effectiveness of the second step of the signaling cascade, trapping activated Gα subunits and augmenting the spatial proximity between them and their effectors.

On the other hand, dissociated Gβγ dimers still maintain a high affinity for membranes with a hexagonal propensity [116], which may also influence their distribution in native membranes. The general observation that prenylated proteins are normally not localized in lipid rafts [128] was also corroborated for Gβγ subunits, which were found to be excluded from synthetic lipid rafts [129]. This indicates that the Gβγ dimer most probably determines the lipid–protein interaction characteristics of the heterotrimeric G proteins. As such, the Gβγ dimer defines the preference of complete Gαβγ heterotrimers

for the hexagonal-phase, thereby masking the lamellar membrane affinity of the Gα-subunit. As a result, one of the functions of the Gβγ dimer could be to shuttle the lamellar membrane phase preferring Gα subunit to the vicinity of the receptor, making it available for instant activation.

In this context, an interesting property has recently been associated with the post-translational modification of Gγ subunits with the isoprenyl moieties farnesol and geranylgeraniol [130]. In model membranes containing PE, these isoprenoids segregate in a multidomain system formed by a lamellar crystalline (Lc) phase and isoprenoid aggregates. In addition, they promoted a temperature-dependent growth of the hexagonal phase that was able to coexist within the lamellar Lc phase. Although the farnesol and geranylgeraniol used in this study were free isoprenoids, a similar behavior was defined in experiments with these isoprenyl moieties bound to the carboxy terminal peptide of the $G\gamma_2$ subunit (manuscript in preparation, P. Escribá). Consequently, it is possible that in vivo the Gβγ dimer not only displays a preference for areas rich in non-lamellar lipids, but that it is also actively involved in the formation or maintenance of hexagonal-prone microdomains with spontaneous negative curvature.

It is tempting to speculate that the preference of the Gβγ dimer for hexagonal (H_{II}) phases could well be important for GPCR desensitization following agonist-mediated activation. Dissociated Gβγ dimers can bind to G-protein-coupled receptor kinases (GRKs), which phosphorylate and inactivate GPCRs. The affinity of the Gβγ dimer for non-lamellar membrane regions where the membrane receptors would also be located could direct these kinases to their target, provoking the termination of the signaling process initiated by binding of the agonist to the receptor. This hypothesis is strongly supported by the involvement of membrane lipids other than PC in the regulation of GPCR phosphorylation and desensitization. Indeed, with the exception of PC, membrane lipids can actually regulate GRK activity [131]. Notably, the retinal GRK1 has a farnesyl moiety covalently attached to its carboxy terminal domain, similar to the Gβγ dimer. Moreover, the presence of this isoprenoid group is essential for light-dependent membrane association of GRK1 [132]. A mutant unfarnesylated form of the kinase remained in the soluble fraction following light exposure and displayed a reduced capacity to phosphorylate rhodopsin. In contrast, a mutant kinase bearing a more hydrophobic geranylgeranyl (C20) isoprenoid moiety was constitutively associated with the membrane although it phosphorylated rhodopsin at a rate comparable to wild-type (farnesylated) GRK1 [132]. Thus, the specific modification found in vivo (farnesylation) ensures that membrane association of GRK1 only occurs in the presence of its activated receptor substrate. However, GRK 4 and 6 are palmitoylated rather than farnesylated [10,133], making it possible that these kinases are regulated distinctly by different membrane lipids.

4.3.3. Interaction of membrane lipids with phosphoinositide-specific phospholipases C

Phospholipases C (PLC) is an enzyme superfamily that is widely distributed in bacteria and eukaryotes. These enzymes catalyze the cleavage of the P–O bond of a phosphoglyceride to yield diacylglycerol (DAG) and a water-soluble phosphoryl derivative (e.g., phosphorylcholine when the parent phosphoglyceride is phosphatidylcholine). Phosphoinositide-specific phospholipase C (PI-PLC) is particularly important because both the end-products produced are potent metabolic signals, phosphoinositols and DAG. PI-PLCs have received special attention in eukaryotes because of their implication in signal transduction. However, several members of the *Bacillus* and *Listeria* genera contain PI-PLC that is structurally simpler while maintaining the activity of their mammalian counterparts. Hence, a number of relevant studies have been carried out on these bacterial isozymes. In all cases, the substrates of these enzymes comprise a part of the lipid bilayer and thus, PLCs must interact with the membrane even though such interactions are mostly transient. PLCs usually belong to the group of the so-called "non-permanent membrane proteins" [134], and they pertain to the subgroup of proteins that interact reversibly with the membrane and cause the covalent modification of the lipids.

4.3.3.1. Studies with Bacillus PI-PLC. An important breakthrough in the field of bacterial PLCs was the elucidation of the crystal structure and the proposal of a mode of action for the *Bacillus* enzyme. The crystal structure of the PI-specific PLC from *B. cereus* in a complex with *myo*-inositol was resolved at a resolution of 2.3 Å by Heinz et al. [135]. The structure consists of an imperfect $(\beta\ \alpha)_8$-barrel similar to that first observed for the triose phosphate isomerase. *Myo*-inositol, a substrate-like inhibitor, interacts with His 32 and His 82, two highly conserved residues in bacterial and eukaryotic PLCs. The involvement of these residues suggested their role in a general acid–base catalytic mechanism of the kind observed for ribonucleases, a hypothesis that received experimental support [136–138]. From these studies, a mechanism emerged by which PI-PLC could catalyze the cleavage of the P–O bond in PI through intramolecular nucleophilic attack of the axial 2-OH group of inositol on the P atom. The catalytic site of PI-PLC would accordingly consist of three main components: His 32 general base; His 82 general acid; and Arg 69 acting as a phosphate-activating residue (Fig. 1).

PI-PLC binding to lipid bilayers has been widely studied in various laboratories. Studies of the interfacial binding of PI-PLC from *B. thuringiensis* (whose sequence is very similar to that of the homologous *B. cereus* enzyme) led to the proposal of a two-stage binding model [139–141]. According to this model, the protein would first bind to the bilayer surface through electrostatic forces and it would only then form a tight membrane protein complex stabilized by hydrophobic forces. In this context, it is relevant that PI-PLC contains two Trp residues (Trp 47 and Trp 242 for the *B. thurigiensis* enzyme) that are common to other membrane proteins and that play a role in lipid binding [142]. A correlation between PI-PLC binding to the phospholipid–water interface, as well as changes in Trp fluorescence emission and catalytic activity have also been observed [143].

One interesting feature of *Bacillus* PI-PLCs is their sensitivity to lipids other than their substrate, particularly PC. This phospholipid augments the affinity of PI-PLC for its

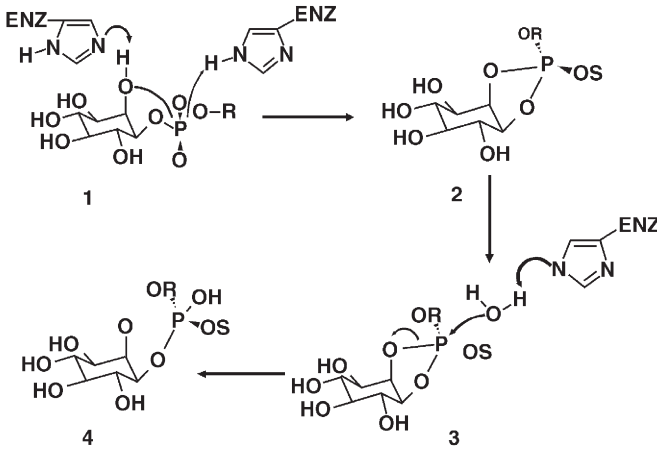

Fig. 1. Reaction mechanism of PI-PLC involving the general acid-general base histidine residues that must be precisely positioned for efficient catalysis. A Ca^{2+} ion (not shown) is involved in stabilizing the phosphonate formation by interacting with the oxygen molecules (O_R and O_S) during formation of the intermediate (2). The reaction is completed by hydrolysis (3) and finally the product is formed (4). [Reprinted with permission from *Biochemistry* 2005, 44, 9980–9. Copyright 2005 American Chemical Society].

substrate following a pattern of allosteric activation, in accordance with which PI-PLC would specifically bind to PC [144–146]. In addition to the catalytic site and the allosteric binding site(s), it is proposed that the enzyme would possess an interface binding surface (or i-face), similar to other interface-acting enzymes. It has been proposed that the interface binding event would be different from the catalytic event [147], although the two may be allosterically coupled. The effect of a variety of non-substrate lipids on the PI-PLC activity has been examined, including that of PC, PE, sphingomyelin, galactosylceramide and cholesterol [148]. Simultaneous measurements of enzyme activity, interfacial enzyme binding, and the fluorescence of different probes, reveal that both enzyme binding and activity diminish with increasing lipid order (decreasing fluidity). These results support a "two-stage model" for binding of PI-PLC to lipid bilayers, and underline the significance of the partial penetration of the enzyme into the membrane's hydrophobic matrix for its catalytic activity.

The catalytic activity of phosphatidylcholine-preferring PLC (PC-PLC) from *B. cereus* induced the aggregation and fusion of large unilamellar vesicles containing PC, PE, and cholesterol [149]. Subsequently, a similar phenomenon induced by *B. cereus* PI-PLC was described in vesicles containing PI, neutral phospholipids, and cholesterol [150]. In both cases, the enzyme end-product diacylglycerol is the essential fusogenic agent. Indeed, vesicle–vesicle fusion was then shown to be limited by the frequency of contact between two diacylglycerol-rich domains [151] (a review on PLC-induced membrane fusion can be found in Goñi and Alonso [152]). Various lines of evidence suggest that PLC-induced fusion may be relevant to the *in vivo* situation. Phospholipases C and diacylglycerol are involved in exocytosis [153,154] and in the acrosome reaction of spermatozoa [155–158]. Diacylglycerol and its formation by phospholipase C also appear to regulate Rab- and SNARE-dependent yeast vacuole fusion [159]. Moreover, if we interpret budding and fission as processes that are the mirror image of fusion, the constant pool of diacylglycerol required by Golgi membranes for the generation of secretory vesicles becomes more noteworthy ([160]; for a review see [161]).

4.3.3.2. Studies with eukaryotic PI-PLCs. By far the most detailed studies on eukaryotic PI-PLCs have been carried out in mammals and thus, with a few exceptions mentioned below, this section will deal essentially with these mammalian PI-PLCs. There are about a dozen known mammalian PI-PLC isoforms that are divided into six groups, namely β, γ, δ, ε, ζ, and η. The PLCβ isoforms are the major PLC isoforms involved in GPCR-mediated calcium signaling, which can also control the cell cycle, while the PLCγ isoforms are the PLC isoforms coupled to tyrosine kinase receptors, such as the EGF and PDGF receptors, and receptors associated with tyrosine kinases, such as the T cell and B cell receptors [162,163]. PLCδ is a potential amplifier of Ca^{++} signals [164]. What is known of the more recently discovered groups, PLCε is an effector of Ras and other GTP-binding proteins [165], PLCζ is extremely sensitive to intracellular Ca^{2+} concentrations [166], and PLCη is implicated in neuronal activity [167].

Virtually all mammalian PLCs contain: (i) the so-called X and Y domains, which form the catalytic core, (ii) an N-terminal Pleckstrin homology (PH) domain, (iii) four EF-hand domains; and (iv) a C-terminal C2 domain [167]. The catalytic core has a triose-phosphate isomerase-like barrel structure, the same structure that is found in the single-*Bacillus* PI-PLC domain [135]. One or more of these domains interact with the membrane lipid–water interface during catalysis. Like the *Bacillus* and other bacterial isoforms, mammalian PLCs cleave the P–O bond via trans-esterification, using the axial OH group of the inositol ring as a nucleophile. Accordingly, they generate a five-membered cyclic phosphate as an intermediate or sometimes as a final product (Fig. 1), and the different enzymes release distinct proportions of the cyclic and the linear phosphate products. The reaction mechanism requires a general acid–base system to facilitate proton transfer, and a positively charged species central to the acid–base pair to promote formation of the transition state phosphate species. The acid–base pair is provided by two essential His residues at the active site. The positively charged species is a Ca^{2+} ion in mammalian and in a few bacterial enzymes, while most other bacterial isoforms utilize a guanidinium group from a neighboring Arg residue [168–170].

The membrane interactions of mammalian PI-PLCs have been particularly well studied in the case of PLCδ$_1$, whose PH domain has been cloned and its membrane activity analyzed. PH domains are about 120 aa, and they are frequently found in proteins involved in cell signaling and cytoskeletal functions. Inositol lipids are important ligands of PH domains [171–174]. The PH domains from different proteins exhibit rather low sequence similarity, but their secondary and tertiary structural motifs are highly conserved. A high-resolution structural model of the rat PLCδ$_1$ PH domain forming a complex with Ins(1,4,5)P_3 has been determined by X-ray diffraction. In this model, the PH domain was proposed to form a seven-stranded β sandwich

formed by two orthogonal, antiparallel β-sheets and a C-terminal amphipathic α-helix [175]. At least in the case of PLC-δ_1, the interaction with PI-4,5-bisphosphate occurs mainly through the loops connecting β1/β2 and β3/β4. In turn, the β5/β6 loop includes a short amphipathic α-helix, the α2-helix, which is not found in other PH domains studied so far.

The conformational changes induced in the PLCδ_1 PH domain upon interaction with a lipid bilayer were first described using solid state NMR [176]. Upon interacting with a lipid bilayer composed of phosphatidylcholine and phosphatidylinositol-4,5-bisphosphate, a local conformational change occurred in the β5/β6 loop mentioned above. According to the NMR data, the short amphipathic α-helix in this loop interacts with the hydrophobic membrane matrix, thus contributing to the membrane binding affinity, as well as the interdomain and intermolecular interactions of PLCδ_1.

A further step in characterizing the interaction of the PH domain with membranes was made by using an isolated recombinant PH domain in studies with lipid monolayers and vesicles [177]. The PH domain from PLCδ_1 (PH-PLCδ_1) displays two modes of membrane binding: (i) a reversible one, that involves the specific recognition of the PI head group, and that does not include membrane penetration; and (ii) a long-lived one that involves membrane insertion promoted by phosphatidylethanolamine and cholesterol, that does not require occupation of the PI-binding site. The long-lived activity of PH-PLCδ_1 is not observed in other PH modules. It is unclear which parts of PH-PLCδ_1 are responsible for its membrane insertion activity although on the basis of prior studies [178], it was suggested that the major contribution to membrane activity comes from superficial hydrophobic residues in the C-terminal portion of PH [177]. Non-specific binding of PH domains to membranes has also been found in PLCβ-PH domains [179]. Molecular modeling studies of all mammalian PLC PH domains [180] point to striking differences in the electrostatic profiles of these PH domains when compared to non-PLC PH domains, as well as to the role of non-specific electrostatic interactions in the membrane localization of the PLCδ-, PLCγ-, and PLCβ-PH domains.

The PH domains of PLCγ target membranes containing PI-3,4,5-trisphosphate, in contrast to the affinity of the PLCδ-PH domain for PI-4,5-bisphosphate [181]. In addition, PLCγ can bind to lipid bilayers via a different mechanism, namely the formation of an intermolecular lipid-binding domain with a canonical transient receptor potential 3 (TRPC3) ion channel [163]. The latter are receptor-operated Ca^{2+} channels, such that formation of the intermolecular lipid-binding domain allows PLCγ to regulate Ca^{2+} entry in the cell. Interestingly, PLCγ has a split PH domain whose C-terminal half (cPH) could give rise to a functional PH-like intermolecular domain when it interacts with TRPC3. A non-canonical PH domain has been predicted within the N terminus of TRPC3 that corresponds to the actual PLCγ-cPH binding pocket [182]. Moreover, *in vitro* experiments show that TRPC3 binds to PI-4,5-bisphosphate only in the presence of PLCγ-cPH. However, it remains to be established whether the PLCγ-cPH/TRPC3 complex does indeed adopt the structure of an intermolecular PH domain, or whether it constitutes a different functionally analogous lipid-binding domain [183].

Although the PH domain appears to be very important for PLC–membrane interactions, other protein domains are also important. Indeed, the EF3 and EF4 motifs of the EF-hand domain are involved in enzyme binding to free fatty acids [184]. While these experiments were performed with the lipid-detergent form of the substrate in mixed micelles, it is likely that EF3 and EF4 are also involved in the enzyme interaction with the phospholipid fatty acyl chains in the bilayer environment. It is noteworthy that the newly described PLCη does not have a PH domain [167] and hence, studies of PLCη–membrane interactions should provide valuable information in this regard.

4.3.4. Interaction of membrane lipids with protein kinase C isoforms

Certain GPCRs use phospholipase C as an effector of signaling, this enzyme producing diacylglycerol (DAG) and inositol phosphate as second messengers. DAG induces activation of protein kinase C (PKC) through both specific lipid–protein interactions and through the induction of non-lamellar phases [185]. Therefore, PKC acts as third messenger in the GPCR cascade, propagating messages from the membrane to the cytosol or other cellular compartments. Non-lamellar phase (H_{II}) propensity has been shown to be involved in the translocation of PKC from the cytosol to membranes, a phenomenon associated with enzyme activation [117,186]. Therefore, membrane protein–lipid interactions regulate the first signaling steps initiated through GPCRs: receptors, G proteins, effectors/second messengers and some third messengers. Obviously, other processes involving the interaction of further messengers with internal membranes may also be regulated by these interactions.

PKC isozymes form a family of proteins involved in several signaling pathways [187]. The interaction of conventional PKCs (α, β and γ) with membranes is not only modulated by DAG and phorbol esters, but it is also under the influence of Ca^{2+} (another second messenger) and phosphatidylserine, one of the main lipids in the cytosolic leaflet of the plasma membrane. Although some studies suggest a specific interaction between PKC with phosphatidylserine [188], other studies indicate that other anionic phospholipids can substitute for this lipid [189]. In any case, PKC activation depends on the presence of a negative charge at the interface region of the membrane. In addition to DAG another non-lamellar prone phospholipid that is abundant in the inner leaflet of the cell membrane, phosphatidylethanolamine (PE), also facilitates the association of PKC with membranes [117,186]. The exact details of the interaction between PKC and membranes with hexagonal-prone lipids are still to be elucidated, but a growing body of evidence points to the possibility that the tendency of a negative curvature to provoke a splayed configuration of phospholipids (see Section 4.2) could be a key property. This may reflect the fact that some phospholipids may extend one of their phospholipid acyl chains out of the membrane when the lateral pressure is high, as in the case of membranes with strong hexagonal propensity, in order

to relieve some of the pressure [95]. Calcium ions are also required for PKC translocation and activation and they consume water at the membrane surface for their own hydration. This leads to the dehydration of the membrane surface which further augments the curvature stress [190] and as a consequence, the formation of a splayed phospholipid configuration. Nonetheless, from an energetic viewpoint a single hydrophobic acyl chain in an aqueous environment is not a very favorable arrangement. The high positive energy of such a position would be largely reduced if a protein could cover the extended acyl chain with a hydrophobic pocket. This would also provide an anchoring point for transiently bound membrane proteins. Indeed, protein kinase C contains such a hydrophobic pocket [191]. Accordingly, it is the non-lamellar phase propensity (or the membrane curvature strain) more than a specific interaction between PE and the enzyme that participates in the membrane recruitment of PKC, both in model and cell membranes. Finally, the number of double bonds of the acyl chains of membrane phospholipids (the degree of unsaturation) also markedly influences PKC activity [192]. However, PE, cholesterol and the size of the liposomes are also able to alter PKC activity. Therefore, it seems that the head group spacing of the membrane phospholipids, a parameter closely related to intrinsic bilayer curvature, mainly governs the ability of PKC to interact with the membrane rather than the lipid acyl chain order.

In general, the wide variety in the composition of cell membranes from the different cell types found in an organism suggests that PKC will display diverse interactions with the membrane (and other peripheral proteins). Moreover, the variety of domains and microdomains in cell membranes implies that lipids may also regulate its location and activity, independent of receptors for activated C kinase (RACK) proteins [186]. In this context, an increase of the non-lamellar phase propensity of membranes favors the binding of purified membrane proteins to model membranes in the absence of other external factors, demonstrating that the cellular localization of PKC is not only regulated by RACK proteins. In living cells, modest H_{II} inducers result in a mild increase in PKC activity, with a persistent increase of PKC expression [186]. In contrast, strong H_{II} inducers (e.g., phorbol esters) result in exaggerated PKC activation followed by enzyme depletion. Membrane lipids may be altered as a consequence of a pathological state [193], dietary fat intake [194], or physiological situations [195]. These include alterations in the membrane concentration of phospholipids classes, the degree of unsaturation of the fatty acyl chains, cholesterol levels, etc., and they have been shown to regulate PKC-membrane lipid interactions and thus, PKC activity [196]. In this context, it is conceivable that inducing changes in the membrane lipid composition through drug treatments aimed at regulating PKC or other membrane proteins may reverse a pathological state. This clinical approach has been termed "membrane lipid therapy" [193]. Indeed, minerval (2-hydroxy-9-*cis*-octadecenoic acid) regulates the membrane structure and the localization and activity of PKC, triggering a series of events associated with its antitumoral action.

5. Conclusion and future perspectives

In spite of the multitude of physical and biochemical influences constantly interfering with lipid bilayer integrity, membrane lipid composition of biomembranes remains amazingly stable. The answer to the question why their composition is so precisely regulated, is simple. Lipids are equally important for biological processes, because they are functionally relevant for the correct and efficient response of membrane proteins to extra- and intracellular stimuli. The fact, that a great part of GPCR-associated signaling takes part in or at the plasma membrane already suggests a great importance for lipid–protein interactions. This review shows the variety of possible interactions, starting with co- or post-translational lipid modifications of key signaling proteins, continuing with the effect of membrane lipid structure and bilayer organization on affinity and localization of signaling proteins, and ending with the direct interaction of specific membrane lipids and effector enzymes. Considering this and the fact that G-protein-coupled signaling proteins form the major target group for pharmaceutical drugs today, it becomes apparent that manipulation of membrane lipid composition might be similarly used in the treatment of diseases. In fact, the nutritional influence of special diets or their components (e.g., the Mediterranean diet or olive oil) showed beneficial effects on the onset or progression of a variety of illnesses while at the same time modifying plasma membrane lipid composition [197–200]. However, it remains to be seen whether the development of drugs aiming to alter lipid–protein interaction will become an equally valuable therapeutical strategy in the future as the classical approach directed against proteins.

Acknowledgments

Work in the laboratory of P.V.E. is supported by grants from the Spanish 'Ministerio de Educación y Tecnología' (SAF2004-05249 and SAF2003-00232). Work in the laboratory of P.B.W. is supported by NIH grants GM56444 and GM62884 and by an Established Investigator Award from the American Heart Association. Work in the laboratory of F.M.G. is supported by the 'Spanish Ministerio de Educación y Ciencia' (Grant No BFU 2004-02955) and by the University of the Basque Country (Grant No 9/UPV 00042.310-13552/2001). Work in the laboratory of O.V. is supported by a grant from the Spanish 'Ministerio de Sanidad y Consumo' (FIS PI051340). O.V. holds a contract from the "Ramón y Cajal" program.

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