

Membranes: a meeting point for lipids, proteins and therapies

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Received: November 24, 2007; Accepted: February 4, 2008

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Abstract

Membranes constitute a meeting point for lipids and proteins. Not only do they define the entity of cells and cytosolic organelles but they also display a wide variety of important functions previously ascribed to the activity of proteins alone. Indeed, lipids have commonly been considered a mere support for the transient or

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permanent association of membrane proteins, while acting as a selective cell/organelle barrier. However, mounting evidence demonstrates that lipids themselves regulate the location and activity of many membrane proteins, as well as defining membrane microdomains that serve as spatio-temporal platforms for interacting signalling proteins. Membrane lipids are crucial in the fission and fusion of lipid bilayers and they also act as sensors to control environmental or physiological conditions. Lipids and lipid structures participate directly as messengers or regulators of signal transduction. Moreover, their alteration has been associated with the development of numerous diseases. Proteins can interact with membranes through lipid co-/post-translational modifications, and electrostatic and hydrophobic interactions, van der Waals forces and hydrogen bonding are all involved in the associations among membrane proteins and lipids. The present study reviews these interactions from the molecular and biomedical point of view, and the effects of their modulation on the physiological activity of cells, the aetiology of human diseases and the design of clinical drugs. In fact, the influence of lipids on protein function is reflected in the possibility to use these molecular species as targets for therapies against cancer, obesity, neurodegenerative disorders, cardiovascular pathologies and other diseases, using a new approach called membrane-lipid therapy.

Keywords: lipid bilayer • lipid composition-structure • membrane lipid organization
• membrane ion channel • membrane receptor • GPCR • G protein • PKC • cell signalling
• heat-shock protein • transmembrane protein • peripheral protein • protein-lipid interactions

Introduction

Most cell functions occur in or around membranes. Membranes not only define the cell's boundary but they also create cytoplasmic compartments into which certain activities can be segregated or to make them more efficient. Membrane proteins have been attributed with the most important roles in membranes, although lipids have also been acknowledged as key elements in numerous processes. The present review shows how membrane lipids, and the structures they form, participate and regulate numerous important cellular activities.

Membrane-spanning (integral, intrinsic) proteins are permanently embedded in the lipid bilayer (Fig. 1). In many cases, the type of lipids that interact with amino acids in the hydrophobic environment of the membrane core and those at the interface are more or less defined, and to a certain extent regulated by the features of the protein. Transmembrane proteins also influence lipid structure in the membrane. Therefore, it is not surprising that changes in the lipid environment of membranes regulate or alter the function of intrinsic membrane proteins (see below). Indeed, in regions rich in a given type of receptor (e.g. synaptosomes, receptor clusters, etc.) these protein-lipid interactions play important roles in both directions.

On the other hand, peripheral (extrinsic) proteins also regulate and are regulated by membrane lipids. G proteins provide a good example of how proteins can affect membrane composition and structure [1]. Like integral proteins, peripheral proteins may regulate membrane composition and structure, and many of these proteins undergo co-/post-translational

modifications, that include the addition or removal of fatty acids or isoprenoid moieties. Recent studies show that these lipid modifications, and the surrounding amino acids are not only involved in the interaction with membranes but that they also regulate: (i) membrane lipid structure; (ii) the formation of lipid domains in membranes and (iii) clustering of G protein peptides [2, 3].

Membrane lipids participate in the interaction of proteins with the cell barrier [4, 5]. They also regulate the distribution and localization of peripheral proteins to membrane domains where they can interact with other signalling proteins [6]. In this context, heterotrimeric and dimeric G proteins prefer hexagonal (H_{II})-prone membranes, whereas monomeric $G_{\alpha i}$ proteins prefer lamellar regions of the membrane. Indeed, both peptide and lipid moieties of these proteins are involved in the reciprocal regulation of structural and functional aspects of the membrane [1, 6–9].

Membranes are formed by a matrix of lipids whose structure and composition is far from simple. First, the number of different lipid molecules found in the plasma membrane of a cell can exceed 1000. Second, the high number of structures formed by lipids *in vitro* indicates that the structural properties of membranes can vary greatly *in vivo*. Third, the interaction of lipid molecules to form membranes is not determined by covalent bonds, like that of amino acids in proteins or bases in nucleic acids. In turn, membrane lipids participate in dynamic interactions that facilitate changes in their relative position in membranes, membrane thickness, surface packing,

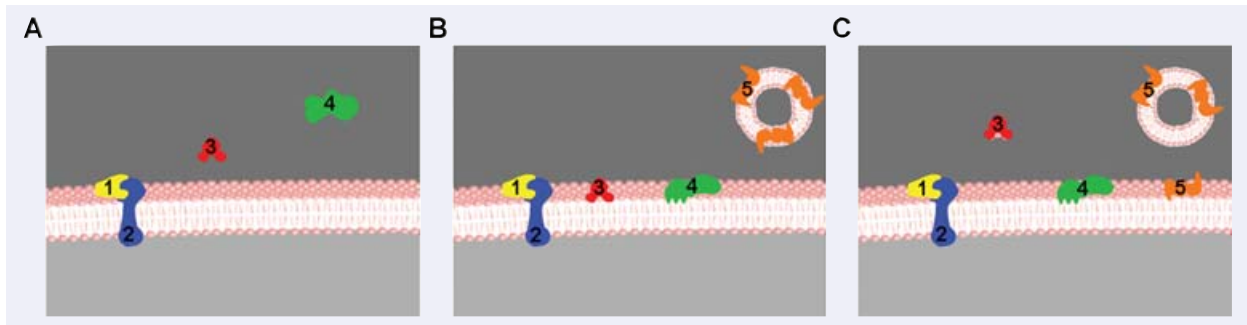


Fig. 1 A simplified drawing representing the various interactions of proteins with lipid bilayers as a function of time: 1, a peripheral or extrinsic protein; 2, an integral or intrinsic protein; 3, a non-permanent protein that interacts reversibly with the membrane, a lipid-transfer protein in this particular example; 4, a non-permanent protein that becomes irreversibly bound to the bilayer once it interacts with it; 5, a non-permanent protein reversibly bound to a secretion vesicle and then transferred to a target membrane. **A, B** and **C** correspond to consecutive stages in the interaction process.

lateral and rotational mobility and other properties that complicate the study of membrane structure. Fourth, in most membranes different regions and domains with defined lipid and protein compositions usually coexist and similar domains are not always entirely equal (*e.g.* two lipid rafts may differ in their size or transient nature, as well as in the proportions of lipids and proteins). Finally, membrane proteins and lipids may both be subjected to regulatory processes in response to pathophysiological situations or nutritional/pharmacological interventions, which in turn may alter the activity and functions of the membrane.

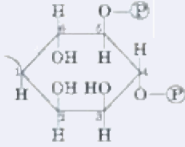
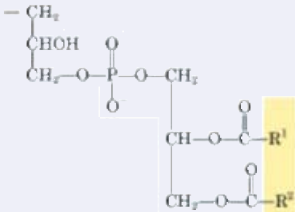
Membranes constitute a meeting point for lipids and proteins, both fulfilling prominent roles in certain cellular processes, equally relevant in many cases. Thus, a given organism may respond to environmental situations not only by regulating protein expression, but also by modulating membrane lipid levels. For example, there are dramatic changes in membrane lipid composition in the brains of fish living in rivers whose temperature varies from 20°C in the summer to 4°C in the winter [10]. If this were not the case, the physicochemical properties of their membranes would not be appropriate to allow cell signalling or to facilitate other important physiological functions. Indeed, all organisms are exposed to stressful conditions such as elevated temperatures and irradiation, as well as physiological stress such as rapid cellular proliferation, oxidative stress due to metabolic reactions, or pathophysiological stress due to infection and inflammation. If unmitigated, such stressful conditions can lead to membrane disintegration, protein misfolding and aggregation, cellular dysfunction and cell death. Significantly, recent stud-

ies strongly suggest that plasma membranes play a critical role in sensing and responding to most stress stimuli, particularly through the activation of specific signal transduction pathways that function in membrane and protein homeostasis [11, 12]. In addition, many diseases are associated with alterations in membrane lipid levels (see sections below). Therefore, therapeutic approaches based on their regulation appear to be useful novel clinical alternatives to other pharmacological strategies [9]. In addition, most drugs currently under development are targeted at G protein-coupled receptors (GPCRs), a ubiquitous family of membrane receptors that control a great number of cellular and physiological functions, whose activity is controlled by their lipid environment (see below). Therefore, membranes also constitute meeting points for therapies that, through regulation of membrane lipids and/or proteins, reverse cellular malfunctions. This fact further shows the relevance of membranes in the control of cell functions, their homeostasis, communication and responses to environmental and pathophysiological situations.

Membrane lipid composition

Biological membranes consist of a lipid bilayer to which proteins and carbohydrates may be associated or covalently linked. Recent advances have provided new perspectives from which the roles of membrane lipids in cells can be evaluated, having evolved from a simple physical barrier to a critical component in cell signalling and other cellular processes.

Table 1 Glycerophospholipid classification according to their polar headgroup

Glycerophospholipid	Headgroup	Formula of headgroup
Phosphatidic acid	-	-H
Phosphatidylethanolamine	Ethanolamine	-CH ₂ -CH ₂ -NH ₃ ⁺
Phosphatidylcholine	Choline	-CH ₂ -CH ₂ -N ⁺ (CH ₃) ₃
Phosphatidylserine	Serine	-CH ₂ -CH ₂ (COO) ⁻ -NH ₃ ⁺
Phosphatidylglycerol	Glycerol	-CH ₂ -CH(OH)-CH ₂ OH
Phosphatidylinositol 4,5-bisphosphate	Myo-inositol 4, 5-bisphosphate	
Cardiolipin	Phosphatidylglycerol	

Membrane lipids can be classified into three main groups: glycerol-based lipids, cholesterol and ceramide-based sphingolipids. Glycerol-based lipids can be divided into two broad categories: glycosylglycerides and phospholipids. Glycosylglycerides form a highly complex lipid family in which the *sn*-3 position of the glycerol backbone is esterified to a glycosyl moiety (*e.g.* galactose, glucose, etc.). They are the most abundant membrane glycerolipids; however, this lipid family is beyond the scope of this review and we will focus our attention on phospholipids. In phospholipids, while their *sn*-1 and *sn*-2 positions are esterified to a fatty acid, the *sn*-3 position is esterified to a phosphate group that in turn is also esterified to a polar headgroup. Although the fatty acid moiety greatly influences their physicochemical properties, these phospholipids are usually classified according to their polar headgroup (Table 1). Cholesterol contains a hydroxyl group that interacts with the phosphate head of phospholipids, whereas the bulky steroid region interacts with phospholipid acyl chains. Among other important physical properties of membranes, these interactions regulate mem-

brane fluidity, membrane packing, non-lamellar phase propensity and the formation of microdomains. Finally, sphingolipids are defined by the presence of a sphingoid-base backbone (*i.e.* 2-aminoalk[ane or ene]1,3-diol with 2S,3R stereochemistry). The main feature that allows the formation of an impermeable lipid bilayer is the amphipathic nature of these molecules, resulting in a highly hydrophobic core and hydrophilic surface, the landmark of biological and model membranes.

Membrane lipid structure

Most phospholipids spontaneously form lipid bilayers in aqueous environments with a pH and ionic strength similar to that of biological systems. However, certain lipids can organize into non-lamellar structures under physiological or non-physiological conditions. Moreover, lipids may not only display different phases under different conditions (lipid mesomorphism), but membranes lipids may also

show distinct finite structures within cell membranes (membrane microdomains). This lipid mesomorphism has been mainly studied *in vitro*, although non-lamellar structures have also been observed *in vivo* [13]. Thus, although lipids usually organize into bilayers their structural versatility implies that the nature of the membranes that form may be diverse.

Membranes are made up of molecules that to some extent preserve their individual characteristics and hence, the particular structure of these molecular bricks influences the structural properties of the membrane. In this context, phospholipids with a bulky polar head, such as phosphatidylcholine (PC), have a cylindrical *molecular* or *effective shape* and they tend to associate with other cylinder-like phospholipids to form planar structures [14, 15]. Other lipids might be prone to form non-bilayer structures. Cone-shaped lipids with bulky polar heads such as lysophosphatidylcholine (LPC), or truncated cone-shaped lipids with small headgroups such as phosphatidylethanolamine (PE), may form spherical micelles or tubular structures with positive (H_I) or negative curvature (H_{II}), respectively. Although these lipids form non-bilayer structures in membranes, the roles of which in general remain to be determined [13], in the last few years some functions have been attributed to non-bilayer prone lipids in planar structures (lipid bilayers). Indeed, these lipids appear to participate in the interaction of several proteins, such as, *e.g.* protein kinase C (PKC) with membranes [4]. Non-lamellar-prone membranes also favour the binding of heterotrimeric G proteins and $G\beta\gamma$ dimers, as well as displaying a lower binding affinity for $G\alpha$ monomers [4–6]. The influence of non-lamellar-prone lipids in facilitating or regulating the docking of amphitropic membrane proteins may have originated from the interaction of a protein with membrane fatty acyl chains leaving the membrane plane, or through the insertion of a protein's hydrophobic domain into a bilayer with 'loose' surface packing (Fig. 2) [16]. This is possibly due to the presence of H_{II} -prone lipids, which generate 'frustrated' bilayers (I_E phase) [15], which may be stabilized by interactions with proteins or lipids (Fig. 2) [9, 16]. Non-lamellar-prone lipids also participate in the formation of the cleavage furrow during cell cytokinesis [17], as well as in other membrane fission and fusion processes. Finally, membrane lipid heterogeneity is responsible for the distinct membrane regions, domains and microdomains that form the spatial organization of, which it is related to, the specific activity at the membrane.

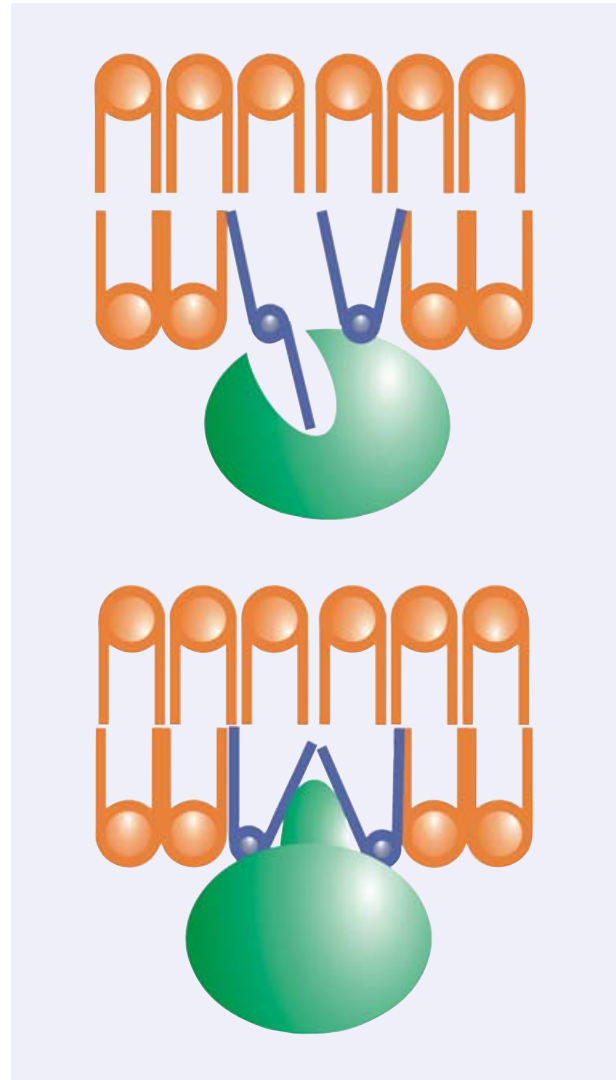


Fig. 2 Non-lamellar-prone lipids with a small polar head-group (*e.g.* phosphatidylethanolamine [PE], blue) induce the formation of non-lamellar-prone regions. These bilayers, with a frustrated (I_E) lamellar phase, can be stabilized by proteins (green) or other lamellar-prone lipids (orange). The loose packing of these bilayers allows some acyl chains to exit the membrane plane and become located in hydrophobic protein sockets (upper scheme). Hydrophobic protein domains, which may correspond to amino acid sequences or lipid modifications, may also be inserted into the membrane. Therefore, non-lamellar-prone lipids facilitate the docking of amphitropic proteins to the membrane. One of these lipids, PE, is abundant in the inner monolayer of the plasma membrane where most peripheral proteins are found.

Table 2 Lipid composition of various types of membranes

	Percentage of phospholipids								Cholesterol ($\mu\text{g}/\text{mg}$ protein)
	PC	PE	PS	PI	PA	CL	LGP*	SM	
Rectal gland plasma membrane	50.4	35.5	8.4	<1	–	–	–	5.7	N.D.
Brush border membrane	33.3	35.6	7.4	8.2	1.2	N.D.	4.1	10.3	50
Cholinergic receptor membranes	37	40.5	17	–	<1	–	<1	<1	135
Plasma membrane	39	23	9	8	1	1	2	16	128
Mitochondria	40	35	1	5	–	18	1	1	3
Micosome	58	22	2	10	1	1	11	1	14
Lysosomes	40	14	2	5	1	1	7	20	38
Nuclear membrane	55	13	3	10	2	4	3	3	38
Golgi membrane	50	20	6	12	<1	1	3	8	78
Sarcoplasmic reticulum	72.7	13.5	1.8	8.7	<1	<1	–	1	12

*Values for lysoglycerophospholipids (LGP) in excess of a few percent should be viewed with caution. High LGP contents of are probably the result of phospholipid degradation during preparation of the material. Adapted from [18].

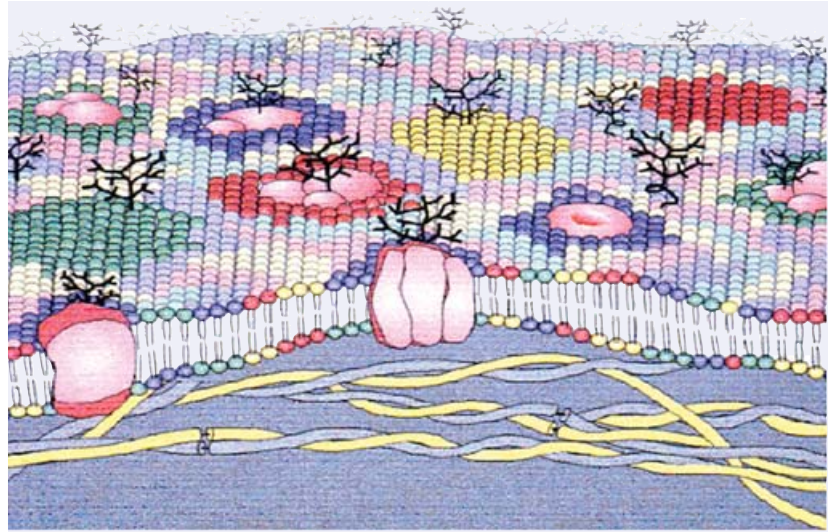
Membrane lipid organization

The specific types of lipid species and their levels in membranes appear to be regulated exquisitely, whereby general patterns emerge that are associated with the type of cell and organelles studied. Each membrane type is highly specialized and its different attributes are determined by specific membrane proteins and lipids. Thus, a given membrane has a stable and specific lipid composition, and although there are many combinations of lipid types and proportions, changes in composition only occur under certain pathological or physiological situations. The wide variety of lipid compositions is shown in Table 2 and the differences observed in the lipid composition of the various membrane types listed, have important consequences on lipid organization in the membrane [18].

The fluid mosaic model proposed that membranes were formed by a fluid bilayer in which proteins and lipids could move freely [19], contributing greatly to

our concept of a cell membrane. Initially, the mosaic model depicted by Singer and Nicolson referred to the random behaviour of the different proteins in the membrane bilayer as if in a 'sea of lipids'. Today, the extended fluid mosaic model contemplates additional structural and functional restraints on membrane organization [5, 20]. In this context, most biological membranes are asymmetrical, both laterally and in cross-section. The cross-sectional asymmetry reflects the lipid composition of each leaflet (Fig. 3) [21]. The external leaflet of the plasma membrane (exoplasmic, E face) and the luminal leaflets of internal organelles are highly enriched in choline-containing lipids such as PC and sphingomyelin (SM). In contrast, the cytoplasmic leaflet (protoplasmic, P face) is rich in amine-containing glycerophospholipids such as PE and phosphatidylserine (PS). Other minor phospholipids, such as phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol-4, 5-biphosphate (PIP₂), are also enriched on the

Fig. 3 Schematic illustration of a biomembrane, depicting membrane lipid asymmetry as well as microdomains enriched in particular lipids and those induced by membrane proteins.



cytoplasmic face of the membrane where they participate in cell signalling [22, 23]. Interestingly, the membrane of the endoplasmic reticulum shows a symmetric lipid distribution and it primarily contains unsaturated glycerolipids that provide flexibility, and that facilitate the incorporation of newly synthesized proteins [24]. This is a clear example of how the same components may combine differently to yield different lipid organizations with features that adjust to their specific needs. Membrane heterogeneity is further achieved by lateral asymmetry in which membrane regions (basal, lateral, apical), and different specialized membrane regions or microdomains (lipid rafts, caveolae, coated pits, synaptosomes, etc.) [9, 25 and references therein] extend the mosaic nature of membranes. The formation of these domains in part results from the non-ideal mixing of lipids in membranes and in some cases, it is enhanced by the participation of certain cytoskeletal structures that underpin the lipid bilayer and that restrict the traffic of proteins and lipids.

Cells possess complex mechanisms to control the specific lipid composition of membrane, imposing directionality and selectivity to the mobilization of lipids. Several specialized enzymes, such as flippases, floppases and scramblases, are responsible for maintaining this precise membrane lipid distribution [24, 26]. Indeed, the heterogeneous distribution of lipids is critical for the correct physiological homeostasis of cells. Accordingly, the loss of transmembrane lipid asymmetry and the concomitant exposure of PS to the external milieu occurs upon induction of

programmed cell death (apoptosis) [27], or on platelet activation and aggregation [28]. Externalization of PS changes the cell surface charge, as its negative net charge at physiological pH alters the electric properties of the external leaflet, mainly influenced by the glycocalyx. The net result of this process is an alteration of cell–cell interactions that might be involved in the conversion to a procoagulating state [29], increased adhesion and aggregation [30] and recognition by phagocytic cells [31]. Although these processes are essential for normal cell development and homeostasis, unregulated loss of PS asymmetry may contribute to the development of heart disease, stroke and diabetes [30].

The organization of specific domains is an important aspect of membrane structure that has recently received increasing attention. Because lipids have significant lateral mobility, one might expect that they would be homogeneously distributed in most membranes. However, both model and biological membranes exhibit non-ideal mixing in systems containing two or more elements [32, 33]. The physicochemical forces involved in this lateral segregation into microdomains are discussed below (Fig. 3). Although various different domains are known to exist, research has focused on *lipid rafts* for many years. From a practical point of view, a lipid raft can be defined as a single, detergent-insoluble glycolipid-enriched (DIG) membrane fraction, with a high content of cholesterol, glycosphingolipids, SM and proteins. Although some authors also consider caveolae to be lipid rafts, others consider them as a different

kind of cholesterol-free sphingolipid containing microdomain with a different protein and lipid composition. Thus, whereas lipid rafts are planar domains with high levels of glycosylphosphatidylinositol (GPI)-anchored proteins and deficient in caveolin, caveolae are cell surface invaginations stabilized by structural proteins such as caveolins and deficient in GPI-anchored proteins [34].

Although still controversial, studies using fluorescence resonance techniques have estimated rafts to have a mean diameter between 30 to 50 nm [35] and several hundred micrometers [36]. It was earlier suggested that rafts moved within the exofacial leaflet of the membrane bilayer [37], although current evidence suggests that rafts also extend through with the cytofacial leaflet due to the organization of lipids associated with the presence of membrane signalling proteins such as rho-A, fyn and the interleukin receptor IL2R- β [38–40]. GPI-anchored proteins are very abundant in rafts [41, 42] but they do not span the membrane bilayer and therefore, they probably have little effect on the organization of the cytosolic leaflet. On the other hand, rafts contain unusual amounts of ethanolamine plasmalogens, PS [43] and the GM3 ganglioside [41].

Caveolae are cell surface invaginations that are usually smaller than lipid rafts, the diameter of their opening at the cell surface typically ranges from 60 to 80 nm. Caveolae contain more free cholesterol than lipid rafts (with respect to sphingolipids) [41, 43], whereas GM3 is practically undetectable [41]. The main feature of caveolae is the presence of caveolin-1, a structural protein present in the form of high molecular weight oligomers, while a second caveolin (caveolin-2) is also often present. When compared to rafts, GPI-anchored proteins are largely or completely absent from caveolin-containing microdomains [41, 42]. Significantly, a wide variety of receptors and signalling proteins have been co-purified with caveolins: receptor kinases, platelet-derived growth factor receptor (PDGF-R), insulin receptor, *shc*, *h-Ras*, etc. Protein kinase A, adenyl cyclase and several isoforms of PKC have also been recovered from subcellular fractions of caveolae [44].

The formation of microdomains within the membrane allows the selective incorporation or exclusion of specific proteins, providing a mechanism to govern protein–protein and protein–lipid interactions [45]. Numerous studies have implicated rafts in the compartmentalization, modulation and integration of sig-

nalling events, providing platforms for the assembly of cell surface receptors and their downstream signalling proteins [46]. Along these lines, SNAREs (soluble N-ethyl-maleimide sensitive factor attachment protein receptor) are known to concentrate in cholesterol-dependent microdomains that define docking and fusion sites for the exocytosis and release of neurotransmitters, hormones, enzymes and other proteins or small molecules [47]. Caveolae have also been implicated in clathrin-independent endocytosis of GPI-anchored proteins and glycosphingolipid-binding toxins [48, 49].

Why so many different lipids?

Lipids are by far the chemically most diverse class of biomolecules, with an average portfolio of a eukaryote cell being comprised approximately 1300–1500 different species. Moreover, different cell types, such as liver parenchymal cells and brain cells have different lipid compositions, as do different cellular organelles. Although some of this diversity is likely to result from diet-induced variation, the overall patterns are actively maintained by the cells, and, accordingly, require significant metabolic energy input and the presence of a large collection of enzymes and transfer proteins, responsible for the active control of lipid compositions and dynamic distribution within a cell. The above, together with the discovery of bioactive lipids (such as platelet activating factor, PAF), and recognition of the key involvement of lipids (*e.g.* PI, diacylglycerol [DAG] and ceramide) in cellular signalling cascades readily makes obsolete the view that lipids are just mere building blocks for making impermeable membranes to provide the cell with distinct compartments.

There are now extensive efforts to establish spatio-temporal, organelle level compositional patterns of lipids and to correlate these to the physiological states of cells, approaching cell behaviour from the point of view of lipidomics. With the emergence of these data it becomes mandatory to understand the mechanisms pertaining to the biological activities of lipids. Lipids represent the paradigm for molecular self-assembly, mainly driven by their amphiphilicity, and biophysical studies conducted during the last four decades or so have demonstrated these assemblies to form, depending on the lipid in question, a range of different phases, sensitive to factors

including temperature, pressure, ions, hydration, small molecules (e.g. metabolites and drugs) and pH. Exploration of the behaviour of lipid mixtures, consisting of only two to three species has further shown membranes to possess a rich scale of 2- and 3-D organization on different length- and timescales. Taken the complexity of biomembrane compositions and the fact, that they are at thermodynamic non-equilibrium (because of membrane potential, for instance), it is obvious that we are still in the early-phases in lacking a true, molecular and *system* level understanding of biomembranes and the roles played by different lipids. Regarding the latter, the effects of lipids can be divided into two fundamentally different categories. The first type is (i) the recognition and binding of lipids as individual molecules by specific proteins, accommodating lipids as ligands. A good example is the PAF receptor, that belongs to the GPCR family [1]. This mode of action of PAF represents a 'classical' protein–small molecule interactions and does not assign any role to the collective membrane properties of the lipid agonist. The other, much more poorly understood area relates exactly to the latter, (ii) lipid bilayer biophysical properties and the impact on these by different lipids, further influencing membrane and membrane protein functions, as well as 2- and 3-D organization of the membrane, including its proteins [50, 51]. A good example is provided by DAG, which can constitute up to 10 mol% of the membrane lipids in transformed cells. Although tentative scenarios have been forwarded to explain the coupling between the physiological state of the cancer cell and the physical properties, such as imparted on cellular membranes by DAG [15, 52, 53], we are still missing conclusive mechanisms. Nevertheless, it is clear that such a high content of DAG necessarily has profound effects on the membranes of transformed cells, including the functions of membrane proteins such as PKC, activated by this lipid. Accordingly, to avoid lipidomics to remain mere cataloguing it is essential to be able to connect the individual lipid species to the overall collective biophysical properties of membranes and their involvement in cellular physiology and pathophysiology, in addition to the recognition of possible novel receptors and effectors for specific lipids. It is the former, biophysical approach on lipidomics, which is outlined and discussed below. This approach thus provides a partial answer to the question on the chemical diversity of lipid structures. For instance, the different

acidic phospholipid species as well as the cationic lipid sphingosine allow complex regulation of the protonation behaviour by surface charge density, affecting electrostatic interactions on membrane surfaces. Moreover, these lipids also differ in their affinity for divalent metal cations, such as Ca^{2+} , thus imparting sensitive to the latter to particular membranes. As membrane electrostatics are further intimately coupled to factors such as acyl chain saturation controlling lipid phase state and lateral organization – these already seemingly simple chemical variations created a rich scale of structures, sensitive to specific environmental variables and controlling membrane protein functions in a highly cooperative manner.

Lipid mixing and demixing

Lipids impact not only the bulk biophysical properties, such as rates of lateral diffusion, but also the dynamic organization of these assemblies. A timely example is the current surge of interest in lipids and their involvement in the lateral heterogeneity and microdomains in biomembranes (Fig. 3), together with the recognition of the large variety of biomembrane functions controlled by this dynamic organization [51, 54]. Membrane microdomains ('rafts') were discovered already in the 1970s and early 1980s using different approaches [55–63] and, importantly, were attributed to the physical properties and organization of lipid mixtures [14, 58, 59]. In this regard, the dependence of the lipid phase transition temperatures on the extent of acyl chain unsaturation and chain lengths as determinants of lipid–lipid and lipid–protein interactions in driving lateral demixing are of importance (e.g. myristoylated *versus* palmitoylated proteins, [54]). Further along these lines of particular current interest are SM and cholesterol, which have been shown to segregate form domains in cells [60] as well as in model membranes [64, 65]. The driving forces for the phase separation in this lipid mixture are still controversial. One possibility is hydrophobic mismatch, which has been demonstrated to drive the formation of microdomains in reconstituted membranes [66, 67]. Accordingly, with the phospholipid acyl chain order augmenting due to cholesterol and thus causing an increase in the bilayer thickness, phase separation would take place, with the formation of the cholesterol-enriched liquid ordered l_o phase [68] decreasing the free energy

penalty due to hydrophobic mismatch (line tension) between these and the liquid disordered (l_d) cholesterol-poor regions [69]. Because of the dynamic nature of the l_o phase and fast exchange between the l_o and l_d domains, these structures can be expected to have a major impact on the lateral organization of integral membrane proteins.

Another example of lipid-driven membrane demixing is provided by ceramide, which is involved in cellular signalling of apoptosis, programmed cell death [70]. This lipid has a pronounced tendency for self-association, caused by intermolecular hydrogen bonding, further promoted by the weak hydration of its headgroup and tight packing of the saturated hydrocarbon chains. The segregated ceramide-enriched phases have significantly elevated chain melting temperatures and are crystalline in nature at physiological temperature [71]. Accordingly, the properties of ceramide- and cholesterol-induced phases are distinctively different and they can be expected to have very different impacts on the lateral organization of membrane proteins, for instance. The tight packing of ceramide further manifests another important property characterizing membranes, bending rigidity, pertaining to the energy required to change the shape (curvature) of a membrane. Although membranes formed by unsaturated PCs, for instance, are very soft, with intense fluctuations in their shape caused by thermal energy, membranes enriched in ceramide are rigid. Ceramide illustrates also another important property of lipids, spontaneous curvature. More specifically, depending on their shapes [14] and more exactly, on their *effective* shapes [52], the curvature of the surfaces formed by lipids can be negative, zero or positive. Because of their small size and tight packing of the ceramide headgroups by intermolecular hydrogen bonding, ceramide-enriched membrane domains have negative spontaneous curvature, ultimately favouring the formation of the inverted hexagonal (H_{II}) phase (Fig. 4). The negative spontaneous curvature of the ceramide-enriched membrane domains, together with their high bending rigidity, have interesting consequences, such as those given below. Ceramide can be enzymatically generated in membranes from SM, which has a strongly hydrated phosphocholine headgroup attached to ceramide. In contrast to ceramide, SM readily mixes at physiological temperatures with liquid disordered, unsaturated PCs. However, upon the conversion of SM to ceramide by

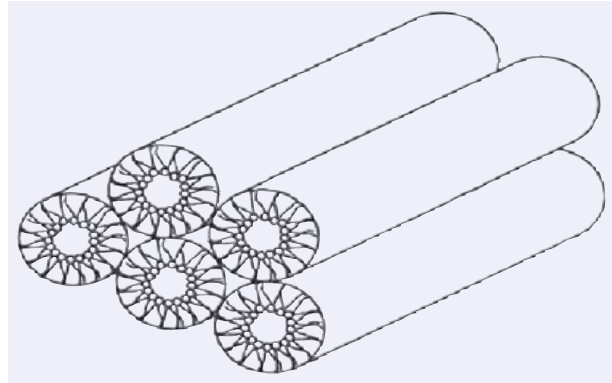


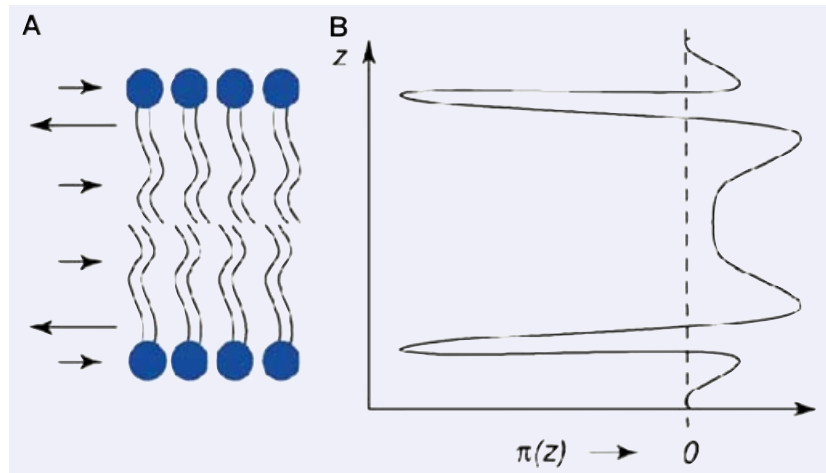
Fig. 4 Inverted H_{II} hexagonal phase composed of water-filled tubes with the lipid acyl chains pointing outwards. Different cellular membranes with a planar geometry invariably contain a variety of lipids and would therefore form such a phase. The presence of these lipids imparts frustration to the membrane, with a high packing density in the hydrocarbon region of the bilayer. Adapted from [51].

sphingomyelinase (SMase), the reaction product segregates into microdomains [72] with negative spontaneous curvature and high bending rigidity. As a consequence, these microdomains start bending the membrane, ultimately causing shedding of ceramide-enriched vesicles from the original bilayer membrane [73, 74]. Accordingly, the formation of ceramide in biomembranes can be anticipated to cause lateral segregation of membrane constituents and be responsible for the membrane blebbing in apoptotic cells, in essence causing both 2-D as well as 3-D reorganization of membrane with its embedded proteins [75].

Lateral pressure

Intimately related to membrane spontaneous curvature is the lateral pressure profile [76]. This concept is highly useful in understanding of several characteristics of membranes. In brief, approaching the surface of a PC bilayer from the water phase and recording the prevailing forces acting on the lipid assembly, there is first a zone with repulsive potential between the strongly hydrated headgroups, such as for PCs and SMs (Fig. 5). Adjacent to the above there is a zone where the hydrophobic effect manifests as

Fig. 5 Lateral pressure profile for a lipid bilayer (left), with surface tension being balanced by steric repulsion between the headgroups and acyl chains. See text for details. Adapted from [51].



interfacial tension, with hydrophobicity of the lipid hydrocarbon chains restricting their contacts with water molecules. Whenever thermal motion and repulsive interactions increased the exposure of the acyl chains to water, its entropy decreased. The resulting tension balances not only the steric repulsion between the headgroups but also the repulsion between the acyl chains, prevailing in the hydrocarbon region of the bilayer. Because of these forces are acting on very narrow zones, the pressure can be considerable, estimated to be hundreds of atmospheres.

The lateral pressure profile can be influenced in a number of ways, *e.g.* by small membrane partitioning molecules as well as by the lipid composition. Introducing a lipid with a large headgroup for example, augments repulsion at this level and therefore reduces the entropic pressure between the chains. Consequently, the acyl chain order and membrane thickness decrease. Introducing a lipid with a small headgroup, such as unsaturated PE (*e.g.* palmitoyl oleoil phosphatidylethanolamine [POPE]) has the opposite effect, with the contribution of the repulsion between the chains in counteracting the interfacial tension becoming more significant. Under these conditions, with moderate contents of POPE, the membrane remains lamellar, yet its free energy increases as the free energy minimum would require negative spontaneous curvature. Such membranes are defined as frustrated phases and this can be expected to have interesting consequences for lipid–protein interactions [15]. It has been demonstrated that it is this frustration, which activates PKC by inverted hexagonal (H_{II}) phase (Figs 2 and 4) forming lipids

(with high negative spontaneous curvature), not the formation of this phase *per se* (for a review, see [15]). High internal pressure within the bilayer can further result in a novel type of lipid–protein interaction, extended lipid anchorage, with one chain of a membrane embedded lipid extending out from the membrane and accommodating into a hydrophobic cavity of a protein, thus attaching the latter to the membrane surface without protein intercalation into the bilayer [77, 78].

Unfortunately, there are no direct experimental techniques available for the quantitative assessment of the lateral pressure profile. Interestingly, recent computer simulations suggest cholesterol to have a profound impact [79]. As the lateral pressure profile should profoundly influence both lipid–lipid and lipid–protein interaction potentials, it is likely that this characteristic property of membranes is under stringent control, as is evidenced by the crucial role of the control of the membrane contents of lipids with negative spontaneous curvature, required for the proper growth of microorganisms [15, 52, 53].

Lastly, it is worth mentioning the difference between the lateral pressure profile and equilibrium lateral pressure, the latter simply being the numeric value where the forces are at equilibrium for a tension-free bilayer. Both theoretical and experimental studies (including relative efficiencies of different phospholipases A_2 in hydrolyzing erythrocyte phospholipids and comparison with their action on lipid monolayers at varying lipid lateral packing densities) have yielded an estimate of approximately 30–35 mN/m for the equilibrium lateral pressure of biomembranes. Although this parameter is unlikely to

vary significantly with lipid compositions found in membranes, their susceptibility to osmotic forces (membrane stretching) has been shown to vary markedly depending on the lipids present [80, 81]. Reflecting lipid acyl chain compositions, for instance, the extent of area increase caused by equal osmotic pressure gradients across a bilayer should greatly increase upon increasing *cis*-unsaturation and shorter chain lengths. As osmotic forces are tightly controlled in cells and regulate proteins such as stretch-sensitive ion channels and phospholipases, these issues would deserve to be investigated in more detail. Of the latter group of enzymes, phospholipase A₂ represents the rate-limiting step in the synthesis of prostaglandins from arachidonic acid, making it a key player in the control of inflammation and thus emphasizing the importance of understanding the biophysics governing biomembrane functional properties. The control of this enzyme by osmotic stretching of the membrane provides an example how a physical property of a lipid bilayer is converted directly into a biochemical signal [82].

Surface electrostatics

From the point of physical chemistry the importance of electrostatics to the assembly of membranes is obvious. Thoroughly studied examples are cationic proteins or proteins with clusters of cationic residues, avidly associating with the negatively charged acidic phospholipids, such as PS and phosphatidylglycerol, PA and cardiolipin. In addition to pure Coulombic attraction specific interactions between particular lipid structures and proteins are expected. The surface electrostatics can be coupled with phospholipid phase behaviour. Accordingly, phase separation of anionic lipids into microdomains with high enough negative surface charge density can be used to control the membrane association of cationic proteins [83]. It is also worth noticing that electrostatics in biomembranes is, in general, dominated by the above negatively charged lipids. Yet, also cationic lipids are found, the most abundant being sphingosine [84] and studies with model membranes have shown its association with acidic phospholipids to regulate peripheral lipid–protein as well as lipid–small molecule interactions [85]. More detailed studies on model membranes have demonstrated the interplay

of surface charge density and the dissociation behaviour of the acidic phospholipid headgroup to exert a pronounced impact on lipid–protein interactions evident as distinctly different binding modes of cytochrome *c* to protonated and deprotonated phospholipid [86, 87].

The importance of understanding in detail the role of electrostatics in lipid–protein interactions is exemplified by studies on the mechanisms of action the so-called antimicrobial peptides (AMPs), constituting the first line of defense of multicellular eukaryotes against invading microbes. More specifically, whereas other modes of action are also involved, it is now thought that one of the principal targets for these short, cationic and amphiphilic peptides are acidic phospholipids in the outer surface of bacteria. Following the association of AMPs to the surface, charge neutralization allows for them to aggregate, leading to the formation of membrane-permeabilizing structures. In this regard, it is of interest that AMPs and acidic phospholipids form Congo red staining fibres, the diagnostic hallmark of amyloids [88, 89]. In the absence of lipids, the formation of these amyloids *in vitro* is generally rather slow and requires slightly acidic pH and low dielectricity (e.g. 30% trifluoroethanol). Accordingly, the relevance of these conditions to the *in vivo* misfolding and emergence of amyloid has been questioned. It should be noted that surface pH of approximately 5.2 has been estimated for membranes containing 20 mol% acidic phospholipids. Simultaneously, the membranes also provide a low dielectricity as well as a highly anisotropic environment, which causes a strong alignment of associated peptides and proteins [90]. In keeping with the above, a fast formation of amyloid fibres in the presence of acidic phospholipid containing liposomes has been demonstrated *in vitro* for a number of cationic peptides and proteins. It is of particular interest that these include, in addition to AMPs, proteins (for instance histone H1, cytochrome *c*, α -lactalbumin, lysozyme and endostatin) that are cytotoxic and trigger apoptosis in eukaryotes. Accordingly, it has been suggested that the formation of amyloid ‘protofibrils’ would underlie the mechanism of toxicity of these peptides and proteins, in addition to possible other modes of action [86, 88–91]. Taking into account that amyloid formation is directly implicated in major diseases such as type 2 diabetes, Alzheimer’s, Parkinson’s and prion diseases, it is obvious that development of detailed understanding

of the molecular level mechanisms involved would be of paramount medical importance.

Role of lipids in cell function

We are still far from formulating a general hypothesis to explain the variety of lipids found in membranes because most membrane functions that we are aware of could be fulfilled with just a few lipid species. However, the ever-increasing number of studies providing information regarding the different functions of specific phospholipids is helping us to understand why membranes are formed by hundreds of different lipid molecules. Phosphoinositides (PIs) are important in cell signalling and vesicle formation, key events in neurotransmission and in the transit of vesicles from the endoplasmic reticulum to Golgi [92]. Moreover, PIs participate in a coordinated manner with PE during cell division, whereby changes in PIP₂ production inside the cleavage furrow occur concomitant with the accumulation of PE in this structure during cytokinesis [93, 94]. This type of association between lipids could be considered as a unique membrane domain that arises transiently to help the cytoskeletal machinery produce two daughter cells. On the other hand, nuclear PS and PI are involved in cell cycle regulation because they stimulate the synthesis of DNA polymerase α [95, 96]. Finally, the presence of anionic phospholipids, in particular PS, is required for the assembly of the prothrombinase complex on membrane surfaces during platelet activation [28] and as mentioned above, exposure of PS at the exofacial leaflet occurs in the initial steps of apoptosis and blood coagulation [97, 98]. These lipids also participate in more complex activities, such as the docking of peripheral proteins to membranes [1, 4, 99]. Cholesterol, SM and gangliosides also participate in the formation of lipid domains. In a similar fashion, membranes have a high number of different fatty acid moieties and although thicker microdomains may require long saturated fatty acid moieties, thinner domains or some membrane proteins may need shorter or unsaturated acyl chains. Likewise, the transmembrane regions of integral proteins may have specific lipid requirements or at least display certain preferences.

An additional degree of complexity in membrane lipid functions lies in the metabolic relationship

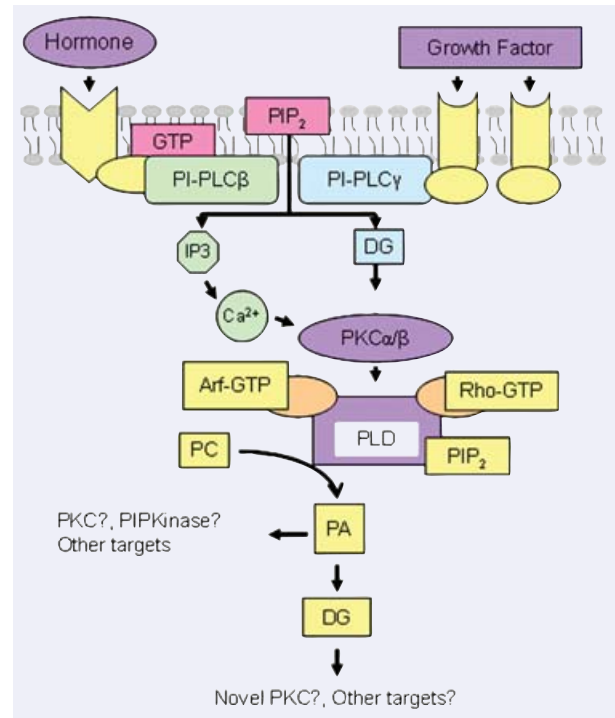


Fig. 6 Phospholipase cascade of PLD activation and amplification of diacylglycerol production. Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; DG, diacylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid; PKC, protein kinase C; PIP₂, PI-4,5-bisphosphate and PIP kinase, PI phosphate kinase. Adapted from [100].

between phospholipid species. There is a relatively large number of phospholipases and phosphatases that specifically participate in the interconversion of phospholipids and that in turn, can modulate the activity of those enzymes. The cascade of phospholipase D (PLD) activation is a clear example of this complexity (Fig. 6) [100]. The initial step of the cascade involves the agonist-induced and GPCR-mediated activation of phospholipase C (PLC) to hydrolyze phosphatidylinositol (PIP₂) into DAG and IP₃. The former activates PKC and the latter induces the release of Ca²⁺ into the cytosol through IP₃-activated channels, which also activates PKC [101]. Interestingly, mammalian PLDs also require PIP₂ as an essential cofactor for their enzymatic activity [102]. PLD is present in Golgi membranes [103] and it hydrolyzes PC to PA, a downstream effector of the small guanosine triphosphate (GTP)-binding protein

adenine diphosphate (ADP)-ribosylation factor (ARF-1) [104]. Finally, PA can also be converted to DAG by PA phosphatases, whose activity is involved in both lipid metabolism and glycerolipid signalling [105]. The complexity of the signalling and metabolic pathways in which phospholipids participate, as well as the cross-talk between these cascades, emphasizes the existence of highly sophisticated regulatory mechanisms that remain to be fully understood. Together, these studies demonstrate the role of membrane lipids in a large variety of cellular functions and emphasize the close relationship between membrane lipid composition and function. In addition, the number of existing human pathologies related to alterations in lipid metabolism is evidence of the importance of membrane lipids and their role in signalling pathways (Table 3).

Lipid influence in transmembrane protein function

The previous paragraphs have been focused on the lipid composition of membranes and how lipids determine the properties of membranes, defining the formation of different types of membrane structures and microdomains. Lipids, and the structures they form, participate in the interaction of proteins with membranes, delineating their functions and modulating their structure. The next paragraphs will describe the interactions between lipids and both peripheral and transmembrane proteins and how lipids intervene in cell functions controlled by such proteins.

Transmembrane proteins are highly diverse but have one property in common: they contain one or more hydrophobic regions that transverse the membrane bilayer and therefore these proteins become intimately exposed to membrane lipids. Such exposure results in a variety of lipid–protein interactions that most frequently reveal themselves as highly relevant to the membrane protein functioning. The observed effects may sometimes be caused by a lipid-induced misfolding or misassembly of the protein within the membrane but more frequently, effects of lipids on the function of properly folded and assembled transmembrane proteins have been documented. Nonetheless, despite the extensive information obtained on the functional dependence of

many membrane proteins by its surrounding lipids [106, 107], this has been a controversial issue over the years and the mechanisms by which such membrane protein modulation is exerted by the different lipid classes still remain unclear. Two general cases could be considered. On the one hand, when the interaction with the lipid is less specific, there are still lipid-associated parameters defining physical attributes of the biological membrane, which are known to modulate membrane proteins. These include lateral pressure [76, 108, 109], membrane fluidity [110], bilayer thickness [111] surface charge distribution [112] or the segregation of membrane microdomains or ‘rafts’ [113], all of which may affect the structure and/or the function of intrinsic membrane proteins. On the other hand, when there is a sufficiently high lipid specificity in the interaction, there could be more direct effects through binding of lipids to defined sites on the transmembrane portion of the protein [114–118], which has led to postulate a possible role of certain lipids as peculiar ‘allosteric’ effectors of the proteins.

In an attempt to illustrate some of these phenomena we have focused on reviewing the subject with regard to ion channels and receptors, two classes of very important integral membrane proteins, which usually allow for very precise monitoring of their functional activities and in some cases, have their structure solved at high resolution.

A prokaryotic potassium channel: KcsA

One bacterial K⁺ channel that has been identified, cloned [119] and its structure determined by X-ray crystallography [120] is the KcsA channel from the Gram-positive soil bacterium *Streptomyces lividans*. KcsA is a homotetramer, each subunit containing 160 residues with two transmembrane α helices separated by a P-loop, and cytoplasmic N- and C-terminal domains.

Previous studies have shown that specific membrane lipids have a strong influence on KCSA assembly and stability [109, 121]. In particular, the presence of anionic lipids like PA or PG influence the stability and folding properties of the potassium channel KCSA [122]. This lipid specificity is also observed in lipid-binding studies [123]. Moreover, the high-resolution structure of KcsA shows tightly bound lipids within the crystal; one modelled as nonan-1-ol and the other located between transmembrane α -helices at monomer–monomer interfaces,

Table 3 Human pathologies and lipid abnormalities

Disease	Membrane abnormality	Proposed molecular mechanisms
Cardiovascular (Hypertension)	Changes in membrane phospholipid and cholesterol levels, changes in fatty acid levels	Regulation of the membrane structure with concomitant alteration of membrane signalling, protein localization and activity
Cardiovascular (Sudden Cardiac death)	Changes in membrane levels of saturated and unsaturated fatty acids	Alterations in δ -6-desaturase activity in the coronary artery wall
Cardiovascular (Cardiac hypertrophy)	Changes in membrane levels of triacylglycerol species and other lipids	Changes in cell signalling and impaired triacylglycerol availability
Cancer (pathologic proliferation)	Changes in membrane fatty acid levels	Altered cell structure and function (including cell proliferation)
Cancer (multidrug resistance)	Alterations in the levels of phospholipid species (PS*)	Reduced drug intake and facilitated drug removal from cancer cells
Respiratory pathologies	Changes in the lipid composition of membrane microdomains	Alterations in mechanotransduction and other signalling processes
Renal Pathologies	Increased lipid peroxidation and augmented proportions of saturated fatty acids caused by haemodialysis	Increased cellular oxidative stress
Alzheimer's disease, Aging and neurodegeneration	Reduced levels of PUFA [†] in brain cell membranes	Altered expression of transthyretin and other genes related to learning, cognitive and integrative functions
Inflammation, autoimmune and related diseases	Release of pro-inflammatory lipids from membranes	Formation of eicosanoids from arachidonic acid, changes in membrane fluidity, changes in membrane lipid–protein interactions
Infectious diseases	Increased ceramide-enriched membrane domains	Modified membrane lipid domains act as platforms for a wide variety of virus, bacteria and parasite infections
Schizophrenia	Decreased proportion of PUFA [†] in membrane phospholipids	Myelin-related and neurotransmitter signalling dysfunctions
Obesity	Changes in membrane lipids	Alterations in membrane protein function
Alcohol-induced fetal damage	Changes in cell membrane composition	Various cell functions alterations
Coagulation (Scott Syndrome)	Defective PS* flip-flop translocation in membranes	Impaired interaction of coagulation factors and blood cell membranes
Triose Phosphate Isomerase deficiency	Lack of symptoms is associated with modification of membrane lipids and lipid fluidity	Changes in membrane protein–lipid interactions and in the activity of certain enzymes

Adapted from [9]

*PS, phosphatidylserine and [†]PUFA, polyunsaturated fatty acids.

modelled as a DAG with one C14 and one C9 chain. Because purified KcsA contains approximately 0.7 phosphatidylglycerol molecules per KcsA monomer, the lipid modelled from the X-ray as a DAG is probably a phosphatidylglycerol whose headgroup is too mobile to be resolved. More recently, such lipids have been found essential for full refolding and tetramerization of unfolded KcsA *in vitro* [124].

Functional studies with KcsA reconstituted into liposomes suggest that a functional channel could only be obtained in the presence of anionic phospholipids, with little or no specificity for the different lipid classes, which could either be phosphatidylglycerol, PS or cardiolipin [125]. More recently, Lee and coworkers have shown positive cooperativity between the open channel probability and the PG content [126].

The functional and structural results mentioned above indicate that the modulation of KcsA by membrane lipids occurs through specific interactions between the protein and tightly bound PG. Interestingly, there are reports on the detection on lipid bound in the crystal structures of other membrane proteins [127–129] and, therefore, it is likely that such proteins might be regulated by lipid in a manner similar to that of KcsA.

Mechanosensitive channels: Msc

The mechanosensitive channels are a structurally heterogeneous protein family widely distributed in archaea, prokaryotes and eukaryotes. These channels are attractive models to study lipid–protein interactions because their function is to couple tension in the lipid membrane to protein conformation [130]. Their topology is very diverse including two pore domain channels, that conduct potassium [131, 132] or sodium ions [133], the TRP (transient receptor potential) channels, such as TRPC1 and TRPY, that are non-selective cation channels [134] and the prokaryotic Msc channels that conduct potassium ions [135].

Bacterial mechanosensitive channels are activated by increasing tension in the lipid bilayer of the cytoplasmic membrane, where they transiently create large pores in a controlled manner. When reconstituted into a bilayer system, the mechanosensitive channel can be opened from its closed state(s) by the addition of LPC to one monolayer of the bilayer. This changes the bilayer curvature and increases the tension, which results in tilting the transmembrane helices and lowering the threshold for channel opening [136]. In similar studies using phospholipids with different acyl chain lengths to change the bilayer hydrophobic thickness, it is observed that thicker bilayers tend to stabilize the closed state of the channel, whereas thinner ones favour channel openings [137]. From these observations it follows that the opened and closed states of the channel are likely to differ in its hydrophobic thickness, such that changing the bilayer thickness should accommodate better to one of such states, and stabilize it accordingly.

In addition to the need of a good hydrophobic matching between the Msc protein and the lipid for channel function, it has been shown that there is a chain-length dependence of the lipid binding to the protein that is different in the two sides of the mem-

brane. Such differences imply that the hydrophobic matching needed for Msc channel function likely involves bending of transmembrane α -helices, rather than simple tilting [138]. Therefore, in Msc channels and similar cases [139, 140] it seems that certain bilayer properties, such as the intrinsic curvature or the extent of hydrophobic mismatch, may influence protein function directly, without any specific interaction arising from a particular lipid class.

A voltage-gated potassium channel: KvAP

Voltage-gated ionic channels (VGICs) are membrane proteins that transiently open a pore through the lipid membrane in response to changes in membrane potential. Kv channels are VGICs and play a critical role in a wide variety of physiological processes, including the regulation of heart rate, muscle contraction, neurotransmitter release, neuronal excitability, insulin secretion, epithelial electrolyte transport, cell volume regulation and cell proliferation. Gating of the Kv channels in response to membrane potential has been correlated with the movement of the positively charged amphipatic S4 transmembrane segment. In the case of KvAP, a prokaryotic voltage-gated channel, diverse studies have demonstrated that the phospholipid membrane provides an appropriate environment for the energetic stability and operation of the voltage-sensing machinery, perhaps by contributing stabilizing interactions between positively charged voltage sensor residues and negatively charged lipid phosphodiester groups [141]. Therefore in the Kv channels and similar cases [142, 143] it seems that a bilayer feature, *i.e.* the charge provided by the phospholipid headgroups, influences protein function by controlling electrostatic interactions at the lipid–protein interface.

Nicotinic acetylcholine receptor (nAChR)

Ligand-gated ionic channels are membrane proteins that transiently open a pore through the lipid membrane in response to neurotransmitter binding. The nAChR is one of the best-understood members of this family. The nAChRs present in the neuromuscular synapses are heteropentamers comprised four

different but highly homologous subunits (for reviews see references in [144]). Each subunit contains an extracellular N-terminal domain (which include the ACh binding sites), four hydrophobic transmembrane domains (M1–M4) and a small intracellular C-terminal domain. Upon activation by agonist, nAChRs transiently open a cationic channel responsible for the initiation of postsynaptic membrane depolarization.

Extensive biochemical studies have demonstrated that the ability of the nAChR to support ion channel function requires the presence of specific lipids. These effects in nAChR function may be exerted through binding to specific sites of the protein or by modification of bilayer physical properties. Previous results have demonstrated that membrane lipids interact differentially with nAChR. For example, sterol, PA and fatty acid spin labels have a relative high affinity for nAChR compared with other spin-labelled phospholipids [145].

Additionally, several lines of evidence demonstrate the existence of more specific distinct lipid-binding sites, namely non-annular sites. McNamee and Lee used brominated lipids to partially quench the intrinsic fluorescence of the nAChR to monitor contacts with the surrounding lipid in reconstituted membranes. They found that receptor quenching by PC was independent of the presence of cholesterol, but there is an additive quenching due to brominated cholesterol derivatives [118]. These results argue strongly for independent binding sites for cholesterol and phospholipids.

Although cholesterol may affect the nAChR directly, it definitely has profound effects on structure of the membrane environment, most notably by changing membrane order or fluidity. In earlier studies both the agonist affinity and ion flux seemed to require an optimal fluidity [110]. However subsequent studies showed that although the ion flux activity of the nAChR was strongly influenced by lipid composition [116], there was no correlation with membrane fluidity, as measured by steady state anisotropy of membrane probes [146]. Measurements of membrane fluidity showed that cholesterol further ordered membranes containing PC and PA, but another sterol, like androstanol, did not, although either one of the two sterols supported similar ion fluxes. Thus, in this case cholesterol exerts its effect on the nAChR through direct interaction with the protein [115].

With respect to PA, *in vitro* studies with nAChR reconstituted in lipid vesicles of controlled composition show that PA is among those phospholipids that

bind the protein with a higher affinity, and it is also most effective in preserving nAChR function [118, 145, 147], possibly through a stabilization of the resting *versus* the desensitized state of the protein [148]. Moreover, in PA-containing membrane, nAChR leads to a dramatic increase in both the lateral packing densities and the gel-to-liquid crystal phase-transition temperatures of the reconstituted lipid bilayers [148, 149]. This strong interaction leads to the segregation of a PA-enriched domain from a complex mixture [149, 150].

The formation of a PA domain is the most likely explanation for the modulatory effects observed *in vivo* upon PA enrichment of oocyte membranes [151]. Moreover, similar PA domains critical for budding of viral particles, have been reported *in vivo* as the result of interaction between the host membrane and envelope viral proteins [152].

Regardless of the *in vivo* possible relevance, lipid modulation of the nAChR seems to be exerted both through specific interactions of cholesterol with non-annular protein sites and by the protein-induced segregation of a PA domains optimal for protein functioning.

G protein-coupled receptors

GPCRs regulate a wide range of cellular processes, including the senses of taste, smell and vision, and control a myriad of intracellular signalling systems in response to external stimuli. These transmembrane proteins interact with extracellular signals, usually through the binding of small signalling molecules, which induce a change in the conformation of the receptor. Such change is transmitted to the cytoplasmic face of the protein and enables a coupling with an intracellular heterotrimeric G protein (GTP-binding protein). The intracellular G protein, in turn, acts as a signal transducer that regulates the activity of 'effector proteins' (*e.g.* adenylyl cyclase, guanylyl cyclase, PLC, potassium channels, etc.). Diseases such as certain forms of blindness, obesity, inflammation, depression, cancer and hypertension, among others, can be linked to malfunctions of GPCRs.

Rhodopsin, perhaps the best-characterized GPCR and the only one to have its structure solved at high resolution, is modulated by membrane lipids. This protein contains a membrane surface recognition domain that adopts an amphiphilic helical structure as a function of membrane composition, PS

being most active in this regard. Such structural change mediated by membrane phospholipids may also contribute significantly to the optimal kinetic functioning of this prototypical G protein [153]. Also, it has been demonstrated that changing the bilayer composition produces changes in the rates of formation of both Metarhodopsin II and Metarhodopsin II-Gt complexes, demonstrating that the diffusion of receptor and G protein are sensitive to the lipid composition of the membrane [154].

Similarly, the angiotensin II receptor, which is also a GPCR, has a carboxy terminus that associates with the cytoplasmic face of the cell membrane *via* a high-affinity, anionic phospholipid-specific tethering that serves to increase the amphipathic helicity of this region [155]. Therefore, such associations with anionic phospholipids seem common in GPCRs and, as in the case of rhodopsin, it is possible that they might be relevant for receptor function. Many GPCRs have a lipid modification in the carboxy-terminal region that creates a fourth intracellular loop in their structure. In this context, palmitoylation/depalmitoylation regulates the coupling of the endothelin B receptor to different G α protein subtypes [156]. Studies on 5-HT_{4a} serotonin receptor and the luteinizing hormone receptor in palmitoylation-deficient mutant mice, further support the importance of receptor palmitoylation as a regulator of GPCR-mediated signal transduction [157, 158], further demonstrating the relevance of protein–lipid interaction in the function of these integral proteins.

As indicated above, most drugs under pharmaceutical development are targeted at GPCRs, because of the great variety of functions they control and their involvement either in the aetiology of diseases or in reversion of pathological states. This fact highlights the relevance of these membrane receptors and the control of cell signalling, exerted at the plasma membrane, in the cell's physiology and human therapy.

Other examples

Table 4 shows other documented cases in which the activities of integral proteins (ion channels and transmembrane receptors) have been shown to be dependent on membrane lipids. Again, such examples include cases where lipid–protein interactions seem very specific, along with others in which the

general properties of the lipid bilayer seem the determinant factor that influences protein function.

Non-permanent proteins in membranes

Association processes of proteins with membranes are not limited to constitutive membrane proteins, but rather include as well those that translocate or insert into a membrane at a specific juncture in their biological functions. The latter proteins are generally soluble and do not fold and assemble into membranes in a constitutive way. They develop instead the ability to insert and/or translocate into membranes under specific conditions and/or when exposed to lipid bilayers [159]. Thus in the living cell, a number of membrane proteins are permanently bound to the lipid bilayer, either as integral or as peripheral proteins (see above), whereas others will contact the membrane only under certain conditions, thereby remaining membrane bound, or returning promptly to the aqueous medium to which they belong (Table 5). This section deals with this kind of proteins that interact briefly with the cell membrane and with those that, becoming only occasionally in contact with the membrane, are irreversibly bound to it when they do. Both one and the other will interact in a more or less specific way with the bilayer and will cause some degree of bilayer perturbation. In both cases the nature of the interaction/perturbation will be directly related to the physiological or pathological role of the proteins. This heterogeneous group of molecules has been designated as *non-permanent* membrane proteins [160]. Figure 1 depicts a schematic representation of the various kinds of permanent and non-permanent membrane proteins.

The subject of proteins that can exist either free or membrane bound has been studied in the past by several workers. Wilson [161] called them 'ambiguitous proteins', and was perhaps the first to present, in a systematic way, the idea that variation in intracellular distribution may represent a regulatory mechanism to suit changing metabolic needs. Burn [162] introduced the concept of 'amphitropic proteins' to encompass the wide group of proteins that associate reversibly with membranes under certain physiological conditions. Later, Nelsestuen and coworkers

Table 4 Additional examples of interactions between ion channel or receptor proteins and membrane lipids

Membrane protein	Observed lipid effect	References
Kv 2.1	Cholesterol depletion favours inactivation	[329]
Kv 1.3	Ceramide inhibits channel activity	[330]
Kv 1.5	Targeted to caveola lipid rafts	[329]
Ca ²⁺ activated K ⁺ channels	Requires anionic phospholipids for channel activation Modulated by lipid bilayer thickness	[140]
K _{ATP} channel	Activation proportional to number of negative charges on the lipid head group	[331]
TREK	Activation by lysophospholipids as a function of acyl chain length and polar head group size	[332]
ENaC	Anionic phospholipids may mediate their regulation	[143]
TRP Ca ²⁺ channels	The existence of a lipid domain modulated the activity of the channel	[333]
NMDA	Correlation between membrane tension (induced by either mechanical or chemical stimuli) and internal Mg ²⁺ block	[334]
α7 nNAcChR	Target to lipid rafts in the somatic spines of ciliary neurons	[335]

Table 5 A classification of non-permanent membrane proteins (modified from [160])

Type	Examples	References
(A) According to the <i>reversibility</i> of the membrane contact		
(1) Proteins that interact reversibly with the membrane	Lipid transfer proteins Ceramide-activated protein kinase	[168, 179, 181]
(2) Proteins with very long-lived (irreversible) contacts	Bacterial toxins (<i>e.g.</i> aerolysin) Blood coagulation factors.	[187, 197]
(B) According to the <i>nature (strength)</i> of the interaction		
(1) Proteins that interact weakly with the membrane (extrinsic-like)	TrwD Protein kinases C	[176]
(2) Proteins that interact strongly with the membrane:		
(2.1) Without covalent modification of the lipid (intrinsic like)	RTX toxins Complement proteins	[196]
(2.2) With covalent modification of the lipid	Phospholipases Enzymes of lipid metabolism	[182]

exemplified in protein kinases C and the annexins the paradigm of proteins that are found either in soluble or membrane-bound forms – their change in location having important physiological consequences [163]. Also among the precedents of this concept, the work by Wimley and White [164] should

be mentioned. The latter authors achieved a quantitative description of the partitioning of peptides into membrane interfaces, by constructing an ‘interfacial hydrophobicity’ scale that has found important applications afterwards. Wimley *et al.* [165] defined as ‘non-constitutive’ the soluble proteins

that exert a biological function by undergoing transient bilayer insertion.

Non-permanent membrane proteins may be classified either according to the *reversibility of the membrane contact*, or to the *nature (strength) of their interaction* with the host membrane (Table 5).

Proteins that interact reversibly with the bilayers

These proteins have in common that, within a timescale compatible with the turnover time of membrane components (up to tens of minutes), the protein binds the membrane, and then comes back to the aqueous medium. This group encompasses a large variety of proteins, with widely diverging kinetics of membrane binding. They are often, but not always, proteins with specific lipid-binding sites. At the limit of the fast exchange we should mention the ceramide-activated protein phosphatases 1 and 2A [166]. In fact, these proteins have never been isolated in a membrane-bound form, but they contain a ceramide-binding site in their catalytic subunit, and strong experimental evidence points towards a direct interaction with ceramide [167].

Other ceramide-binding proteins are known to exist transiently in the membrane-bound form, such as ceramide-activated protein kinase [168], some isoenzymes of PKC [169, 170] or c-Raf-1 [171]. It has been proposed that ceramide binds proteins in this group through their cysteine-rich domains [172]. Recently, ceramide-1-phosphate has been shown to bind a cytosolic phospholipase A2 *via* interaction with its C2 domain [173].

A large number of proteins are known that bind transiently the cell membranes, and have a specific binding site for DAG (see [174, 175] for reviews). Chief among these are the PKC isoenzymes belonging to the so-called 'conventional' and 'novel' families. In these enzymes, that otherwise are found in the cytosol, DAG binding induces their docking to membrane, in addition to causing protein activation. The movement from cytosol to membrane is called translocation and constitutes an important event in signal [176].

Some heat-shock protein (Hsps) are associated with membranes, although they do not contain transmembrane domain or signal sequences, thus they can also integrate the category of non-permanent membrane proteins (see below). Recent studies indi-

cate that these proteins play an important role in membrane quality control and thereby potentially contribute to the maintenance of membrane integrity, especially under stress conditions [177]. The bacterial Min protein system prevents the aberrant localization of the cell division machinery at the cell poles. Min happens to be a non-permanent, or amphitropic protein, that binds anionic phospholipids and undergoes dynamic oligomerization on the membrane surface [178]. In this line, membrane lipid reorganization has been shown to play an important role.

Another important group of proteins that bind the cell membranes transiently is constituted by the so-called 'lipid transfer proteins' [179]. These are intracellular proteins with the capacity to bind a lipid from one membrane and release it to a different one. Some recent results have revealed their role as mediators between lipid metabolism and cell functions. For example, the Sec14-superfamily of PI transfer proteins are important in linking phospholipid metabolism, membrane traffic and intracellular signalling networks [180], and the ceramide transport protein transports the sphingolipid to the *trans*-Golgi apparatus, where it is converted into ceramide-1-phosphate, in turn an activator in the synthesis of prostaglandins [181].

Several enzymes involved in phospholipid metabolism are, by conventional standards, cytosolic, although they exert their catalytic properties in the membrane-bound state. The various phospholipases are often good examples of cytosolic or extracellular proteins that become transiently docked to membranes to perform their catalytic roles. All of the above enzymes possess lipid-binding sites, either regulatory or catalytic, thus their transient binding to membranes must be mediated by those specific sites. This is not the case, however, of the transiently membrane-bound CTP:phosphocholine cytidyltransferase, an important enzyme in the synthesis of PC whose substrates and products are water soluble, and has no specific lipid-binding site [182]. Studies in which membrane binding and activity of the purified enzyme were measured in lipid vesicles [183] showed that membrane binding of the cytidyltransferase required anionic lipids.

Bacteriophage M13 offers an interesting and rather unique example of a protein that becomes inserted into the cell membrane in the way of the integral proteins, yet insertion is reversible. During viral replication the major coat protein of M13 (protein

VIII) accumulates in the host cell membrane, in the form of an integral protein [184]. Inside the cell, the newly synthesized phage DNA is protected transiently by protein V. Viral extrusion occurs without lysis of the host cell and, simultaneously, protein V is released to the cytoplasm and protein VIII is taken up from the membrane. Protein VIII appears to exist in two conformations, α , or viral form, and β , or membrane form [184].

Proteins that interact irreversibly with the bilayers

In some cases, proteins that are not permanent constituents of the membrane become associated with it in an irreversible way. This occurs most often with proteins that are not encoded by the own cell genome, *i.e.* proteins from parasitic or toxic organisms. To mention a few examples, this is the case of equinatoxin II from the sea anemone *Actinia equina* [185], α -haemolysin from *Escherichia coli* [186], or the variety of 'pore-forming protein toxins' reviewed by Parker and Feil [187].

In all these cases, after insertion, the proteins behave exactly like any other integral protein in the cell membrane. Note that the toxins are released as soluble proteins to the aqueous medium, and the mechanism by which they undergo the transition from soluble (water soluble) to integral (lipid soluble) proteins is a fascinating mystery, and a difficult one to unravel.

The above concepts can be illustrated with the example of aerolysin, a 47-kD channel-forming protein that contributes to the pathogenicity of *Aeromonas hydrophila*, a bacterium associated with intestinal and deep wound infections [188]. The toxin is secreted in the form of an inactive precursor called proaerolysin that can be proteolytically processed to aerolysin by a number of proteases including trypsin and furin. The active form of the toxin is a water-soluble molecule that can spontaneously oligomerize, producing heptamers that may then insert into lipid bilayers, giving rise to discrete hydrophilic channels. On the basis of the crystal structure of proaerolysin [189], which reveals extensive β -structure, it seems likely that the oligomeric form of the toxin contains an amphipathic β -barrel analogous to that observed in the heptamer of *Staphylococcus aureus* α -toxin [190]. Aerolysin has

no apparent affinity for lipids until it has oligomerized, but the oligomer contains an exposed hydrophobic surface, presumably the outside of the amphipathic barrel, and can insert directly from solution. How such a large structure as the barrel penetrates a lipid bilayer is a largely unexplored puzzle. Alonso *et al.* [191] have proposed that the aerolysin oligomer may overcome the barrier of the polar interfacial region by destabilizing the bilayer locally, causing the formation of non-lamellar structures. Using liposomes as the host membranes, those authors found that the inclusion of lipids that facilitate the lamellar-to-inverted hexagonal phase transition enhanced aerolysin insertion, whereas the opposite occurred when lipids that stabilize the lamellar phase were present.

An additional aspect that complicates the taxonomy of non-permanent membrane proteins is that there are examples of proteins that can bind lipid bilayers either reversibly or irreversibly, depending on the composition and physical properties of the bilayer. This is the case of *E. coli* α -haemolysin that binds reversibly bilayers in the gel state and irreversibly those in the liquid-crystalline state [192, 193]. Also, recent data [194] suggest that a fraction of α -haemolysin is secreted from the bacterium bound to outer membrane vesicles, from which the toxin is transferred to the target cells, thus α -haemolysin is reversibly bound to the secretion vesicles before becoming irreversibly inserted into the target membrane. Equinatoxin II binds reversibly pure PC bilayers, but irreversible insertion occurs when SM is incorporated in the bilayer composition [185, 195]. However, binding to cell membranes is almost always irreversible, and this is why these toxins are best classified within the group of non-permanent membrane proteins that become irreversibly bound.

In the previous paragraphs we have seen examples of bacterial or viral proteins that become part of the host cell membrane. The opposite may occur as well, when the host tries to attack an unwanted parasite, as in the case of complement-mediated bacterial killing. Complement proteins, of which more than 20 are known, exist in blood as part of the innate immune system. Proteins 5b and 9 of the complement system form the so-called 'membrane attack complex', that binds the outer membrane of Gram-negative bacteria. The bactericidal activity of complement is dependent upon C9, but currently it is not

understood how this protein translocates across the periplasm and dissipates the potential across the inner membrane [196].

In some cases, proteins encoded by the same organism may bind irreversibly its cell membranes. This is the case of several proteins involved in blood coagulation, *e.g.* factor X [197], factor VIII [198] or factor V [199]. To mention but one example, factor V circulates in plasma as a single chain procofactor (330 kD), from which derive the two chains, respectively, 94 and 74 kD, of the active form, factor Va. Factor Va facilitates activation of prothrombin to α -thrombin by factor Xa. For this purpose, the serine protease factor Xa and factor Va assemble on a membrane surface in the presence of calcium ions. Insertion of factor Va in lipid bilayers containing neutral and acidic phospholipids has been studied by Majumder *et al.* [200]. Membrane association of factor Va appears to be a complex process involving both chains of the protein, changes in lipid packing, and in protein conformation. Interestingly, membrane binding also facilitates proteolytic degradation of factor Va [199].

Non-permanent membrane proteins can also be classified according to the *nature (strength) of their interaction* with the host membrane (Table 5).

Proteins that interact weakly with the membrane

These are proteins that remain membrane-bound through non-covalent forces other than hydrophobic interactions. Electrostatic and polar forces are the most relevant in this case. This group of proteins overlaps almost exactly with those that bind reversibly the cell membranes: many ceramide- and DAG-activated proteins involved in cell signalling belong to it. However, phospholipases and other enzymes of lipid metabolism that induce covalent modification of membrane lipids should not be included here, because for the most part they bind the membrane through strong, through transient, hydrophobic forces.

Proteins that interact strongly with the membrane

The proteins in this group are bound to the membrane mainly, but not exclusively, through hydropho-

phobic forces. This does not mean that hydrophobic forces are particularly strong. When hydrophobic interactions are individually considered they are actually rather weak (of the order of 5 kJ/mol), their strength coming from the fact that, within the non-polar membrane matrix, a multitude of hydrophobic interactions join their forces providing an overall strong bond for the incoming protein. Among the non-permanent proteins that interact strongly with the membrane, an important distinction must be made between:

- 1 Proteins whose interaction does not lead to covalent modification of the membrane lipids and
- 2 Proteins whose interaction with membranes does lead to covalent modification of the lipids.

In other words, strongly bound non-permanent membrane proteins may or may not have an enzyme activity on the lipids. Among the proteins devoid of enzyme activity on lipids are the toxins mentioned above: α -haemolysin, equinatoxin II, aerolysin and many others, but note that there are bacterial toxins with phospholipase activity. The latter, together with the physiological phospholipases (including SMase) and other enzymes of lipid metabolism constitute the second subgroup.

From a thermodynamic point of view, cell membranes constitute an open system, far from equilibrium, in constant exchange of matter and energy with their environment. Molecules as important (qualitatively and quantitatively) as proteins may become in contact with the membranes, either for short or for long periods. The term 'non-permanent proteins' has been suggested [160] to encompass the variety of such molecules that, at some stage, come to interact with any of the cell membranes. Some of them are originated by the own cell genome, others arise from a foreign genome. New methods to study the interaction of the membrane with these 'visitors', particularly with those that favour very short visits, will have to be developed. Our view of the structure and dynamics of cell membranes will have to be gradually broadened, to encompass the increasing number of proteins that, being only transitorily part of the membrane, are not less worthy of attention than the more permanent ones. The important observation has been made [201] that non-permanent proteins can generate large local tensions in the membrane, that frequently relax into a variety of physical effects along lateral and transverse planes of the membrane. In turn these effects can mediate molecular and structural changes

modifying membrane topography and dynamics, and ultimately membrane biochemical reactivity.

In the next paragraphs, we will review the molecular bases of the interaction of specific proteins with membranes as examples of the general principles depicted in this section.

G proteins and their interactions with membranes

Interactions with lipids regulate the localization and activity of many amphitropic proteins. These proteins can interact with various types of membrane lipid species or lipid structures in defined membrane microdomains, depending on their transient structural status, on their tertiary structure or on the modifications in the number of oligomeric subunits in the complexes. Therefore, the messages they propagate into the cell will somehow depend on these protein–lipid interactions. Here, we shall review the interactions of several types of amphitropic proteins with membranes and the role these interactions play in the cell's physiology.

GPCRs constitute the largest gene family of membrane receptors (see above sections) and they are involved in controlling a wide number of cellular and physiological processes, such as: vision, taste, smell, blood pressure, metabolism, cell proliferation, body weight, neurotransmitter release, affective status, etc. [202, 203]. Agonist-mediated activation of these receptors propagates signal transduction through heterotrimeric G proteins. Hence, the wide range of receptor types and the relevance of the functions they control, makes *via* G protein signalling one of the most important cell signalling pathways in the cell (for a review, see [202, 203]). GPCRs undergo conformational changes upon receptor activation that activate the associated heterotrimeric G proteins. However, recent evidence indicates that some GPCRs are able to trigger signalling events in cells without the intervention of G proteins [204, 205], and that G proteins can be activated by means of GPCR-independent activators of G protein signalling proteins (AGS proteins) [206, 207]. There are at least 16 types of $G\alpha$ subunits, 5 of $G\beta$ subunits and 12 types of $G\gamma$ subunits encoded by the G protein gene family [208]. $G\alpha$ subunits can be divided at least into four structurally related categories: $G\alpha i/o$, $G\alpha s$, $G\alpha q/11$ and $G\alpha 12/13$, although other classifications based

on their functional properties have been also proposed. Among all the G proteins, the GTPase domain is highly conserved whereas the helical domain that directs the coupling with the GPCRs varies [208].

The association between an extracellular agonist ligand and the GPCR molecule induces the productive coupling to intracellular heterotrimeric ($G\alpha\beta\gamma$) G proteins. This coupling promotes the exchange of guanosine diphosphate (GDP) for GTP on the $G\alpha$ -subunit and the $G\alpha$ subunit then dissociates from the $G\beta\gamma$ dimer, the GPCR moving to other membrane domains. Both, $G\alpha$ and $G\beta\gamma$ proteins exert their action by modulating the activity of different intracellular effectors and kinases, the latter being responsible for receptor phosphorylation and inactivation. While the GPCR remains active, several G protein molecules can be activated by a single receptor molecule, resulting in the amplification of the incoming message. For effective signal amplification, a large number of G proteins must be available near to the receptor molecule, which is achieved by specific protein–lipid interactions. Termination of $G\alpha$ signalling activity occurs when the γ -phosphate of GTP is removed by the intrinsic GTPase activity of the $G\alpha$ -subunit, leaving GDP in the nucleotide-binding domain of $G\alpha$. As a result, the $G\alpha$ -GDP re-associates with the $G\beta\gamma$ complex and the G protein trimer is again ready to be activated by a receptor molecule [209]. Activated $G\alpha$ monomers regulate the activity of effector proteins, such as adenylyl cyclase, PLC, K^+ channels, etc., which in turn control the cytosolic levels of second messengers. Most effector proteins stimulated or inhibited by G protein subunits are to some extent membrane-bound proteins and therefore, they are associated with lipids. In this context, G protein–lipid interactions may also play a role in driving G protein subunits to specific membrane domains where their protein targets reside. G protein-mediated signalling is further regulated by the binding of regulators of G protein signalling (RGS) to $G\alpha$ subunits. RGS proteins constitute a large group of proteins that stimulate the GTPase activity of $G\alpha$ subunits [209, 210]. In the past few years, it has become clear that the role of RGS in G protein signalling is not the mere inactivation of $G\alpha$ subunit but rather, they act as mediators of signalling linking G proteins with signal transduction pathways that influence cell motility, intracellular trafficking and important responses such as cell growth and differentiation [210].

G proteins bind tightly yet reversibly to cell membranes, and lipid modifications to their α and γ subunits assist in their membrane docking [1]. Indeed, both lipid–lipid and protein–lipid interactions participate in the binding of G proteins to membranes [3, 99]. $G\alpha$ subunits are modified at their N-termini by myristoylation and/or palmitoylation, whereas farnesyl or geranylgeranyl isoprenoid moieties can be added to the C-termini of γ subunits. Myristoylation occurs co-translationally on glycine residues of $G\alpha$ proteins [211, 212], whereas other $G\alpha$ proteins (with few exceptions) are post-translationally and reversibly palmitoylated on cysteine residues [211, 212]. The regulation of the state of palmitoylation by GPCR-mediated activation influences the cellular localization of $G\alpha$ subunits and the propagation of signals through these membrane receptors [8, 213–216].

All 12 known $G\gamma$ proteins are isoprenylated on carboxy-terminal cysteine residues *via* a thioether bond. Either a farnesyl moiety on the $G\gamma_1$, $G\gamma_9$ and $G\gamma_{11}$ subunits (CVIS motif) or a geranylgeranyl moiety on the $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, $G\gamma_5$, $G\gamma_7$, $G\gamma_8$, $G\gamma_{10}$, $G\gamma_{12}$ and $G\gamma_{13}$ subunits (CAIL motif) is recognized by the corresponding enzyme [1]. Isoprenoids bind with high affinity to non-lamellar-prone lipids (*e.g.* PE) and increase their hexagonal (H_{II}) phase propensity [2]. This lipid modification is involved not only in G protein–membrane interactions but also in the formation of non-lamellar-prone membrane domains [2, 3]. Moreover, regions with high non-lamellar-phase propensity are important in the activity of the various forms of G proteins produced during their cycle of activity: Both $G\alpha\beta\gamma$ heterotrimers and $G\beta\gamma$ heterodimers prefer non-lamellar prone domains (PE-rich), whereas $G\alpha$ subunits prefer lamellar regions (*e.g.* lipid rafts) [6]. The behaviour of these proteins would explain the accumulation of heterotrimeric G protein molecules near receptors, where they are needed in a molar excess to amplify GPCR-mediated signals. Similarly, these preferences explain how $G\alpha$ subunits leave the receptor microdomain upon activation and dissociation from the $G\beta\gamma$ complex, facilitating their encounters with signalling effectors found in other membrane domains. This model also explains why $G\beta\gamma$ dimers remain around GPCRs where they may recruit GRK to phosphorylate and inactivate the receptor protein. Thus, lipid structures fulfil an active role in signal propagation, generating first a pool of $G\alpha\beta\gamma$ proteins near GPCRs, and later

defining the localization of $G\alpha$ and $G\beta\gamma$ proteins to different membrane regions [6].

In addition to lipid–lipid interactions, G proteins are also associated with membranes *via* electrostatic interactions between specific amino acids and membrane phospholipids. The inner leaflet of the membrane contains relatively large amounts of PS, so that protein–lipid interactions between this anionic phospholipid and the cationic amino acids from G proteins may also participate in the docking of this transducer to membranes [3, 99].

Although GPCRs have gathered the attention of academia and industry to delineate therapies, G proteins themselves have not been on the focus of the design of therapeutic drugs. This is in part due to the fact that small-molecule ligands for GPCRs differ among receptor types and specific molecules with high subtype recognition have been or can be synthesized, whereas all G proteins bind the same ligands: GDP, GTP and their synthetic analogues. However, G proteins show a greater variation in their membrane–lipid interactions. Moreover, a given $G\alpha$ protein interacts differentially with membranes in its monomeric and trimeric forms or when it has or lacks a palmitic acid moiety, which results in different activity states. This possibility to regulate G protein activity through regulation of their lipid environment has been recently used to develop drugs for treatment of cancer, obesity, hypertension, etc. [2, 3, 9, 16].

Small monomeric G proteins: the Ras and Ras-like family

Many growth factor receptors activate Ras and Ras-like proteins, most of them belonging to the tyrosine kinase receptor family. Mammalian Ras proteins (H-Ras, K-Ras4A, K-Ras4B, N-Ras) have a molecular weight of 21 kD and except for K-Ras4B (188 amino acids), they contain 189 amino acids. The N-terminal 85 residues of the four members are identical and their similarity remains as high as 90% over the following 80 residues. Accordingly, the principal source of divergence among Ras isoforms is restricted to the 24 C-terminal amino acids, which display less than 15% homology [217]. This diversity is related to the different membrane anchors used by the Ras isoforms to interact with the plasma membrane. H-Ras forms transient interactions with lipid rafts when bound to GDP, whereas when bound to GTP it

aggregates with cholesterol-insensitive, galectin-1-dependent, non-raft domains. On the other hand, K-Ras is clustered in cholesterol-insensitive, non-raft domains that differ from the activated H-Ras microdomains [218, 219]. H-Ras, N-Ras and K-Ras4A are palmitoylated on cysteine residues in the hypervariable domain, and they are isoprenylated at their C-terminal cysteine (Cys186). In contrast, K-Ras4B is not palmitoylated but rather it is targeted to membranes by the combination of a polybasic domain and isoprenylation. Polyisoprenylated but non-palmitoylated H-Ras protein is biologically fully active and it associates weakly with cell membranes. However, palmitoylation increases the membrane binding of H-Ras and enhances its transforming activity [220].

The Ras-like small GTPase family is made up of several members, such as Ras, Rap1, Rap2, R-Ras, Ra1, Rheb, M-Ras and TC21 [217]. Ras-like proteins play important roles in various cellular signal transduction pathways, regulating differentiation and proliferation through their interaction with several signal transduction proteins known as Ras effectors. These effectors include a large number of protein kinases, lipid kinases and guanine nucleotide exchange factors, such as SOS and CD25 [218–224].

Like the γ subunit of heterotrimeric G proteins, Ras and Ras-like proteins contain a CAAX isoprenylation motif at their carboxy terminus (C for cysteine, A for aliphatic and X for any amino acid, see above) [81, 225]. This post-translational modification favours the anchoring of proteins to membranes and modifies the structural properties of membranes. In this way, non-lamellar-prone regions are generated that further increase the affinity of this lipid for membranes, and the segregation of membrane microdomains enriched in isoprenoids [2, 3, 226–228]. Prenylation of Ras and related proteins is a complex process, frequently followed by proteolytic cleavage by zinc metalloproteases such as AFC1 and RCE1 [229, 230]. This process removes the last three amino acid residues, altering the isoprenyl-modified cysteine and making this C-terminal lipid modification permanent.

Several proteins of this family are also palmitoylated at 1 or 2 cysteines near the farnesylated carboxy-terminus [231]. In recent years, the post-translational lipid modification of Ras and other membrane-associated proteins has been associated with processes

other than membrane anchorage. Lipid modifications, such as isoprenylation and N- and S-acylation also play important roles as specific recognition elements for protein–protein interactions, as well as hydrophobic switches that permit the temporal regulation of docking to sub-compartments like lipid rafts, caveolae [232] and other cellular locations [233]. Palmitoylation augments the affinity of Ras proteins for membranes and activates the mitogen-activated protein kinase (MAPK) pathway [234]. In contrast to farnesylation, palmitoylation of Ras and Ras-like proteins is reversible, reflecting its connection to regulatory phenomena [228, 234]. Ras activation is the first step in the MAPK pathways, an important and conserved signal transduction mechanisms in eukaryotes [235, 236], exemplified by the number of different and interconnected MAPK signal transduction pathways that coexist within cells [235, 236]. In this context, Ras activates Raf (MAP kinase kinase, MAPKKK) through interactions that occur at the plasma membrane. Activated (phosphorylated) Raf activates MAPKK (MEK), which in turn activates MAPK (also called extracellular signal-regulated kinase, ERK). The transmission of intracellular signals is then produced by sequential phosphorylation (and activation) of the components specific to any respective cascade [235, 236]. In mammals, a number of MAPK pathways coexist, including the ERK1/2, c-Jun N-terminal kinase (JNK) and p38 MAPK cascades [237, 238]. These signalling pathways regulate critical events in the cell, such as proliferation, apoptosis and the cell's response to a variety of stimuli. Because the first steps in propagating signals *via* these cascades are associated with membranes, protein–lipid interactions are crucial in the regulation of distinct cellular activities.

Mutations of Ras proteins have been found in about one third of all human cancers. Because their tumourigenic potential is lost if their interactions with membrane lipids are impaired, some therapies have aimed inhibition of Ras-membrane interactions. However, cancer cells can bypass farnesyl transferase inhibition through addition of a geranylgeraniol moiety to the C-terminal cysteine that it is usually farnesylated. In any case, future treatments involving combined farnesyl and geranylgeranyl transferase inhibition or even alternative ways to control Ras-lipid interactions through other membrane-lipid therapy approaches might be of clinical interest.

Protein kinase C

Since its identification in the bovine cerebellum [239], membrane-associated PKC isozymes have been shown to be fundamental signal transduction molecules, involved in a huge variety of events including cell-cycle regulation, cellular survival, malignant transformation and apoptosis [240]. PKC isoforms can be categorized into three groups depending on the cofactors that regulate their activity: conventional (c) PKC isoforms (α , β _I, β _{II} and γ) that require Ca^{2+} and DAG; novel (n) PKC isoforms (δ , ϵ , ζ , η and μ) that are only activated by DAG; and atypical (a) PKC isoforms (χ , ι and its murine homologue, λ) that do not require Ca^{2+} or DAG. The distinct PKC isoforms exert different responses depending on the cellular context, highlighting the necessity to understand the signalling events controlled by PKCs in each cell type [241].

A common feature of all PKC isoforms is that their activation is dependent on establishing a close interaction with membrane lipids. Indeed, current models for the interaction of PKC with phorbol esters consider lipids to be essential cofactors of the enzyme [242, 243]. PKC can interact with different lipids in a variety of ways although the regulatory region of (c) PKCs contains two membrane-targeting domains, C1 and C2. Whereas the C1 domain is composed of two cysteine-rich zinc finger motifs that bind DAG and phorbol esters (C1a and C1b), the C2 domain is responsible for Ca^{2+} -dependent membrane binding [244]. DAG is a by-product of the digestion of PI by PLC and it activates this kinase both through specific protein–lipid interactions and through the induction of non-lamellar phases [175]. In fact, non-lamellar (H_{II}) phase propensity has been shown to be involved in the translocation of PKC from the cytosol to membranes, a phenomenon associated with enzyme activation [4, 245]. On the other hand, some studies suggest that a specific interaction between PKC and PS occurs [246–248], although other studies have indicated that different anionic phospholipids [249], or even neutral phospholipids like PC [250], can also activate PKC. These works demonstrate the importance of PKC–lipid interactions in the activity of this enzyme, and have been related to the therapeutic activity of some drugs against cancer, such as Minerval [9, 245].

It has been proposed that substrate binding can induce the translocation of PKC α to the membrane

and that removal of its pseudosubstrate domain may be coupled to a conformational change that results in exposure of hydrophobic groups [251]. Alternatively, myristoylation of PKC substrates promotes their attachment to the membrane, and the associated enzyme–substrate co-localization would be reflected in more efficient catalysis [252–254]. In this regard, myristoylated peptides mimicking the pseudosubstrate regions of a number of PKC isoforms have been used as specific and efficient enzyme inhibitors [252–255]. These studies highlight the relevance of protein–lipid interactions in co-localizing proteins that must physically interact to yield the productive propagation of signals. Thus, lipids also regulate protein activity by modulating their cellular localization.

In addition to membrane phospholipids, *cis*-unsaturated fatty acids such as arachidonic, linoleic and oleic acid can also activate PKC independently from DAG [250, 256, 257]. These lipids increase the non-lamellar (H_{II}) phase propensity of membranes [258], which in turn favours the translocation of PKC to membranes [4]. In this regard, the 18:1/22:6 species of PE but not those of PC cause an increase in the rate of histone phosphorylation by PKC beyond that caused by other less unsaturated PEs [259]. Similarly, in model systems of pure membrane-forming phospholipids and purified PKC α , non-lamellar-prone membranes of dioleoyl phosphatidylethanolamine have a greater capacity to bind PKC α than membranes containing only the lamellar-forming phospholipid dioleoyl phosphatidylcholine [245]. The importance of this phenomenon has been highlighted by the development of a new anti-cancer drug (Minerval) that increases PKC binding to natural and model membranes containing PE [245].

Membrane microdomains and lipid mediators in the control of the heat shock protein response

Induced by a wide range of stressors, ranging from temperature stress to hypoxia, inflammation, infections or environmental pollutants, stress proteins, also termed Hsps, play key roles in all living systems [260]. Their major conserved classes are grouped according to their molecular weights (Hsp100, Hsp90, Hsp70, Hsp60 and the ‘small Hsps’, sHsps)

and some Hsps are encoded by more than one gene [261]. Acting as molecular chaperones, Hsps are able to recognize unfolded and/or damaged proteins and further sort them for repair (refolding) or proteolysis. Hsps can regulate the life or death of cells by directly modulating certain apoptotic signalling events, or indirectly, by participating in antigen processing [261]. Hsps reside not only in the cytosol, but also in the plasma membrane, lysosomes, nucleus and mitochondria. The glucose-regulated Hsp prologues (Grps) reside in the ER. Surprisingly, some Hsps are localized in the extracellular space [261].

Stress sensing and signalling: the membrane sensor theory

Due to their multiple and vital functions briefly highlighted above, Hsps play fundamental roles in the aetiology of several human diseases [262]. Aberrantly high levels of either the overall array of Hsps, or certain Hsp classes are characteristic in different cancer cells and the converse situation applies typically for type 2 diabetes, neurodegeneration, cardiovascular diseases or aging [11, 16]. In certain cancers, development and metastatic potential favours tumours that express a higher level of sHsps and a lower level of Hsp70 in their plasma membranes [263]. Accordingly, it is of key importance to understand the mechanism whereby cells can elicit a Hsp response and regulate the cellular translocation and membrane association of various Hsp classes [11, 16]. As a commonly accepted paradigm, it was earlier suggested that stress-induced protein denaturation serves as a primary stress-sensing machinery that triggers Hsp gene expression [264, 265]. During the past decade, a new, but not exclusive model, the 'membrane sensor' model, has emerged, which predicts the existence of a membrane-associated stress sensing and signalling mechanism from prokaryotes to mammalian cells [12, 266–273]. In favour of this model, the exposure of mammalian cells to various membrane fluidizers, or compounds with the ability to interact with certain membrane lipids, substantially modulate Hsp expression without inducing protein unfolding [266]. It was recently documented in a cellular melanoma model that changes in the physical state (fluidity) and the concomitant destabilization/reorganization of cholesterol-rich membrane microdomains may indeed serve as

such a 'molecular switch' and is sufficient for the operation of these 'cellular thermometers' [11, 16, 270]. Thus, although many inducers/silencers of the Hsp response may function through a protein-unfolding pathway, some inducers/silencers may work through a distinct mechanism.

The plasma membrane, which is the barrier to the external environment, is well suited for sensing stress and acts as an important regulatory interface. As detailed elsewhere, even subtle alterations in its lipids (by causing 'membrane defects') may influence membrane-initiated stress-signalling processes by changing the global fluidity, the membrane thickness, the local organization of microdomains and thereby the clustering of receptors or other proteins localized in the plasma membrane [11, 16, 262].

Hsp signalling in cancer and diabetes

We recently suggested that, in such prominent disease states as insulin-resistant diabetes and cancer, where the directions of Hsp dysregulation and membrane fluidity run in parallel but opposite manners, there must exist a conserved signalling cascade which is uniformly controlled by membrane hyperstructures and ultimately affects the level of Hsp expression as well [11]. Signalling from the transmembrane growth factor receptors to Hsp genes fulfils such a criterion. PI3K, Akt and GSK3, the latter as a negative regulator of HSF1 activation [274], are central components of such a signalling cascade. Insulin resistance and type 2 diabetes are known to be associated with low Hsp levels, and with decreased PI3K and enhanced GSK3 activities (see references in [275]). Correction of a low Hsp state improves insulin resistance [275]. A close chemical relative of the membrane-intercalating drug candidate, the hydroxylamine bimoclomol [276, 277], BRX-220, has been reported to improve insulin sensitivity of both Zucker diabetic fatty rats and streptozotocin-diabetic rats [278]. Another hydroxylamine analogue, the Hsp co-inducer BGP-15, has also successfully passed a phase IIa human clinical trial as an insulin-sensitizer compound [279]. Using BGP-15 to achieve an elevation of Hsp72 protein, protection against diet or obesity-induced hyperglycaemia, hyperinsulinemia, glucose intolerance and insulin resistance was observed. This protection was tightly associated with the prevention of JNK phosphorylation [280].

As documented by Khaleque *et al.* [281], Hsp elevation in tumour cells can be induced by the malignant growth factor heregulin β_1 (HRG β_1), which causes homo- and heterodimerization between each member of the four ErbB receptor molecules *via* 'horizontal signalling' in the plane of the plasma membrane. The formation of raft-associated growth factor receptor dimers is followed by tyrosine phosphorylation of their intracellular domains. The major downstream signalling pathways include the Ras-Raf1-Mek-ERK and PI3K-PDK1-Akt pathways [281]. HRG β_1 appears to be linked to Hsp expression by its activation of HSF1 through inhibition of the constitutive kinase GSK3. It should be noted that the transmission of a proper signal from the cell surface to Hsp genes is uniformly dependent on precise regulation by the lipid composition of the membranes. The localization of growth factor receptors to distinct microdomains possessing a well-balanced ratio of inner and outer leaflet lipids appears to modulate both their ligand binding and tyrosine kinase activities [282]. Moreover, the major signal termination mechanism, *i.e.* the lateral movement of dimerized receptors in the plane of the plasma membrane and the delivery of activated receptor-containing endosomes, is totally lipid dependent [283, 284].

The role of membrane microdomains

The exact role played by lipid microdomains in membrane-directed stress sensing and signalling is far from clear. The related studies have been hampered by the lack of suitable physical methods for the visualization of membrane microdomains in intact cells [20, 285]. Membrane structures in mammalian cells display an enormous variety of lipids, functions, localizations, associations and intimate links with neighbouring membranes, and this makes their study extremely difficult. It is tempting to speculate that one of the major roles of the more than one thousand lipid molecular species in mammalian membranes is to provide an on-off switch for signalling events at the membrane level ([286] and see references within). Membrane lipids are among the molecules that adapt best in response to various environmental perturbations. Even subtle changes in the compositions of acyl chains or head groups can alter the packing arrangements of lipids within a bilayer. As a chain reaction, altered lipid packing properties change the

balance between bilayer and non-bilayer lipids, affect the bilayer stability and fluidity, and ultimately alters the lipid-protein interactions and microdomain organizations. External factors, including temperature, chemicals, ions, radiation, pressure, nutrients, the growth phase of cultured cells, etc., are all capable of changing the membrane packing, order and lipid composition (see references in [286]). Our understanding of the plasma membrane has changed considerably as our knowledge of membrane microdomains, rafts, has expanded.

We should point out that the 'lipid building blocks' of cell membranes can also serve as a source of those mediators which can be involved in the activation or attenuation of Hsp signalling pathways. Lipids, lipid kinases and lipid phosphatases have not received the same amount of attention as proteins in studies of signal transduction [287]. Thus, stresses and clinical conditions can induce alterations in the raft organization, but also give rise to changes in the metabolism of membrane lipids in producing a unique set of lipid mediators with the potential of retailoring the pre-existing Hsp profile [11, 16, 20, 262, 286].

Lipid mediators of the stress response

Many of the lipid mediators (*e.g.* leukotrienes, prostaglandins and certain lysophospholipids) leave the host cells and bind to GPCRs in the surface membrane of the same or neighbouring cells. Other lipid signals converge on PLC, PIs or Ca^{2+} . As highlighted in Fig. 7 the typical non-raft phospholipids and raft lipid classes (*i.e.* cholesterol and SM) can equally serve as stress-response modulating factors. Phospholipids are reservoirs of arachidonic acid. Phospholipase A₂ is known to be activated by different stressors, and the concomitant release of arachidonic acid can stimulate heat shock factor1 (HSF1)-DNA binding, increase the phosphorylation of HSF1 and ultimately up-regulate some Hsps [288]. On the other hand, arachidonic acid is used in the synthesis of prostaglandins *via* cyclooxygenases, some of which are also potent Hsp inducers ([289] and see references in [16, 287]). Moreover, certain lipoxygenase products of arachidonic acid, such as 12-hydroxyeicosatetraenoic acid, have also been shown to induce the expression of individual Hsps in human leucocytes [290]. Upon exposure to

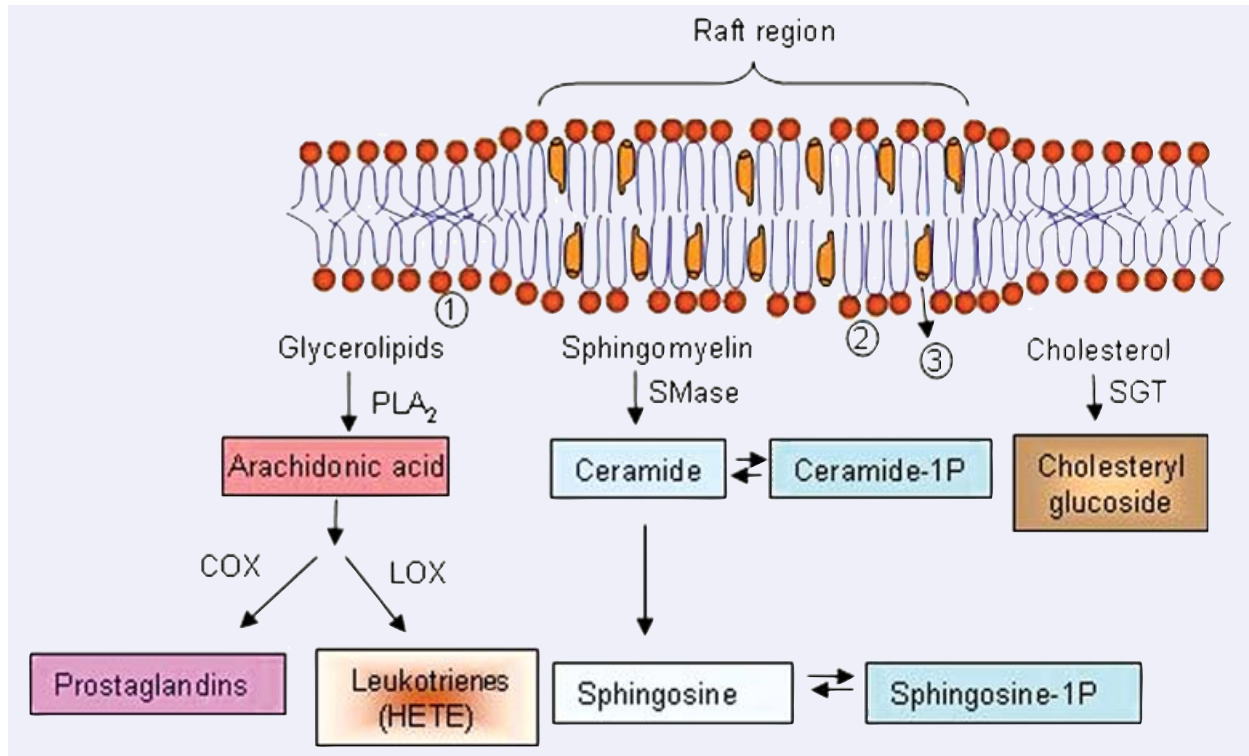


Fig. 7 Some typical lipid mediators capable of altering the Hsp response. The parental molecules of a variety of lipid mediators (boxes) are: (1) glycerolipids of the bulk membrane; constituents of rafts such as (2) SM and (3) cholesterol. PLA₂: phospholipase A₂; SMase: sphingomyelinase; SGT: sterol glucosyltransferase; COX: cyclooxygenase and LOX: lipoxygenase.

environmental stress, cholesterol can rapidly transform to cholesteryl glucoside, and its production is followed by the activation of certain protein kinases also engaged in the induction of Hsps [291]. Similar to cholesterol, SM may play both structural and functional roles as a raft lipid, and in parallel it takes part in the generation and transduction of Hsp signals. Within the large SM family of lipid mediators, the reactions are reversible and the lipids are interconvertible. Ceramide and sphingosine can be released by the sequential cleavage of SM under various stress conditions [292]. Ceramide, ceramide-1-phosphate, sphingosine and sphingosine-1-phosphate have all been shown to carry out second messenger functions linked ultimately to refinement of the cellular Hsp pattern [293].

In conclusion, the above observations indicate that Hsp expression in response to various stressors is regulated by differential control mechanisms rather

than by a uniform mechanism. It appears that one type of signal in the multistep activation pathway of HSF might arise from the exposure of hydrophobic domains of cytosolic proteins and in such cases typically the entire array of stress proteins is induced. There must exist, however, 'alternative' sensing-signalling pathways, which cause the expression of varying Hsp profiles obtained with different stressors. We propose that changes in the composition and microdomain organization of membranes offer the solution to the problem and represent a new theory: subtle changes of membrane lipids can cause remarkable alterations in the expression pattern of Hsps. Changes in lipid composition and membrane microheterogeneity during stress or disease states could trigger selective stress signalling responses either through global effects on the physical state of the membrane matrix, or *via* specific chemical interactions of boundary lipids with membrane proteins.

Various membrane imaging technologies, real-time detection and the monitoring of rafts and combination of these technologies with lipidomics and computational methods may allow the identification and characterization of the hypothetical stress-sensory membrane domains. Hsps could be induced or inhibited by drugs that specifically change the activity of such sensory lipid–protein membrane hyperstructures according to the principles of membrane-lipid therapy [9, 11].

A subpopulation of Hsps can interact with and translocate through membranes

The presence of Hsps in membranes and lipid rafts is widely documented. On the basis of prokaryotic models, we suggested earlier that a lipid-selective association of a subpopulation of Hsps (GroEL and sHsps) with membranes, leading to increased molecular order, may in turn result in down-regulation of Hsp gene expression [177, 269, 294–297]. Such hypothetical ‘cross-talk’ between the membrane primary stress sensors (see above) and Hsps suggests a feedback loop mechanism in the regulation of Hsp genes [11, 16]. In addition, interactions between certain amphitropic Hsps and specific lipid domains can remodel the pre-existing architecture and physical order of membranes. In parallel with the microdomain reorganization and thermal stabilization, we have shown that highly saturated ‘heat shock lipids’ can accumulate in membranes and exhibit a preferential interaction with sHsps [298]. Non-bilayer-forming lipids are known to be important controlling factors of cell signalling because they regulate the localization (and thereby activity) of key signalling proteins such as PKC and G proteins [4, 9]. It has been demonstrated that, in membranes composed of non-lamellar lipids, sHsps inhibit the formation of inverted hexagonal structures and are thereby important determinants of the membrane lipid secondary structures. Evidence from FTIR and DSC studies has indicated that the interactions of sHsps are preferential by/with anionic lipids and affect both the polar headgroup region and the hydrophobic core [177, 295, 297]. We inferred from these results that the association between sHsps and membranes may constitute a

general mechanism that preserves the membrane integrity under fluctuating, stressful conditions. Moreover, we reasoned that the specific Hsp–lipid interactions may serve as an unrecognized means for the spatial separation and distinct compartmentalization of Hsps to lipid domains which are thought to be involved in various stress signalling pathways [11, 16, 286].

Hsp90 in eukaryotic membranes

The presence of Hsps in plasma membranes and lipid rafts of mammalian cells is widely documented. Much less is known, however, about the mechanism controlling the translocation of Hsps to membranes and the modes of their interactions with membrane lipids, membrane proteins or both. It has been shown that Hsp90 is an iron-binding protein associated with the plasma membrane of HeLa cells [299]. Moreover, the interactions of STAT3 with caveolin-1 and Hsp90 in plasma membrane rafts have been revealed to play a role in the preservation of cytokine signalling during fever [300]. Further, the interaction of Hsp90 with the heterotrimeric G protein $G_{\alpha 12}$ has been demonstrated to target $G_{\alpha 12}$ to lipid rafts [301]. Hsp90 is present in membrane microdomains, together with CD14 and other molecules, following lipopolysaccharide-induced cell activation. Lipid raft integrity is essential for the process [302]. In fact, treatment with the Hsp90 inhibitor geldanamycin ameliorates the response to LPS in murine macrophages by decreasing CD14 surface expression, presumably due to the improper folding and arrest of CD14 within the endoplasmic reticulum [303]. ErbB2, a member of the EGF receptor family of tyrosine kinases is overexpressed on many tumour cells of epithelial origin and colocalized with Hsp90 in plasma membrane. It is the molecular target of trastuzumab (Herceptin), the first humanized antibody used in the therapy of solid tumours.

Hsp70 in cell membranes

Direct and specific interactions of Hsc70 (the constitutive form of the 70 kD Hsp) and Hsp70 with membrane lipids have been suggested to play a role in the folding of membrane proteins and the translocation of polypeptides across membranes [304]. The

interactions of Hsp70 with lipids are additionally indicated by their intrinsic capacity to open ion conductance channels or by aggregating liposomes. The interactions of Hsp70 with lipids are highly dependent on the presence of PS. In line with this, Hsp70 expresses toxicity towards cells presenting PS on their surface [305]. The possible role of Hsps as enhancers of endocytosis has been speculated to be part of the cellular stress response for rapid remodelling of the plasma membrane [306].

Anti-inflammatory drugs cause the differential up-regulation of cytosolic and membrane-bound Hsp70 in tumour cells [307]. It has been suggested that such an increase in membrane-bound Hsp70 corresponds to an enhanced sensitivity to granzyme B-induced apoptosis and natural killer cell-mediated killing. This finding provided a biological rationale for combining anti-inflammatory drugs with immunotherapy in cancer treatment. Moreover, the cell surface-bound Hsp70 has been postulated to mediate perforin-independent apoptosis by the specific binding and uptake of granzyme B [308]. Su *et al.* demonstrated, that constitutive Hsp70 (Hsc70) attenuates hydrogen peroxide-induced membrane lipid peroxidation [309]. The finding that Hsc70 reduces lipid peroxidation suggests that this protein (and maybe other Hsp members like sHsps) may act through a general mechanism to interfere with the reactions of lipid oxidation. The fact, that Hsp70 is capable to form complexes with the acidic glyco- and phospholipids further implicates that Hsp70 plays various, hitherto unidentified and lipid-mediated functions on the membrane surfaces [310].

Hsp27-membrane interactions

It has been shown that Hsp27, which associates with membranes *via* specific lipid interactions [297] has a potent protective effect against α -synuclein-induced cell death in mammalian neuronal cells [311] where the association of α -synuclein with membranes leads to disruption of the membrane bilayer structure. After preconditioning of rat heart (a single episode of 5 min. global ischaemia followed by 5 min. of reperfusion), HSP27 redistributed from the cytosol to the sarcomere and recovery of the contractile function was significantly enhanced, which suggests that translocation of HSP27 to the sarcomere may be involved in the cardioprotective mechanism afforded

by ischaemic preconditioning in rat heart [312]. In contrast with Hsp27, Hsp70 does not exert such an action.

Secreted Hsps

Despite lacking a secretory signal, some Hsps are released from cells through physiological secretory mechanisms [261]. Thus, extracellular stress proteins including glucose-regulated proteins are emerging as important mediators of intercellular signalling and transport. Among others, immunological 'danger signals', physical trauma or behavioural stress are capable of triggering the release of these proteins from cells [313]. Evidence has been presented that the 'leaderless' stress proteins may be secreted into the circulation *via* lipid raft-, granule- or exosome-mediated exocytosis, for instance in haematopoietic or tumour cells. Hsp70 surface-positive tumour exosomes are able to stimulate the cytolytic and migratory capacity of resting natural killer cells [314]. Hsp70 is released from prostate carcinoma cells through an active secretory mechanism that has been documented to involve the endolysosomal compartments, and is taken up by other cells ([313] and see references within). It has been demonstrated that neither the common secretory pathway nor the lipid raft-mediated pathway is involved, however, in the release of Hsp70 from human mononuclear cells, perhaps the best-studied model of Hsp secretion [261]. In stressed Caco-2 cells, the amount of Hsp70 is increased specifically in the lipid rafts and this is correlated with the robust stimulation of Hsp70 release. Manipulation of the lipid composition of rafts resulted in a concomitant modulation of Hsp70 release, suggesting that in this case the lipid raft may represent a cellular mechanism for membrane delivery and the release of Hsps [315].

Table 6 illustrates this newly acknowledged dual function of Hsps: while maintaining cellular homeostasis intracellularly during protein- and/or membrane-damaging stresses, they may protect cells also beyond their borders. We will next highlight a few of the representative cases listed in Table 6.

Hsp47, a collagen-specific molecular chaperone, is involved in the processing and secretion of procollagens, and its expression is increased in various fibrotic diseases. The overexpressed Hsp47 has been observed in the muscle membrane only in the case of active inflammatory myopathy. In particular,

Hsp47 is strongly expressed in the membrane of regenerating fibres, implying that it may be involved in the repair or regeneration of muscle fibres in addition to the fibrotic change in the connective tissue [316].

Other studies have reported that hypoxia results in disassociation of the Hsp60-Bax complex, with translocation of cytosolic Hsp60 to the plasma membrane and Bax to the mitochondria [317]. The interaction between Hsp60 and Bax may be critical in preventing apoptosis in the normal cell. The surface presentation of Hsp60 on the myocyte combined with serum antibodies to this protein, may be one mechanism fuelling the downward spiral in heart failure [318]. It is possible that membrane Hsp60 may be recognized by macrophages and thus mark the myocyte for destruction. Whether translocation of Hsp60 to the membrane might possibly stabilize its structure and exert a protective effect is unknown, as is the mechanism(s) controlling this translocation. Hsp60 is released *via* exosomes and within the exosome Hsp60 is tightly attached to the exosome membrane. Hsp60 is released from adult cardiac myocytes in both the basal state and following mild stress through an exosome-mediated process. Lipid rafts participate in this process, as inhibition of lipid raft formation reduces the release of Hsp60 [318].

Insulin-treated hearts display elevated levels of Hsp70, particularly in the membrane fraction. In contrast, heat-shocked hearts exhibit elevated levels of Hsp70 in the cytosol, membrane and pellet fractions. After insulin treatment, Hsp70 is mostly colocalized to the plasma membrane with dystrophin. In contrast, after heat shock, Hsp70 is localized mainly between the cardiomyocytes in apparently vascular or perivascular elements. The localization of Hsp70 is dependent on the inducing stimulus of either heat shock or insulin treatment. The cell membrane *versus* vascular localization of Hsp70 suggests the interesting possibility of functionally distinct roles for Hsp70 in the heart, depending on whether it is induced by insulin or heat shock treatment [319].

It was reported earlier, that Hsp70 acts as a potent survival protein whose depletion triggers massive caspase-independent tumour cell death [320]. This Hsp70-mediated protection against various death stimuli in Hsp70-expressing human tumour cells and in immortalized Hsp70 transgenic murine fibroblasts occurs at the level of the lysosomal permeabilization. The cell death induced by Hsp70 depletion is preceded by the release of lysosomal enzymes into the

cytosol and inhibited by pharmacological inhibitors of lysosomal cysteine proteases. However, Hsp70 does not inhibit cytochrome c-induced, apoptosome-dependent caspase activation *in vitro* and Fas ligand-induced, caspase-dependent apoptosis in immortalized fibroblasts. Immunoelectron microscopy has revealed that endosomal and lysosomal membranes of tumour cells contain Hsp70. The permeabilization of purified endosomes and lysosomes by digitonin fails to release Hsp70, suggesting that it is physically associated with the membranes. Finally, Hsp70-positive lysosomes display increased size and resistance to chemical and physical membrane destabilization. Taken together, these data identify Hsp70 as a 'survival protein' that functions by inhibiting the death-associated permeabilization of lysosomes [320].

As outlined above, the extracellular factors that regulate the quantity and phenotype of the exosomes produced are poorly understood, and the properties of the exosomes that dictate their immune functions are not yet clear. Studies on the effects of cellular stress on the exosomes produced by B-lymphoblastoid cell lines have revealed that, under steady-state conditions, the exosomes are positive for Hsp27, Hsc70, Hsp70 and Hsp90. Exposure of cells to heat stress results in a marked increase in these Hsps, whereas the expressions of other stress proteins, such as Hsp60 and Grp96, remain negative [321]. It has also been elucidated that Hsps are located within the exosome lumen, rather than at the exosome surface, suggesting that such exosomes may not interact with target cells through cell-surface Hsp receptors. It has been concluded that specific alterations in exosome phenotype are a hitherto unknown component of the cellular response to environmental stress, and their extracellular function does not involve the direct activation of dendritic cells [321].

To investigate the putative association of Hsp27 (known at that time as 'barbed-end microfilament capping protein') with plasma membranes, bovine endothelial cells expressing the human wild-type or a mutant non-phosphorylatable 27-kD Hsp were subjected to subcellular fractionation and subsequent immunoblot analysis [322]. The 25-kD endogenous bovine homologue and both exogenous gene products partitioned with cytosolic or plasma membrane components, indicating that phosphorylation is not required for membrane association. Phorbol ester treatment results in phosphorylation of only

Table 6 Hsps in membranes and in the extracellular space

HSP	Localization	Tissue/cell type	Conditions	References
Hsp47	Basement membrane	Muscle	Muscular dystrophy	[316]
Hsp60	Plasma membrane	Human endothel Cardiac myocyte	After stress Hypoxia	[336] [317]
	Exosomes, attached to the membrane Exosomes	Rat adult cardiac myocytes Mouse B lymphoma cells	Unstressed, hypoxia Unstressed, heat shocked	[318] [340]
Hsp70	Plasma membrane Exosomes (independent of rafts)	Rat heart PBMC, 3T3-L1, L6 myotubes, B cells	Insulin treatment Heat shock	[319] [337]
	Extracellular space, (unconventional release)	In many tumour cells Under pathological conditions of normal cells Human tumour cells	Unstressed, heat shocked During necrotic cell death Cell death	[321] [338]
	Lysosomal membrane		Cytokines, anticancer drugs, irradiation, oxidative stress, photolysis.	[339] [320]
Hsp90	Exosomes	B cells	Unstressed, heat shocked Unstressed, heat- shocked	[321]
		Mouse B lymphoma cells		[340]
		H460 cells	γ -irradiation	[341]
		Human mesothelioma cells	Unstressed	[342]
α -B crystalline	Plasma membrane	DRM myotubes	Dexamethasone treatment	[343]
		Bovine Ocular lens	Increased upon mutation	[344]
HSPB1 (Hsp27)	Outer mitochondrial membrane	U937 cells	During apoptosis	[324]
	Plasma membrane Exosomes	Bovine endothelial cells	Overexpressed human Hsp27 Dexamethasone treatment	[322] [343]
		DRM myotubes		[343]
		B cells	Unstressed, heat- shocked	[321]
HSPB2	Outer mitochondrial membrane	C2C12 cells, KNS-81 cells	Under normal conditions	[323]

membrane-associated 25-kD and wild-type 27-kD Hsp and does not induce redistribution [322].

The sHsp HspB2 colocalizes with mitochondria in differentiated C2C12 cells, KNS-81 glioma cells and NIH3T3 cells transfected with a HspB2 [323]. Together with the fact that HspB2 does not possess a mitochondrial targeting signal sequence, these

results suggest that HspB2 is not located inside the mitochondria. Instead, it is attached to the surface of the mitochondria by weak interactions with some outer membrane components. The amount of HspB2 in the mitochondrial fraction increases when the cells are subjected to mild heat shock, suggesting that this association can be altered by stress conditions.

Association of another sHsp family member, Hsp27, occurs with the mitochondrial outer membrane in U937 cells. It has been suggested that this pool of Hsp27 negatively regulates cell death by interacting with cytochrome *c* released from the mitochondria into the cytosol in apoptotic pathways [324].

In conclusion, we assume that aging and various pathological states which lead to abnormal alterations in the lipid metabolism give rise to abnormal global (fluidity, surface charge, permeability, etc.) and specific (raft remodelling) changes within membranes. The formation of 'defects' in response to stress within the membrane microdomains is causally linked to the dysregulated expression and, at least in part, to the abnormal intra or extracellular localization of specific Hsps. In this context, membrane microdomains represent new therapeutic targets. In fact, a targeted and distinct reorganization of membrane microdomains with drug candidates acting according to the principles of 'membrane-lipid therapy' [9] best represented by the lipid-interacting hydroxylamines, has been shown to be coupled with the simultaneous normalization of the dysregulated expression and cellular localization of Hsps in such prominent disease states as type 2 diabetes and amyotrophic lateral sclerosis.

Concluding remarks

Membrane lipids, and the structures they form, not only play structural roles but also participate actively in numerous cellular processes. Hundreds of lipids form complex and heterogeneous membranes, where stable (*e.g.* synaptosomes) or dynamic (*e.g.* caveolae) microdomains define the structural and functional properties of a given bilayer. Membranes are the meeting point of many proteins and lipids, and they are the structures where most cellular activities take place. Lipids are not simply the physical scaffold for membrane proteins, forming barriers that isolate and define cells and organelles, but rather, they are also active in many cellular functions. In this context, they regulate the co-localization of proteins to receive and propagate messages that regulate hormone and neurotransmitter release, protein secretion, the cell division cycle, cell differentiation, contractility, gene expression, etc. In addition,

certain membrane lipids act as ligands or substrates of signalling proteins and enzymes. Therefore, protein–lipid and lipid–lipid interactions have a great impact in the physiology of cells. Similar to regulation of gene expression, the presence and levels of lipids species can change in response to diet, physiological and environmental conditions. In this context, in several human pathologies there have been described changes in membrane lipids, that have been associated either with adaptive responses or with the aetiology of the disease [9, 325, 326] (Table 3). This fact highlights the prominent role of lipids in cells and suggests that they may also be appropriate targets for therapeutic interventions. Currently, most clinical drugs are targeted to proteins. Moreover, over a half the drugs currently under development are directed against GPCRs [327] and many others are designed to modulate the activity of other membrane proteins. Because the activity of membrane proteins can be regulated by lipids, it is feasible that lipid treatments could modulate the activities of these proteins and/or associated signalling mechanisms. This approach, called membrane-lipid therapy [9], has been used to develop molecules with low toxicity and high efficacy, to treat cancer, cardiovascular diseases, obesity, etc. In this sense, interventions on the composition and structure of membranes may impinge on the physical and structural properties of the lipid bilayer and concomitantly on the interaction and activity of signalling proteins with lipids or with other membrane proteins. Moreover, whereas the *cis*-MUFA oleic acid regulates membrane structure and cell signalling, its *trans*-isomer, elaidic acid, and its saturated fatty acid analogue, stearic acid, do not regulate neither lipid structure nor GPCR-mediated function [328]. This fact is only one example of the many available showing the relationship between lipid structure and cell activity and it might be related to the beneficial (*e.g.* *cis*-MUFA) and detrimental (*e.g.* *trans*-MUFA and saturated fats) effects that lipids have on human health. Our current understanding of lipids, the structures they form, their roles in cells, and the physicochemical and biological properties of membranes are the result of many years of research. The following years present a thrilling scenario for the study of membranes, in which many issues that remain poorly understood are likely to be successfully resolved, thanks in part to new discoveries and the development of new experimental approaches.

Acknowledgements

The present work was financed in part by Grants SAF2003-00232, SAF2004-05249 and BFU2007-61071 (Ministerio de Educación y Ciencia, Spain); and Grants PRIB-2004-10131, PCTIB-2005GC4-07 and PROGECIC-3C (Govern de les Illes Balears, Spain). Grants RET OMFB 00067/2005 and OTKA 68379 (Hungary) to I.H. and L.V., the Finnish Academy of Sciences, the Marathon Foundation and the Sigrid Juslius Foundation also financed in part the present study.

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