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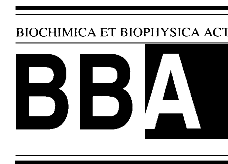

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Review

Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile

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Received 5 May 2004; received in revised form 16 June 2004; accepted 24 June 2004
Available online 23 July 2004

Abstract

Nonbilayer lipids can be defined as cone-shaped lipids with a preference for nonbilayer structures with a negative curvature, such as the hexagonal phase. All membranes contain these lipids in large amounts. Yet, the lipids in biological membranes are organized in a bilayer. This leads to the question: what is the physiological role of nonbilayer lipids? Different models are discussed in this review, with a focus on the lateral pressure profile within the membrane. Based on this lateral pressure model, predictions can be made for the effect of nonbilayer lipids on peripheral and integral membrane proteins. Recent data on the catalytic domain of Leader Peptidase and the potassium channel KcsA are discussed in relation to these predictions and in relation to the different models on the function of nonbilayer lipids. The data suggest a general mechanism for the interaction between nonbilayer lipids and membrane proteins via the membrane lateral pressure.
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Keywords: Protein–lipid interaction; Nonlamellar lipid; KcsA; Leader Peptidase; Oligomer; Ion channel; Phosphatidylethanolamine; Curvature; Lipid polymorphism

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Abbreviations: CF, carboxyfluorescein; C_o, spontaneous curvature; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DLPE, dilauroylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; Δ2–75, catalytic domain of Leader Peptidase; g_c, curvature stress (bending energy per unit area); H_{II}, hexagonal phase; κ, mean curvature modulus; L_α, liquid crystalline lamellar phase; L_β, lamellar gel phase; MD, molecular dynamics; MGDG, monogalactosyldiacylglycerol; NMR, nuclear magnetic resonance; π, lateral pressure; PC, phospatidylcholine; PE, phosphatidylethanolamine; PENMe, *N*-methylphosphatidylethanolamine; $PENMe_2$, *N*,*N*-dimethylphosphatidylethanolamine; PG, phosphatidylglycerol; *R*, radius of curvature; R_o, intrinsic radius of curvature; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; τ, torque tension; TFE, 2,2,2-trifluoroethanol; z, depth in the lipid bilayer

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0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2004.06.010

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In this review, we will focus on the role of the so-called nonbilayer lipids in biological membranes. First, it will be explained what is meant by the term nonbilayer lipids, based on the shape-structure concept of lipid polymorphism. In literature, different suggestions have been given for the role of these lipids. For example, it has been suggested that the presence of nonbilayer lipids is important for the local formation of nonbilayer structures. More often, it is stated that nonbilayer lipids affect the function of membrane proteins. Different models have been described to explain these lipid–protein interactions. An overview and discussion of these different models will be given below, with a focus on the lateral pressure model. Subsequently, an overview is given of recent data on both peripheral and integral membrane proteins, and the effect of nonbilayer lipids on their structure and function. These data are related to the lateral pressure profile within the membrane. Finally, biological implications of these data will be discussed.

1. Lipid polymorphism and nonbilayer lipids

Isolated lipids can assemble in different structures when they are dispersed in an aqueous environment. A simplified way to rationalize the preference for a particular structure is the so-called shape-structure concept of lipid polymorphism [1], as illustrated schematically in Fig. 1 [2–4]. According to this concept, lipids have an overall cylindrical shape when the cross-sectional area of the lipid headgroup is similar to the cross-sectional area of the acyl chains. This is for example the case for phosphatidylcholine (PC). Such lipids can self-assemble into a liquid crystalline lamellar phase (L_α), similar to the phase of a biological membrane. Another possible bilayer structure is the lamellar gel phase (L_β), which is a highly ordered and tightly packed bilayer structure that occurs below the main transition temperature [5,6]. When the cross-sectional areas of the lipid headgroup and acyl chains are very different, other aggregate structures will be preferred. If the cross-sectional area of the headgroup is larger than that of the acyl chains (type I lipids), aggregate structures with a positive curvature are favored, for example micelles [1]. On the other hand, when the cross-sectional area of the headgroup is smaller, resulting in an overall conical shape (type II lipids), aggregate structures with a negative curvature are preferred, such as the inverted hexagonal phase (H_{II}). Another nonlamellar phase is the cubic phase, which has characteristics that are intermediate between an L_α and H_{II} phase [7]. Within the shape-structure concept of lipid polymorphism, the overall shape of the lipid is not only defined by the chemical structure of the lipid headgroup and acyl chains, but also by environmental parameters, such as the pH, salt concentration, temperature, presence of divalent cations, and hydration [1,8]. For example, charged lipid headgroups show repulsions and

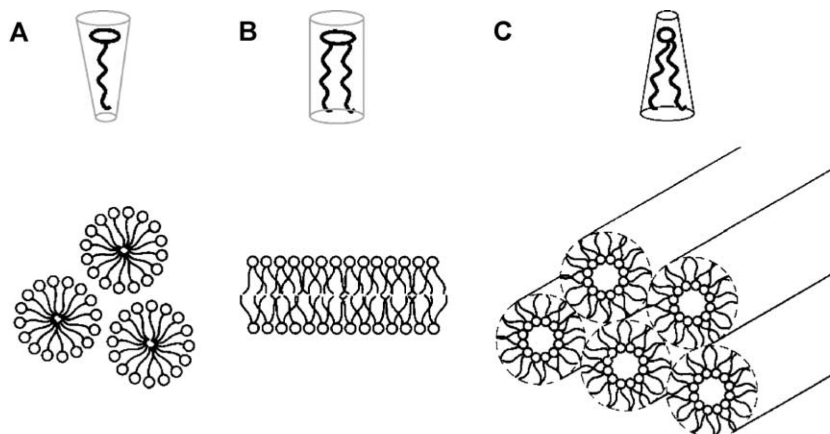

Fig. 1. Illustration of the shape-structure concept of lipid polymorphism, indicating the overall shape of the molecules and examples of the aggregated structures. (A) Molecules that have an overall inverted conical shape, such as detergent molecules, form structures with a positive curvature, such as micelles. (B) Cylindrical-shaped lipid molecules preferentially form bilayer structures, for example the liquid crystalline (L_α) phase. (C) When lipid molecules have an overall conical shape, structures with a negative curvature are preferred, such as the hexagonal (H_{II}) phase.

thereby have a relatively large headgroup cross-sectional area. This can be decreased by the presence of salt, which is able to neutralize the charge to some extent [8]. Another example is cardiolipin, which normally adopts an L_α-phase, but in the presence of divalent cations forms a complex with a preference for the H_{II} phase [9]. Although nonbilayer lipids prefer organization in curved structures, such as the inverted hexagonal phase, there is an energy cost to formation of these structures. Under some conditions, this energy cost is sufficient to inhibit the formation of nonlamellar phases. Thus, the monolayers tend to curve in opposite directions, but are forced to remain organized in a bilayer: a so-called frustrated bilayer [10,11].

A more quantitative description related to the shape concept is the intrinsic radius of curvature (R_o) [2,12]. R_o is defined as the radius of curvature of the lipid–water interface in a situation that the lipid monolayer is elastically relaxed [12]. By definition, R_o is negative for type II lipids and positive for type I lipids [8]. The spontaneous radius of curvature of a certain lipid can be determined experimentally by X-ray diffraction[1] [13–15]. R_o is low for lipids that tend to form highly curved monolayers, but increases for bilayer forming lipids. The spontaneous curvature (C_o) is the inverse of the spontaneous radius of curvature (C_o=1/R_o) [7].

Type II lipids, which have an overall conical shape, are often called nonbilayer lipids, since they have a preference for nonbilayer structures such as the H_{II} phase. Although biological membranes are bilayers, most of them contain a substantial amount of these nonbilayer lipids. For example, the *Escherichia coli* inner membrane contains about 75% of the nonbilayer lipid phosphatidylethanolamine (PE) [5]. Therefore, the question is rising: why are these nonbilayer lipids present? The balance between bilayer and nonbilayer lipids is tightly regulated [16] and has been shown to be essential for viability [17], suggesting an important role for the nonbilayer lipids.

2. Formation of nonbilayer structures

One possible role of nonbilayer lipids involves the local formation of nonbilayer structures. It has been shown for the microorganisms *Acholeplasma laidlawii* and *E. coli* that these cells always try to maintain the physical state of the membrane lipids close to a bilayer–nonbilayer phase transition [16,18,19]. This allows the membrane to readily undergo temporal local rearrangements, for instance for cell division [11]. In addition, it has been suggested that the transient formation of inverted structures within bilayers can provide a pathway for transbilayer transport of lipids and polar solutes [20] or be involved in membrane fusion [21,22]. Also stable nonbilayer structures have been suggested to occur in biological membranes, e.g., for the organization of tight junctions between mammalian cells [23]. Although the inverted hexagonal phase itself has never been convincingly demonstrated to be present in biomembranes under physiological conditions, membrane structures resembling inverted cubic phases are frequently encountered [24]. Importantly, in all cases mentioned above, the formation of nonbilayer structures seems to occur only locally at specific places. This suggests that there should be another reason for the presence of such a high concentration of nonbilayer lipids.

3. Effect of nonbilayer lipids on membrane proteins

Another possible function of nonbilayer lipids is that they modulate an important general property of the lipid bilayer. This could be of direct influence on the membrane function, for example by affecting the flexibility or barrier properties of the bilayer, or it could result in an indirect effect on the function of membrane proteins. Indeed, it has been shown for an increasing number of membrane proteins that the presence of nonbilayer lipids influences their function. For several peripheral membrane proteins it has been shown that their activity is increased in the presence of nonbilayer lipids (in most cases PE). Examples are protein kinase C [25,26], phospholipase A_2 [27], CTP:phosphocholine cytidylyltransferase [27,28], monoglucosyl diacylglycerol synthase [29] and diglucosyl diacylglycerol synthase [27,30,31]. Also for several integral membrane proteins it has been shown that nonbilayer lipids stimulate their activity. Examples are the bacterial translocase secYEG [32], cytochrome P450SCC [33], rhodopsin [34,35], the mitochondrial adenine nucleotide transporter [36], the osmosensing ABC-transporter OpuA [37,38], ubiquinol-cytochrome *c*-reductase [39], the Mg^{2+}-ATPase and Na^+/Mg^{2+}-ATPase from *A. laidlawii* [39], H^+-ATPase [39]. Furthermore, nonbilayer lipids have been shown to stimulate the conductance of the calcium activated potassium channel [40] and the channel-forming peptides alamethicin [41] and gramicidin [42]. On the other hand, for some enzymes that are involved in the synthesis of nonbilayer lipids, for example phospholipase C [27,43] and diacylglycerol kinase [44], it has been shown that they are inhibited by the presence of these lipids.

Nonbilayer lipids can also influence the folding of proteins, as was shown for bacteriorhodopsin [45] and lactose permease [46–48]. Finally, the presence of nonbilayer lipids was found to be important for functional reconstitution of membrane proteins [49], for example for cytochalasin B [50]. All these examples show that many membrane proteins are able to somehow sense the presence

[1] For this a certain lipid is mixed with a large amount of nonbilayer host lipid (e.g., PE). The radius of curvature (R) of the resulting HII phase can be determined by X-ray diffraction. The intrinsic radius of curvature (R_o) of this lipid can be calculated from the difference between this measured radius of curvature (R) and the known intrinsic radius of curvature of the host lipid (R_o, PE).

of nonbilayer lipids. However, the mechanism by which this occurs is not known, although several models have been described in literature. A problem in this field is that many different terms are used and that the definitions and the relations between them are not clear. Therefore, an overview is given here of the different models that are described in literature. First, the concept of curvature stress is explained, which is the basis of all other models.

3.1. Curvature stress

Curvature stress (or curvature strain or elastic stress) is an energy term that is often used in discussions about non-bilayer lipids. When a lipid monolayer contains nonbilayer lipids, this monolayer will have a tendency to adopt a certain curvature. When such lipids are present in a planar lipid bilayer, two monolayers are forced to flatten, resulting in a bilayer with two 'frustrated' monolayer leaflets containing stored curvature stress [51]. This curvature stress energy can be expressed and relaxed by forming an H_{II} phase [52], but can also be released by coupling with other membrane processes [53], such as the partial membrane insertion of a peripheral membrane protein [28]. The curvature stress can be calculated as the bending energy per unit area (g_c). This is for a flat monolayer $g_c=2\kappa C_o^2$, where κ is the mean curvature modulus (sometimes also called the bending modulus [51] that can be determined experimentally, for example by X-ray diffraction [13]) and C_o is the spontaneous curvature (see above) [51,54]. The curvature stress can be measured only indirectly, e.g., using the changes in the L_α-to-H_{II} transition temperature [52] or a calorimetric detection of the incorporation of detergent molecules into a lipid bilayer [53]. The curvature stress is an overall property of the membrane, which can be modulated in different ways. For example, the curvature stress of a certain bilayer can be increased either by changing the lipid headgroup into a smaller one or by increasing the chain unsaturation [55]. This increased curvature stress has been suggested to influence the structure and activity of membrane proteins. The curvature stress can be considered as a source of energy that may affect the free energy of membrane binding of a peripheral membrane protein or provide energy for a conformational change of a protein [34,56,57]. We suggest that this occurs via a mechanism as described in one (or more) of the following models. Although this way of presenting suggests that these models are completely independent, this is not necessarily the case. In Sections 4 and 5, the different models will be discussed in relation to recent data on peripheral and integral model proteins.

3.2. Hydrophobic mismatch

This model of how membrane proteins may sense the presence of nonbilayer lipids only applies to proteins, of which the length of the hydrophobic membrane-spanning domains is different from the hydrophobic bilayer thickness, resulting in hydrophobic mismatch. Since exposure of the hydrophobic regions of either lipids or proteins to a water phase is energetically unfavorable, a membrane will try to adapt to this mismatch situation. It can be expected that the bilayer structure will be adapted locally, with a negative or positive curvature, depending on the mismatch situation. When the hydrophobic length of a transmembrane protein is too large to match the hydrophobic bilayer thickness, this will result in a local negative curvature, while a short transmembrane protein will give rise to a local positive curvature, while the overall structure is still a bilayer. Therefore, it can be expected that the presence of nonbilayer lipids will influence the ability of a membrane to adapt to a mismatch situation. Such effects are especially important for proteins that can occur in two conformational states with different hydrophobic lengths. Examples of this are gramicidin [42,58] and rhodopsin [59]. For both of these proteins it has been shown that the presence of nonbilayer lipids affect the transition between their different conformations [42,59]. It has been calculated that these mismatch effects are coupled to curvature stress [60].

3.3. Packing defects

A second model of how nonbilayer lipids may influence protein structure and function is related to the lateral packing of lipids, as it is sensed, in particular, at the membrane surface. Several studies have suggested that the presence of nonbilayer lipids in a membrane gives rise to the formation of bilayer packing defects, which could influence the function of mainly peripheral membrane proteins. Bilayer packing defects are defined as imperfections in molecular packing, but different suggestions have been given for the nature of these defects. Sen et al. [61] and Hui [62] suggested that these defects are similar to defects that are formed when a bilayer undergoes a L_β–L_α phase transition, related to the pre-transition molecular packing stress (curvature stress). This would promote membrane binding of peripheral membrane proteins and also increase the substrate availability for phospholipase A_2 [61]. In contrast, Senisterra and Epand [25] noted that not the coexistence of the L_β and L_α phase, but only the presence of nonbilayer lipids increased the activity of protein kinase C, indicating a difference between the L_β–L_α-related defects and the packing defects induced by the presence of nonbilayer lipids. Soulages et al. [63] suggested the presence of 'hydrophobic spots' in the headgroup region induced by the presence of diacylglycerol, which would promote the membrane binding of the peripheral membrane protein apolipophorin II. Similar defects were suggested by Davies et al. [56] for the membrane binding of CTP:phosphocholine cytidylyltransferase. Although the exact nature of these defects is not clear from these studies, they probably refer to the same phenomenon as discussed in the studies of Sen et al. [61] and Hui [62]. An alternative type of packing defect was proposed by Kinnunen [64] and Tuominen et al. [65],

who suggested that a membrane can relieve the curvature stress by allowing some of the lipids to adopt a so-called extended conformation, in which one of the acyl chains extends out from the bilayer into a hydrophobic cavity of a (peripheral) membrane protein, such as cytochrome *c*. However, this suggestion is still under discussion, since the observations leading to this suggestion may also be explained by the unfolding of cytochrome *c* at the membrane interface, which was observed previously [66,67].

3.4. Lateral pressure profile

A more general model of how proteins sense the presence of nonbilayer lipids deals with the lateral pressure profile within the membrane. The lateral pressure (π, sometimes also called lateral stress [6]) varies with depth in the membrane. A large negative pressure due to the cohesive hydrophobic interfacial tension is localized at the polar–apolar interface between lipid acyl chains and headgroups. This negative pressure is balanced by a positive lateral pressure in the acyl chain region due to repulsions between the hydrocarbon chains. There will also be a repulsive (or possibly sometimes even attractive) lateral pressure acting between the lipid headgroups, e.g., due to electrostatic interactions [6,68,69]. The lateral pressure profile is schematically shown in Fig. 2. Since membranes are self-assembled in a tension-free state, the overall lateral pressure (i.e. the integral of the lateral pressure profile across the monolayer leaflet) is equal to zero [68,70]. The curvature stress is related to the lateral pressure profile via the torque tension (τ), which is the first moment of the lateral pressure, $\tau=\int\pi(z)z\ \mathrm{d}z=2\kappa C_\mathrm{o}$, where π is the lateral pressure, z is the depth in the lipid bilayer, κ is the mean curvature modulus and C_o is the spontaneous curvature (see above). An increase in the lateral pressure in the acyl chain region will be correlated with an increased curvature stress [21]. Other changes in physical properties of the membrane, such as the bilayer thickness, may be an indirect consequence of a changed lateral pressure profile [21,68].

Direct and unambiguous measurements of lateral pressures in the lipid bilayer are not available yet. Templer et al. [54] used dipyrenyl PC, with the pyrene-moieties attached to acyl chains of different lengths. The pyrene excimer fluorescence is an indication for the lateral pressure in the membrane. However, it cannot be excluded that the results of these measurements are affected by the large size of these fluorescent probes and by the presence of double bonds in the lipid acyl chains in close proximity to the pyrene-moieties [54]. Another possible method is to determine the order parameter profile by ^{2}H-NMR, although the exact relation between the acyl chain ordering and the lateral pressure is not clear [71]. In addition, the monolayer technique, in which the surface tension is measured, can be used to obtain information about the free space in the lipid headgroup region, related to the interfacial lateral pressure [72]. Other studies have determined the lateral pressure profile based on different computational methods, such as mean field theory [73–82], dissipative particle dynamics simulations [83], molecular dynamics simulations [82,84,85] or Monte Carlo simulations [81]. The calculations using mean field theory are restricted to the acyl chain region of the lipid bilayer, which is considered as a well-defined hydrophobic region. Best known are the studies of Cantor, based on statistical thermodynamics calculations [68,73–77,86]. The lipid headgroup region has been included in calculations using molecular dynamics or dissipative particle dynamics, but the data for this region are less reliable, because of computational difficulties, such as uncertainties in intermolecular potentials [81]. Therefore, such calculations have been performed until now only for a coarse-grained lipid model [83] and for DPPC [84] and DLPE [85], which are often used for MD simulations. Strikingly, the calculated lateral pressure profiles based on MD simulations indicate the presence of an additional peak in the center of the bilayer [84,85], which may be due to interdigitation of lipid acyl chains. This is in contrast to what is shown in Fig. 2 and what appears from other computational studies [73–83], indicating that also for the

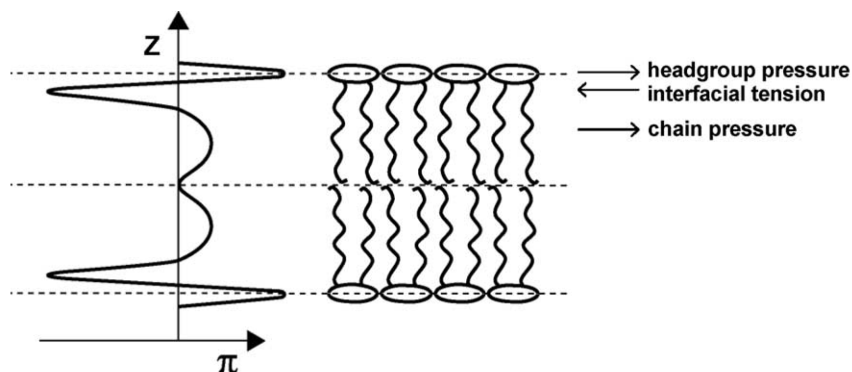

Fig. 2. Schematic representation of the lateral pressure profile in the lipid bilayer. The lateral pressure (π) is indicated as a function of the depth (z) in the membrane. A negative pressure due to the interfacial tension is located at the polar–apolar interface, compensated by a positive lateral pressure in the headgroup and acyl chain region (due to repulsions). Lateral pressure profile was adopted from Ref. [87].

acyl chain region of the lipid bilayer the exact shape of the lateral pressure profile is not sure yet.

The lateral pressure profile can be changed by variations in the lipid composition [76]. For conical-shaped nonbilayer lipids, it was suggested that these lipids increase the lateral pressure in the acyl chain region, and decrease the lateral pressure among the lipid headgroups [10,11,21,87]. This suggestion is not yet confirmed by computer simulations, but is supported by fluorescence measurements [54] and ^{2}H-NMR studies [88–90]. In addition, the fluorescence measurements suggest that incorporation of nonbilayer lipids also affect the lateral pressure distribution within the acyl chain region, shifting the lateral pressure towards the center of the lipid bilayer [54]. This is in agreement with calculations of Cantor, which indicate that variations in the lipid composition can induce large changes of the lateral pressure profile within the acyl chain region [68,73–77,86].

Also small solutes, such as alcohols, can induce changes in the lateral pressure profile [73,74,91], which were therefore also used for part of the research discussed in this review. Although many small alcohols are miscible with water, they partition to a certain extent into the lipid bilayer and in that way they can influence the lipid bilayer structure and lateral pressure and thereby they can indirectly influence membrane proteins. However, small alcohols can also influence the structure of proteins via direct interactions, as has been reported for trifluoroethanol (TFE) [92–94].

It has been calculated that changes in the membrane lateral pressure can influence structural properties of membrane proteins, such as their conformational state or their oligomeric structure [68,77]. However, changes in the lateral pressure profile will only affect those membrane proteins whose function involves a conformational change that is accompanied by a depth-dependent variation in the cross-sectional area of the protein [68].

Based on the lateral pressure profile model, predictions can be made for the effect of nonbilayer lipids on peripheral and integral membrane proteins, as illustrated in Fig. 3. Fig. 3A shows a suggestion for the lateral pressure profiles for a membrane composed of only bilayer lipids, and for a membrane containing a large fraction of nonbilayer lipids. It can be expected that the differences in the lateral pressure profile, due to the presence of nonbilayer lipids, will affect membrane proteins, and that different components of this lateral pressure profile are important for different types of membrane proteins. The decreased lateral pressure among the lipid headgroups could affect the membrane binding of peripheral membrane proteins, as illustrated (in extreme) in Fig. 3B. It is likely

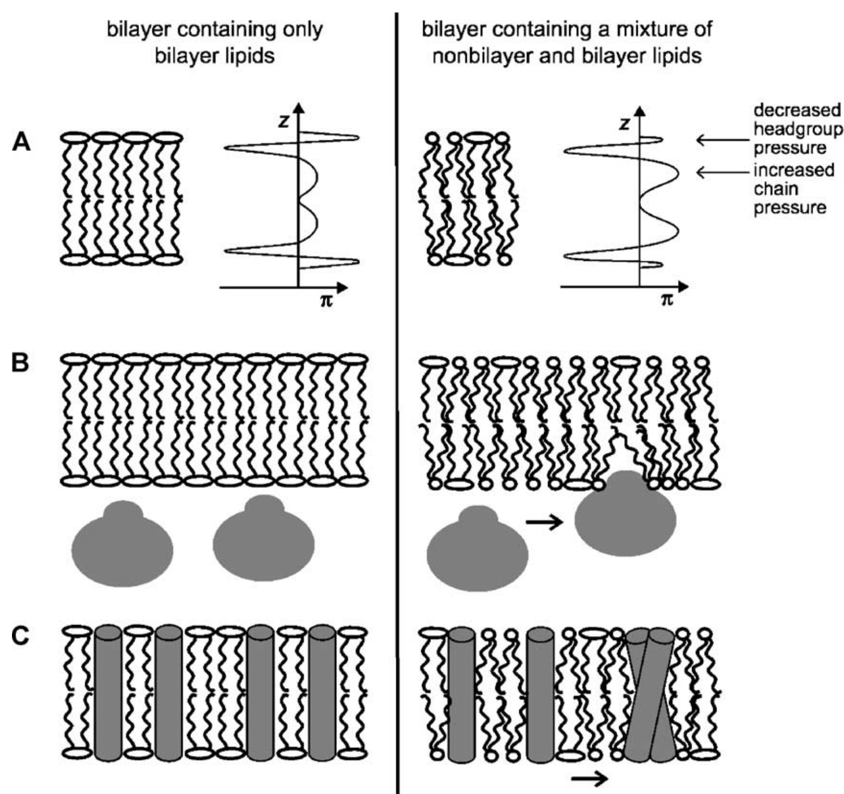

Fig. 3. Predictions on the effect of nonbilayer lipids on peripheral and integral (oligomeric) membrane proteins via the lateral pressure profile. (A) The lateral pressure profile is indicated for a bilayer containing either only bilayer lipids (left) or a mixture of nonbilayer and bilayer lipids (right). The effect on both peripheral membrane proteins (B) and oligomeric integral membrane proteins (C) is indicated below in extreme: nonbilayer lipids are expected to support membrane binding of peripheral membrane proteins and stabilize the oligomeric structure of integral membrane proteins. Lateral pressure profiles were adopted from Ref. [87].

that this will have consequences for the function of these proteins. On the other hand, the higher lateral pressure in the acyl chain region could influence the (oligomeric) structure of integral membrane proteins, as indicated (in extreme) in Fig. 3C, which might also affect their function. Below, recent data on both peripheral and integral model membrane proteins will be discussed and related to the suggestions and models mentioned above.

4. Effect on the peripheral membrane protein Leader Peptidase Δ2–75

E. coli Leader Peptidase is an integral membrane protein consisting of two transmembrane helices and a large periplasmic domain [95]. This protein plays a role in the protein secretion pathway: it removes the N-terminal signal peptide from proteins that are translocated across the membrane by the Sec machinery [95]. The active site of Leader Peptidase is located in the periplasmic domain [96]. It has been shown that it is possible to isolate this periplasmic domain (Δ2–75) without the two transmembrane segments [97]. This variant is water-soluble and catalytically active [97,98]. The protein contains a large exposed hydrophobic surface, which includes the substrate binding site and catalytic centre [99] and was suggested to be the membrane-association surface. It was shown by van Klompenburg et al. [100] that this protein is indeed able to insert into phospholipid monolayers, with a preference for PE over PC and phosphatidylglycerol (PG). Because of the presence of a hydrophobic membrane binding interface and the observed binding to phospholipid monolayers, this catalytic domain of Leader Peptidase has been used as a model protein to study the effect of nonbilayer lipids on the insertion of peripheral membrane proteins into the membrane. Both with lipid monolayers and vesicles, an increased membrane binding of Δ2–75 was observed for the nonbilayer lipid PE, compared to PC. Increased membrane binding was also observed for other nonbilayer lipids (diacylglycerol (DAG) and monogalactosyldiacylglycerol (MGDG)), indicating that no specific interactions were involved [72]. On the basis of these results, it was suggested that nonbilayer lipids create insertion sites due to a reduced packing density at the membrane–water interface [72]. Based on monolayer insertion experiments, it was even possible to estimate the size of these insertion sites, as shown in Fig. 4. The size of the insertion sites was maximally 15±7 Å^2/lipid molecule for PE relative to PC [72]. These data for the catalytic domain of Leader Peptidase support the suggestion that nonbilayer lipids indeed create interfacial insertion sites that stimulate the membrane binding of peripheral membrane proteins, as was predicted above (Fig. 3). These insertion sites can be considered as a reduced packing density in the lipid headgroup region, resulting in exposed hydrophobic sites, which can favorably accommodate membrane active compounds or proteins [72]. This decreased packing density results in a low headgroup lateral pressure. Due to this low lateral pressure, it is easier for membrane active molecules to insert between the lipid headgroups and contact the hydrophobic acyl chain region of the membrane. This is even more favorable because of the hydrophobic nature of these insertion sites. Although the term ‘insertion sites’ suggests the presence of distinct cavities in the headgroup region of the lipid bilayer, this reduced packing density should be more considered to be a bulk property of the membrane. Only for the occasion of membrane insertion of a membrane active molecule, such as the membrane binding domain of a protein, the lipid headgroups will move aside to create a large cavity in which this molecule can insert. While this insertion is affected by the membrane lateral pressure, the other way around is probably also true, that the membrane lateral pressure is changed when molecules insert into these insertion sites. It is likely that when a large number of peripheral protein molecules insert into a lipid bilayer interface, this will give rise to an increased headgroup lateral pressure, most likely compensated by a decreased acyl chain lateral pressure, and resulting in a decreased curvature stress. In addition, indications have been obtained that alcohols change the lateral pressure profile in a similar way, due to their insertion in the interfacial part of the lipid bilayer [101,102].

The definition of the insertion sites is very similar to the packing defects as mentioned in Section 3.3, especially

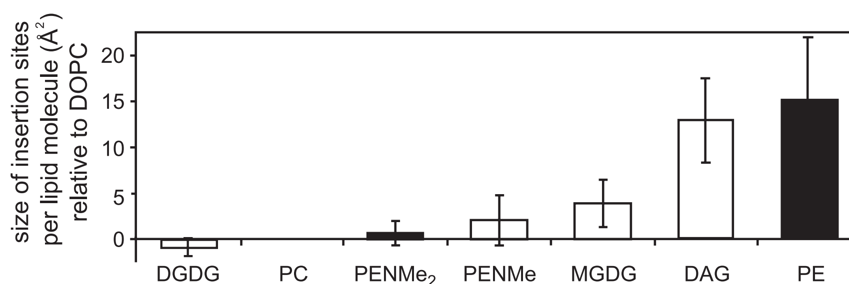

Fig. 4. Calculated size of the insertion sites (Å^2/lipid molecule) relative to phosphatidylcholine (PC). This was done for phosphatidylethanolamine (PE) and its *N*-methylated derivatives *N*-methylphosphatidylethanolamine (PENMe) and *N*,*N*-dimethylphosphatidylethanolamine (PENMe$_2$) (black bars), as well as for other (non)bilayer lipids digalactosyldacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG) and diacylglycerol (DAG) (white bars). Error bars represent the standard deviation. Figure is derived from Ref. [72].

the defects according to Soulages et al. [63] and Davies et al. [56], which are defined as 'hydrophobic spots' in the bilayer headgroup region. These were proposed to explain the stimulatory effect of diacylglycerol on the membrane binding of apolipophorin III [63] or the stimulatory effect of diacylglycerol and PE on the membrane binding and activity of CTP:phosphocholine cytidylyltransferase [56]. Also the packing defects according to Sen et al. [61] and Hui [62], defined as imperfections in molecular packing at the onset of the transition to the hexagonal phase, seem to be related. To measure the molecular packing stress related to this type of packing defects, they used the fluorescent dye Merocyanine 540, which inserts between the lipid headgroups. The fluorescence properties of this dye were found to be sensitive to packing changes in the headgroup region [103], indicating that the packing stress is related to the presence of insertion sites. It can be concluded that insertion sites and packing defects (according to the definitions mentioned above) are two different terms for the same phenomenon, and are related to the lateral pressure profile, as explained above. Davies et al. [56] propose for CTP:phosphocholine cytidylyltransferase that such packing effects are less important than the membrane stored curvature strain energy, suggesting them to be two independent parameters. However, since the curvature stress is related to the membrane lateral pressure, as explained above, it can be concluded that also these insertion sites and the curvature stress are most likely not two independent bilayer properties, but interrelated via the membrane lateral pressure. This is in agreement with the analysis of Attard et al. [28], who found a correlation between the curvature stress and the activity of CTP:phosphocholine cytidylyltransferase, which depends on its interfacial membrane binding. Also for phospholipase A2, such a correlation with curvature stress has been suggested [27]. These findings support the suggestion that the membrane binding of peripheral membrane proteins is affected by changes in the membrane lateral pressure, which is related to the formation of insertion sites and the storage of curvature stress.

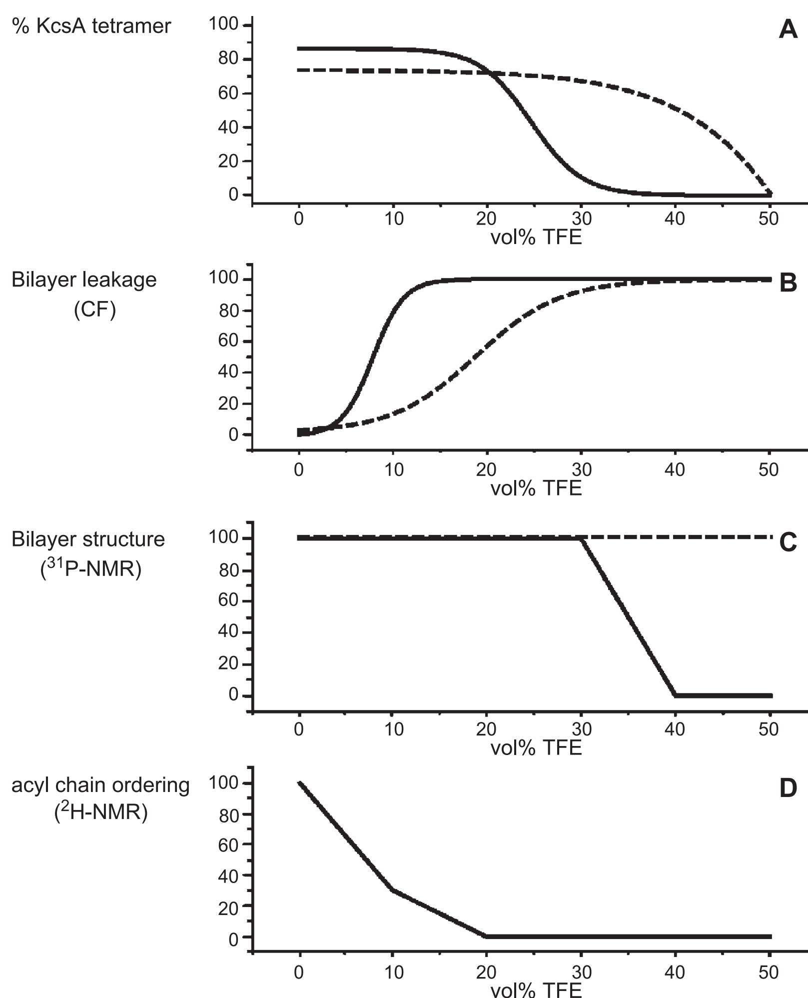

Fig. 5. Effect of TFE on the structure of the KcsA tetramer and the surrounding lipid bilayer of DOPC (black) or DOPE/DOPG (7:3, dashed line). (A) relative amount of KcsA tetramer as function of the TFE concentration. (B) TFE-induced bilayer leakage, as measured by fluorescence, using carboxyfluorescein-loaded vesicles. (C) Relative amount of intact lipid bilayer, as determined by ^{31}P-NMR. (D) Relative acyl chain ordering, as determined by ^{2}H-NMR using $^{2}H_4$-DOPC, labeled at the C_{11}-position. Derived from Ref. [101].

5. Effect on the integral (oligomeric) membrane protein KcsA

The integral membrane protein KcsA has been used as a second model protein to study the role of nonbilayer lipids. KcsA is a potassium channel from *Streptomyces lividans*. The channel is a symmetric tetramer with each monomer consisting of an N-terminal helix at the membrane interface, a transmembrane helix, followed by a short pore helix leading to the selectivity filter in the tetrameric structure, a second transmembrane helix and a large C-terminal domain in the cytoplasm. The structure of the transmembrane domain of KcsA was determined by X-ray crystallography [104], while the structure of the N- and C-termini was modeled based on EPR-measurements [105]. KcsA is often used as a model protein to study ion channel function [106–109] and membrane protein assembly [110–115]. The KcsA tetramer is extremely stable, even in a detergent like SDS [116], but can be dissociated by heat treatment (>60 °C), high pH (>12) [117] or by incubation with trifluoroethanol [118,119]. It was shown that the lipids PE and PG are important for efficient membrane association and tetramerization of KcsA [113] and that KcsA preferentially interacts with both these lipids [113,118–120]. Because of the relatively simple tetrameric structure and the possibility to study the formation and dissociation of the tetramer by SDS-PAGE [116], this protein was used as a model protein to study the effect of nonbilayer lipids and of changes in the membrane lateral pressure on the stability of the oligomeric structure of the protein.

The dissociation of the tetrameric potassium channel KcsA induced by trifluoroethanol (TFE) was studied in the presence of different lipids [101]. It was found that TFE induces tetramer dissociation in all tested lipid systems and that it also changes the secondary and tertiary structure of the protein [101]. The tetramer stability was found to be dependent on the shape of the surrounding lipids, with an order of stabilization of (inverted conical) detergent< (cylindrical) PC ≈ (cylindrical) PC/PG (7:3)<(conical) PE/PG (7:3) [101]. Similarly, the presence of the nonbilayer lipid PE stabilized the lipid bilayer structure, while there was no effect of PG [101]. NMR-experiments indicated that TFE inserts mainly in the headgroup region of the lipid bilayer, thereby decreasing the acyl chain ordering [101]. A summary of the described data for PC and PE/PG (7:3) is shown in Fig. 5, indicating that the presence of the nonbilayer lipid PE increases the tetramer stability of KcsA, decreases the membrane leakage and stabilizes the bilayer structure. Comparison of the different panels in this figure leads to the conclusion that the KcsA tetramer dissociation (panel A) is not triggered by membrane leakage (panel B) or dissolving the lipid bilayer (panel C), but most likely related to the membrane lipid acyl chain packing or acyl chain ordering, which was strongly reduced by the presence of TFE (panel D). In a subsequent paper [102], similar effects were observed for other small alcohols depending on their membrane affinity. Based on these data, it was proposed that small alcohols increase the lateral pressure in the lipid headgroup region by partitioning into this region. This is then compensated by a decreased lateral pressure in the acyl chain region, resulting in a destabilization of the KcsA tetramer, as shown in Fig. 6, panel I. Furthermore, it was proposed that the presence of nonbilayer lipids results in an increased lateral pressure in the acyl chain region, leading to a higher KcsA tetramer stability (Fig. 6, panel II).

Changes in the lateral pressure profile will only affect membrane proteins whose function depends on a transition between conformational states that is accompanied by a

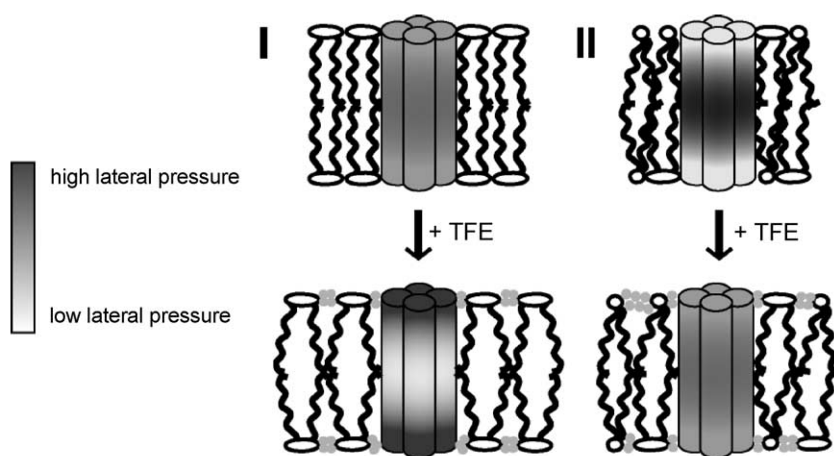

Fig. 6. Model of the effect of the surrounding lipids and TFE on the lateral pressure acting on KcsA (taken from Ref. [101]). For simplicity, a KcsA monomer is represented as a cylinder. The coloring of the cylinders indicates the positive lateral pressure acting on the KcsA tetramer, with dark coloring representing a high positive lateral pressure, and light coloring indicating a low positive lateral pressure. For simplicity, the negative lateral pressure at the interface between the headgroups and acyl chains is not indicated in the model. The KcsA tetramer is shown in a bilayer containing bilayer lipids (I) or nonbilayer lipids (II). The effect of addition of TFE (±20 vol.%) on the lateral pressure is also shown for both systems. The data suggest that at a concentration of ±20 vol.% TFE, the acyl chain lateral pressure in a bilayer of PE/PG (7:3) is similar to the acyl chain lateral pressure in a membrane of PC/PG (7:3) without TFE, since in both systems an additional 25 vol.% TFE is needed to induce KcsA tetramer dissociation (see Ref. [101]).

depth-dependent change in the cross-sectional area [68,87]. It can be expected, for example, that a transition from a cylindrically shaped membrane protein structure towards an hourglass-shaped membrane protein structure is stimulated by the presence of nonbilayer lipids, as was suggested previously [68,87]. Therefore, the question is arising on how the shape of KcsA is changed during the tetramer formation and dissociation. Above, a simplified model was used to describe the oligomerization of KcsA (Fig. 6), in that in these figures the shape of the membrane embedded part of the protein was kept constant. The crystal structure of KcsA shows that the transmembrane domain of the KcsA tetramer has an inverted conical (also called inverted teepee) shape [104], as is schematically shown in Fig. 7A. However, the N-terminal domain of the protein is an amphipathic helix that seems to be inserted in the membrane interface [105]. As a result, the narrowest part of the protein is probably located in the lower part of the transmembrane domain, just below the center of the lipid bilayer. The structure of the KcsA monomer is unknown. However, it was shown recently that the pore region of monomeric KcsA is exposed outside the membrane during the topological membrane insertion [115]. We suggest that also during the disassembly of the KcsA tetramer, induced by the small alcohols, the pore domain of the monomeric KcsA becomes exposed, as is schematically shown as a speculative model in Fig. 7B. Furthermore, we suggest that after dissociation of the KcsA tetramer induced by the alcohols, the N-terminal domain of the protein is not inserted in the lipid bilayer anymore, because of the partitioning of the alcohol in the lipid bilayer and the direct interactions of the alcohol molecules with the protein (see Fig. 7B). It is not known if the transmembrane helices in the monomer are tilted and associated, but most likely the overall shape is more cylindrical compared to the KcsA subunit in the tetramer, due to the flipped-out pore-region and N-terminal domain. On this basis, we suggest that alcohol-induced dissociation of KcsA is accompanied by a transition from an hourglass-shaped structure towards a more cylindrical-shaped structure. Such a transition would be inhibited by the presence of nonbilayer lipids, which is indeed what was observed [101]. The observation that the narrowest part of the tetrameric structure is located in the lower part of the transmembrane domain might be related to an enrichment of nonbilayer lipids in the inner leaflet of the membrane, as has been observed for the plasma membrane of eukaryotes [121,122], in which many ion channels function. In addition, the presence of these nonbilayer lipids would facilitate the membrane insertion of the N-terminal part of the protein, due to the presence of insertion sites.

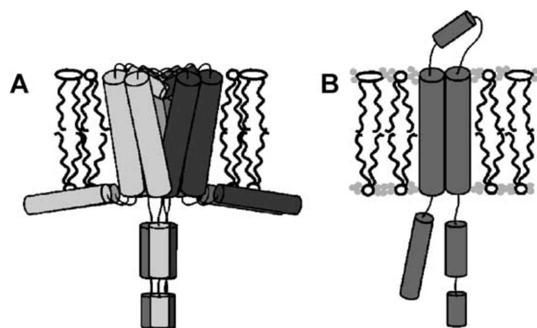

Fig. 7. Simplified model for the structure of the KcsA tetramer (A) and a speculative model of the KcsA monomer after the alcohol-induced tetramer dissociation (B). See text for further comments. Derived from Ref. [102].

Another model to explain the effect of nonbilayer lipids on integral membrane proteins deals with hydrophobic mismatch, as explained in Section 3.2. This model only applies to proteins that occur in two (or more) conformations with different hydrophobic lengths. It has been suggested that nonbilayer lipids will stabilize the conformation with the largest hydrophobic length [34,42,87]. This model was used to explain the observation that nonbilayer lipids stabilize meta II state of rhodopsin, which is suggested to be longer than the meta I state [34]. Although the exact structure of the KcsA monomer is not known, the model in Fig. 7 suggests that the hydrophobic length of monomeric KcsA is similar or larger than that of tetrameric KcsA. In that case, the mismatch-model would predict a destabilizing effect of nonbilayer lipids on the KcsA tetramer, which is in contrast with our observations. This suggests that in the case of KcsA there is no or only a small effect of hydrophobic mismatch, but that the effect of the membrane lateral pressure is dominating.

6. Biological implications

Although we described here only the study of two specific membrane proteins, these proteins can be considered as model proteins for a large class of peripheral and integral membrane proteins. We suggest that the observed effects of nonbilayer lipids reflect a general principle on the role of these lipids, namely that nonbilayer lipids stimulate membrane binding of peripheral membrane proteins and affect the stability of (oligomeric) complexes of integral membrane proteins via changes in the lateral pressure profile, which will be important for the function of these proteins. Examples of membrane proteins that follow this principle are not only the ones described here, but probably also the proteins listed in Section 3, which were activated by the presence of nonbilayer lipids. We expect that in the future for much more membrane proteins such a role of nonbilayer lipids will appear, for example for peripheral membrane proteins that are bound to the inner leaflet of the plasma membrane of eukaryotes, since this leaflet of the membrane is strongly enriched with the nonbilayer lipid PE [121,122]. Although we propose this effect of nonbilayer lipids on membrane proteins via the lateral pressure profile to be a general principle, it might be that for some membrane proteins other effects, for example due to electrostatic interactions, are more dominating.

We focused in this review mainly on the structural aspect of this principle. However, we suggest that also for the

specific proteins that were tested, this will have functional consequences. The first model protein *E. coli* Leader Peptidase cleaves off the signal peptide of translocated proteins. It was observed that nonbilayer lipids stimulate the membrane binding and insertion of the catalytic domain of this protein [72]. Since signal peptides are too short to span the membrane [123] and are anchored at the cytosolic side of the membrane [124,125], the catalytic domain of Leader Peptidase likely has to insert quite deeply into the membrane to optimize contact with the signal peptide cleavage site. This would be facilitated by the presence of nonbilayer lipids, which are abundant in *E. coli* (75% of the total lipid composition is PE). This was recently confirmed by the observation that the enzymatic activity of wild-type *E. coli* Leader Peptidase reconstituted in a PC bilayer strongly increases in the presence of PE [126].

The second model protein *S. lividans* KcsA functions as a potassium channel. It was observed that nonbilayer lipids stabilize the tetrameric structure of KcsA, which could be destabilized by small alcohols [101,102]. Since the KcsA tetramer is extremely stable, the oligomer formation seems to be structural and not part of functional regulation. However, it was suggested previously that the initial assembly of the potassium channel is stimulated by nonbilayer lipids, as was shown for the nonbilayer lipid PE [127]. In addition, we expect that also the gating mechanism of KcsA will be influenced by the presence of nonbilayer lipids. Although the crystal structure of KcsA only shows the closed structure of the protein, several studies indicate that the opening of the KcsA tetramer is accompanied by a conformational change, especially in the lower part of both transmembrane helices [108,128,129]. Since this is the narrowest part of the transmembrane domain of KcsA in the closed conformation (see above), the opening of the channel results in a more cylindrical overall shape. Therefore, we expect that non-bilayer lipids, which increase the lateral pressure in the hydrophobic part of the bilayer, will have a an inhibiting effect on the channel opening of KcsA, while the gating would be stimulated by a decrease in the acyl chain lateral pressure. Similarly, it has been shown that the channel opening of the mechanosensitive channel McsL is induced by a decreased acyl chain pressure, created by the asymmetric incorporation of inverted cone shaped lipids (opposite to nonbilayer lipids) [130,131]. For other types of potassium channels, the effect of nonbilayer lipids may be different. For example, for the voltage-gated potassium channel KvAP, it has been shown that opening of the channel is accompanied by a movement of the voltage-sensing helices from the inner leaflet towards the outer leaflet of the membrane [132], resulting in a totally different overall shape of the protein complex. In contrast to KcsA, it has been shown for several other membrane proteins that oligomer formation and dissociation can be used as a regulatory mechanism, for example for G protein-coupled receptors [133]. A similar mechanism has been proposed for transporter proteins, such as the Na^+–H^+ transporter NhaA [134]. We suggest that nonbilayer lipids affect the function of these proteins by affecting oligomer formation.

Possibly, the interaction between nonbilayer lipids and membrane proteins provides the cell an additional means to regulate the processes that are carried out by these membrane proteins, for example by variations of the lipid composition. A similar suggestion was done earlier for mitochondrial respiration that might be controlled by thyroid hormones at the level of CL synthase [135]. Since the lateral pressure is an overall property of the membrane, such adaptations in the lipid composition will have a general effect on all membrane proteins in a particular membrane. However, the existence of membrane domains, with a specific lipid and protein composition, may provide the possibility to influence the function of only a limited group of membrane proteins. Not only variations in the lipid composition, but also the presence of small solutes, such as neurotransmitters [136] and anesthetics like alcohols, might be used control the function of membrane proteins via the membrane lateral pressure.

Acknowledgements

We thank the Dutch Foundation for Fundamental Research on Matter (FOM) for financial support. We also want to thank the other members of the section Biochemistry of Membranes at the Utrecht University for their various contributions to the work described in this review.

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