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Regulation of membrane protein function by lipid bilayer elasticity—a single molecule technology to measure the bilayer properties experienced by an embedded protein

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Abstract

Membrane protein function is generally regulated by the molecular composition of the host lipid bilayer. The underlying mechanisms have long remained enigmatic. Some cases involve specific molecular interactions, but very often lipids and other amphiphiles, which are adsorbed to lipid bilayers, regulate a number of structurally unrelated proteins in an apparently non-specific manner. It is well known that changes in the physical properties of a lipid bilayer (e.g., thickness or monolayer spontaneous curvature) can affect the function of an embedded protein. However, the role of such changes, in the general regulation of membrane protein function, is unclear. This is to a large extent due to lack of a generally accepted framework in which to understand the many observations. The present review summarizes studies which have demonstrated that the hydrophobic interactions between a membrane protein and the host lipid bilayer provide an energetic coupling, whereby protein function can be regulated by the bilayer elasticity. The feasibility of this 'hydrophobic coupling mechanism' has been demonstrated using the gramicidin channel, a model membrane protein, in planar lipid bilayers. Using voltage-dependent sodium channels, N-type calcium channels and $GABA_A$ receptors, it has been shown that membrane protein function in living cells can be regulated by amphiphile induced changes in bilayer elasticity. Using the gramicidin channel as a molecular force transducer, a nanotechnology to measure the elastic properties experienced by an embedded protein has been developed. A theoretical and technological framework, to study the regulation of membrane protein function by lipid bilayer elasticity, has been established.

(Some figures in this article are in colour only in the electronic version)

Contents

List of abbreviations

c_0	monolayer spontaneous curvature
C_A	aqueous concentration
CMC	critical micellar concentration
D	dimer
d_0	bilayer hydrophobic thickness
DHA	docosahexaenoic acid
DOPC	dioleoylphosphatidylcholine
DOPS	dioleoylphosphatidylserine
DPhPC	diphytanoylphosphatidylcholine
F	bilayer disjoining force on the gramicidin channel
f	channel appearance rate
FMM	fluid mosaic model
g	gramicidin channel conductance
gA	gramicidin A
GX100	Genapol X-100
HCM	hydrophobic coupling mechanism
k_a	association rate constant
K_a	area compression modulus
K_c	bending modulus

K_D	channel dimerization constant
k_d	dissociation rate constant
K_G	Gaussian curvature modulus
l	hydrophobic length
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPI	lysophosphatidylinositol
LPL	lysophospholipid
LPS	lysophosphatidylserine
M	monomer
N	number of conducting channels, channel activity
PUFA	polyunsaturated fatty acid
r_0	bilayer inclusion radius
rTX100	reduced Triton X-100
SOPC	1-stearoyl-2-oleoyl-phosphatidylcholine
TMD	trans-membrane domain
TX100	Triton X-100
u_0	linear extent of monolayer deformation
VDSC	voltage-dependent sodium channel
V_{in}	voltage of half maximal inactivation
ΔG_{cont}	bilayer deformation energy described by continuum elastic properties
ΔG_{def}	bilayer deformation energy
ΔG_{gA}	energetic cost of gA channel formation
$\Delta G_{int,gA}$	intrinsic energetic cost of gramicidin channel formation
$\Delta G_{packing}$	energetic contribution from changes in local lipid packing
$\Delta G_{protein}$	energetic cost of protein conformational change
ΔG_a^*	activation energy for monomer association
ΔG_d^*	activation energy for monomer dissociation
ΔG_{int}	intrinsic energetic cost of protein conformational change
βOG	β octyl glucoside
γ	interfacial tension
τ	gramicidin channel lifetime

1. Introduction

Membrane protein function is regulated by the molecular composition of the cell membrane lipid bilayer. There are numerous examples of such regulation [1–7], but despite extensive research the underlying mechanisms have long remained enigmatic. In some cases lipids (e.g., phosphoinositides [8]) regulate membrane protein function by specific, stoichiometric, binding to their target proteins. More often specific interactions have not been identified, and the apparently non-specific regulation of protein function is poorly understood. A similar situation applies to the regulation of membrane protein function by other amphiphiles that are adsorbed to lipid bilayers; some cases involve binding to specific receptors, but many such compounds regulate a number of unrelated proteins with little apparent specificity (e.g., [9]).

Early studies noted that many amphiphiles, which prevent excitation of excitable cells and regulate membrane protein function in a rather unspecific manner, protect red blood cells

against osmotic hemolysis [10]. This led to the hypothesis that such 'membrane stabilizing' compounds could regulate membrane protein function by altering the physical properties of the host lipid bilayer [10, 11]. The notion of membrane stabilizing compounds is still used (e.g., [12, 13]), but the possible connection between the effects on membrane protein function and bilayer physics has not been established.

It is today generally accepted that a change in bilayer molecular composition *can* alter protein function in a manner which depends on changes in the bilayer physical properties rather than on specific interactions [1, 2, 4–7]. However, the underlying mechanisms, and the role of such changes in the more general regulation of membrane protein function, have long remained unclear.

Considerable research effort is invested in studying the regulation of membrane protein function by biological and pharmaceutical amphiphiles, very often at concentrations that may affect the physical properties of lipid bilayers (cf [9, 14]). The importance of lipids in cellular signalling [3, 15–17] and major diseases, such as cardiovascular diseases and type II diabetes [18, 19], is increasingly appreciated. Membrane proteins account for ∼70% of all known drug targets [20], and a considerable amount of pharmaceuticals are amphiphiles. Pharmaceutical development thus to a large extent involves studying the effects of amphiphiles on membrane protein function. In studies of the biological effects of amphiphiles, the role of specific interactions can be analysed within a general framework involving well-described theories of ligand–receptor interactions and a number of technologies. However, there is no generally accepted framework in which to study the role of changes in the physical properties of the host lipid bilayer. The conclusion that changes in bilayer physics could be involved thus generally has to rely on diagnosis of exclusion; i.e., effects for which specific interactions have not (yet?) been identified.

In order to understand the promiscuous regulation of membrane protein function by lipids and other amphiphiles, a general theoretical and technological framework to study the role of changes in bilayer physics needs to be established. Many amphiphiles alter the (pseudo) equilibrium among protein conformational states, and such a framework should be based on equilibrium energetic principles and allow for quantitative predictions, which can be experimentally tested.

Many membrane proteins are regulated by collective or 'general' physical properties of the host lipid bilayer, such as bilayer thickness or monolayer spontaneous curvature (cf [2, 4–7, 21, 22]). Though well-established this type of regulation is not well understood, as is evident from the variety of descriptors that have been proposed for the underlying mechanisms, for example, changes in: bilayer coupling [23]; bilayer compression energy [24]; curvature frustration energy [25]; acyl chain packing [26]; bilayer deformation energy [27, 28]; bilayer stiffness [9, 14, 29, 30]; lateral pressure profile across the bilayer [31]; lipid packing stress [2]; and bilayer free volume [32, 33] or bilayer fluidity [34].

Part of the difficulties arise from the complex structure of membrane proteins and biological membranes, as well as the limited structural information about the conformational changes involved in the membrane protein function. However, the fundamental uncertainties pertain even to 'simple' model systems involving peptides of known structure in lipid bilayers of a well-defined composition. First, because the relation between bilayer molecular composition and physical properties has to be inferred from measurements in protein-free lipid systems, which is problematic [9, 14, 35]. Second, because even in model systems, there has been no generally accepted framework in which to analyse the many observations.

Given the diversity of lipids and other amphiphiles in cellular membranes (e.g., [17]), it is unlikely that just a single organizing principle can account for all the non-specific effects on the membrane protein function. However, an important element, which has not

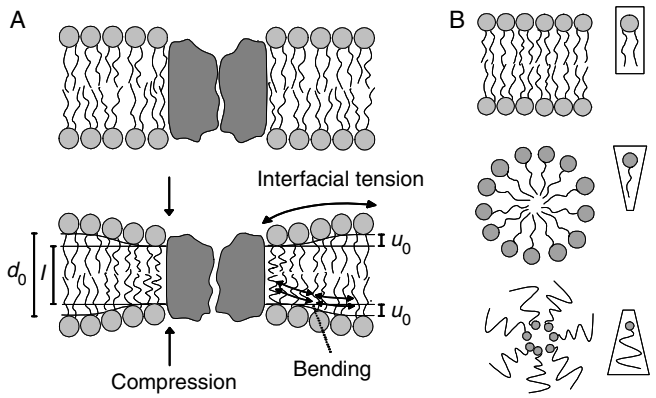

Figure 1. (A) Hydrophobic coupling between a membrane protein and the surrounding lipid bilayer. A protein conformational change causes a local bilayer deformation. (B) Top panel: lipids with an effective cylindrical shape form lipid bilayers with a monolayer spontaneous curvature of zero. Middle panel: amphiphiles with an inverted cone shape form micelles with a positive monolayer curvature. Bottom panel: cone-shaped amphiphiles form H_{II}-phases with a negative monolayer curvature. Modified from [14].

been considered in much of earlier work, is the energetic cost of the bilayer deformation associated with a protein conformational change at the protein–bilayer hydrophobic interface. Lipid bilayers are elastic bodies and due to the protein–bilayer hydrophobic interactions, such a conformational change may cause a local bilayer deformation (figure 1(A)). As the bilayer deformation energy contributes to the total energetic cost (free energy change) of the conformational change, the protein conformational distribution will depend on the bilayer elastic properties [1, 14, 27–29, 36–39]. The protein–bilayer hydrophobic interactions therefore provide an energetic coupling between protein conformation and bilayer elasticity—and thus between protein function and bilayer molecular composition. A number of theoretical studies have predicted the existence of such a regulatory mechanism [27, 36–39]. The present review summarizes studies which have investigated the practical feasibility of this 'hydrophobic coupling mechanism' [14] has been investigated. Based on measurements of lipid bilayer elasticity using a bilayer-embedded molecular force transducer, a general framework to study the regulation of membrane protein function by bilayer elasticity has been developed [9, 14, 28–30, 40–42].

1.1. The fluid mosaic model

The present understanding of the structural arrangement of biological membranes is based on the fluid mosaic model (FMM) [43]. This model rests on the generalization that the gross structure of biological membranes is organized by a single physical principle: the need for optimizing hydrophobic interactions among the amphiphilic membrane-forming molecules. For membrane lipids this entails an arrangement similar to a conventional lipid bilayer, in which the acyl chains and/or other non-polar groups are buried in the hydrophobic bilayer core, while charged or polar head groups are exposed to the aqueous environment. For membrane proteins it means burying non-polar residues in the bilayer core, while exposing charged or polar residues to the environment. A cell membrane lipid bilayer is thus viewed as a two-dimensional fluid (mosaic), in which molecular lateral diffusion and structural changes, e.g., protein conformational changes, are allowed, but molecular escape from the bilayer is

(relatively) prohibited by the energetic cost of exposing a hydrophobic surface to the aqueous environment. Recent findings strongly suggest some lateral organization of the molecules in cell membrane lipid bilayers, as described below. Nevertheless, the fundamental generalization employed in the FMM, which is that the gross structure of a cell membrane lipid bilayer is organized by the hydrophobic interactions among the bilayer-forming molecules, while detailed molecular structure can be ignored, is a central paradigm in biological sciences.

1.2. The hydrophobic matching principle

A mismatch between the thickness of a lipid bilayer hydrophobic core (hydrophobic thickness) and the length of the hydrophobic part of an inclusion (hydrophobic length) is associated with an energetic penalty due to exposure of hydrophobic surface to the aqueous environment [24]. To avoid such hydrophobic mismatch membrane protein trans-membrane segments must be of sufficient length and hydrophobicity to span the bilayer core. Evidence of long hydrophobic stretches in membrane proteins began to accumulate at the time when the FMM was proposed [44–47]. These and later similar findings led to the Kyte–Doolittle method for predicting membrane protein trans-membrane segments based on their hydrophobicity and length, rather than by their specific amino acid sequence [48]. More recent prediction methods also consider the specific amino acid composition of segments; but the fundamental principle employed in the Kyte–Doolittle method, which is to recognize the organizing potential of the energetic penalty due to hydrophobic mismatch, provides the basis for present methods.

The FMM and the Kyte–Doolittle method describe the average structural organization of a cell membrane lipid bilayer by the hydrophobic interactions among the bilayer-forming molecules. They do not consider the dynamic regulatory potential of these interactions. In the FMM, the lipid component of the bilayer is treated as a thin sheet of liquid hydrocarbon—the point being that a fluid lipid bilayer allows for membrane protein conformational changes. In the Kyte–Doolittle method, the bilayer is implicitly treated as a rigid structure—the point being the importance of hydrophobic mismatch. A lipid bilayer, however, is an elastic macrostructure, and the energetic penalty due to hydrophobic mismatch with an embedded protein may be minimized by a local bilayer deformation to match the protein hydrophobic length (figure 1(A)). The energetic cost of such a deformation depends on the bilayer collective elastic properties given by the molecular composition [27, 30, 36, 49–51]. A change in lipid bilayer hydrophobic thickness, e.g. during a temperature-induced lipid phase shift, will involve a change in the bilayer deformation energy associated with local adjustment of the hydrophobic thickness to match an embedded protein of a fixed hydrophobic length [24]. Based on this notion Mouritsen and Bloom (1984) [24] proposed a thermodynamic model to describe the phase diagrams of lipid–protein mixtures by the energetic cost of the bilayer compression to match the embedded proteins. The hydrophobic interactions among the bilayer-forming molecules thus allow the bilayer elasticity to regulate the structural organization of the system. This 'hydrophobic matching principle' [24] has served as a guiding principle in much of the later research on lipid–protein interactions, e.g., [6, 21].

1.3. Energetics of lipid bilayer elastic deformations

Lipid bilayers are liquid crystals that over length scales from $\sim$10 nm to several μm behave as rather uniform bodies with well-defined elastic properties [25, 52–55]. Using the continuum theory of elastic liquid-crystal deformations, the deformation of a lipid bilayer may be described as occurring in three independent modes: compression–expansion; bending; and

changes in surface area[1]. The bilayer energy density (energy per unit area) for each mode is to the first significant order given by:

Compression–expansion energy density [53]:

$$\frac{1}{2}K_{\mathrm{a}}\left(\frac{\Delta d}{d_0}\right)^2, \tag{1}$$

where K_{a} is the area compression–expansion modulus and $\Delta d/d_0$ is the relative change in bilayer thickness.

Bending energy density [52]:

$$\frac{1}{2}K_{\mathrm{c}}\left(\frac{c_1+c_2}{2}-c_0\right)^2+K_{\mathrm{G}}c_1c_2, \tag{2}$$

where K_{c} is the mean curvature bending modulus, and c_1 and c_2 are the curvatures associated with the principal radii, $R_1 = 1/c_1$ and $R_2 = 1/c_2$. The monolayer spontaneous curvature, c_0, is equal to $1/R_0$. In the following these radii will refer to a neutral plane, where bending and compression are energetically uncoupled [56–58]. K_{G} is the Gaussian curvature modulus.

Energy density due to changes in interfacial area [50]:

$$\gamma\frac{\Delta A}{A_0}, \tag{3}$$

where γ is the bilayer interfacial tension, and $\Delta A/A_0$ is the relative change in bilayer interfacial area.

The energetic cost of locally adjusting the hydrophobic thickness of a lipid bilayer to the hydrophobic length of a cylindrical inclusion has been analysed using the continuum theory of elastic liquid-crystal deformations [27, 30, 49–51]. Following these authors the bilayer deformation energy (ΔG_{def}) may be described as:

$$\Delta G_{\mathrm{def}} = \Delta G_{\mathrm{cont}} + \Delta G_{\mathrm{packing}}, \tag{4}$$

where ΔG_{cont} is the bilayer deformation energy described by the continuum elastic properties, and $\Delta G_{\mathrm{packing}}$ is the contribution from local changes in lipid packing. ΔG_{cont} may be approximated as:

$$\Delta G_{\mathrm{cont}} = \Delta G_{\mathrm{compression}} + \Delta G_{\mathrm{bending}} + \Delta G_{\mathrm{tension}}. \tag{5}$$

For a symmetrical lipid bilayer with an unperturbed hydrophobic thickness, d_0, and an inclusion with a hydrophobic length, l, the linear extent of the deformation in each monolayer, u_0, is $(d_0 - l)/2$ (figure 1(A)). ΔG_{cont} may be approximated as the surface integral of the energy densities due to compression–expansion, bending and changes in interfacial area (cf equations (1)–(3), (5)):

$$\Delta G_{\mathrm{cont}} = \int_{r_0}^{\infty}\left(\frac{1}{2}K_{\mathrm{a}}\left(\frac{2u_0}{d_0}\right)^2+\frac{1}{2}K_{\mathrm{c}}\left(\frac{c_1+c_2}{2}-c_0\right)^2-\frac{1}{2}K_{\mathrm{c}}c_0^2+K_{\mathrm{G}}c_1c_2+\gamma\frac{\Delta A}{A}\right) \times 2\pi r\,\mathrm{d}r, \tag{6}$$

where r_0 is the radius of the inclusion [50, 51]. As in equation (2), K_{c} and K_{G} refer to the bilayer elastic moduli. The term $\frac{1}{2}K_{\mathrm{c}}c_0^2$ represents the bending energy in the unperturbed planar bilayer. The contribution from interfacial tension is due to the change in interfacial area associated with the bilayer deformation. Equation (6) has provided a basis for interpretation of experimental work. However, a strict separation of the different energetic contributions to ΔG_{cont} is problematic, and their interplay is complex [50, 51]. Further, the bending

[1] A fourth mode, lipid tilt [52, 209], will not be considered here.

Hamiltonian in the form used here relates to a uniformly bent monolayer, in which all dividing surfaces are similarly curved. Nevertheless, equation (6) considers a deformation in which the neutral plane of each monolayer is bent, whereas the hydrophobic plane (facing the other monolayer) is flat (cf figure 1(A)).

1.4. The hydrophobic coupling mechanism

The hydrophobic matching principle describes how the hydrophobic interactions between a lipid bilayer and an embedded protein allow the bilayer elasticity to regulate the structural organization of the system. The protein–bilayer hydrophobic interactions allow for a similar 'hydrophobic coupling mechanism' (HCM), whereby membrane protein can be regulated by the bilayer elasticity [14]. Due to the energetic penalty for exposing a hydrophobic surface to an aqueous solution (hydrophobic exposure) a protein conformational change that involves the protein–bilayer hydrophobic interface, e.g., a change in protein hydrophobic length, will cause a local bilayer deformation (cf figure 1(A)). The total energetic cost of the conformational change, $\Delta G_{\text{protein}}$, may be expressed as

$$\Delta G_{\text{protein}} = \Delta G_{\text{int}} + \Delta G_{\text{def}}, \tag{7}$$

where ΔG_{int} is the intrinsic energetic cost of the conformational change (defined as all contributions not included in ΔG_{def}). The distinction between ΔG_{int} and ΔG_{def} may not be unambiguous, but equation (7) provides for a framework in which the bilayer protein interactions may be examined. As ΔG_{def} contributes to $\Delta G_{\text{protein}}$, a change in bilayer elasticity will alter the protein conformational distribution. The protein–bilayer hydrophobic interactions therefore provide an energetic coupling between protein conformation and bilayer elasticity, and thus between protein function and bilayer molecular composition. A number of studies have predicted the existence of such a regulatory mechanism based on similar thermodynamic considerations [27, 36–39]. We have investigated the practical feasibility of the HCM [9, 14, 28–30, 40–42], as will be described in the following.

For a membrane protein with two interconverting conformational states, regulation by the HCM may be described as:

$$\ln\left\{\frac{n_{\text{state2}}}{n_{\text{state1}}}\right\} = -\Delta G_{\text{protein}}/RT = -(\Delta G_{\text{int}} + \Delta G_{\text{def}})/RT, \tag{8}$$

where the ratio $n_{\text{state2}}/n_{\text{state1}}$ describes the equilibrium distribution between the number of molecules in each of the two states, and R and T are the gas constant and temperature in kelvin, respectively. In order for the HCM to be important for protein function, three conditions would have to be fulfilled:

(A) Protein function should involve conformational changes at the protein–bilayer interface.
(B) ΔG_{def} should make a significant contribution to $\Delta G_{\text{protein}}$.
(C) Changes in bilayer molecular composition should significantly alter ΔG_{def}.

(A) Protein conformational changes at the protein–bilayer interface. Ample evidence shows that membrane protein function involves conformational changes at the protein–bilayer interface. Early, low-resolution structures of gap junction channels [59] and the nicotinic acetylcholine receptor [60] show that channel function is associated with alterations in subunit tilt within the bilayer, suggesting that the channel hydrophobic length is altered. Similar subunit rearrangements, based on high-resolution structures of extra-membranous domains, have been proposed in glutamate-activated channels [61]. High-resolution structures of protein trans-membrane domains provide evidence for structural reorganization in stretch-activated MscL channels [62, 63]; bacteriorhodopsin [64, 65]; H^+- and Ca^{2+}-gated potassium

channels [66, 67]; the sarcoplasmic Ca^{2+}-ATPase [68]; and members of the Major Facilitator Superfamily of transport proteins [69, 70]. Further, chemical cross-linking and spectroscopic studies provide evidence for movement of membrane-spanning α-helices relative to each other [71–73], and studies using the substituted cysteine accessibility method show that $GABA_A$ receptor function involves conformational changes at the trans-membrane domain (cf [74]).

((B), (C)) Contribution from ΔG_{def} to $\Delta G_{protein}$—and changes in ΔG_{def}. Could ΔG_{def} make a significant contribution to $\Delta G_{protein}$—and could this contribution be considerably altered by changes in bilayer molecular composition? Membrane protein conformational changes can perturb the structure of the surrounding lipid bilayer (e.g., rhodopsin [75] and Na^+, K^+-ATPase [76]), but ΔG_{def} cannot be measured directly. The fact that many membrane proteins are regulated by bilayer continuum elastic properties, such as thickness or monolayer spontaneous curvature [2, 4–7, 21, 22], suggests that ΔG_{cont} is important for protein function. However, there are a number of difficulties in studying the role of ΔG_{cont}. In the continuum theory of liquid crystal deformations a bilayer deformation induced by an inclusion is decomposed into three different modes, and the corresponding elastic parameters, in principle, should be transferable from measurements in protein-free lipid systems. However, a strict separation between the energetic contributions to ΔG_{cont} is problematic [50, 51], and a change in bilayer lipid composition will always alter more than one of the elastic parameters [14]. Therefore such transferal is difficult. Moreover, membrane protein conformational changes are complex, and even in cases where the protein–bilayer interface is known to be involved, there is insufficient structural information to estimate the impact on the bilayer (and thus on ΔG_{cont}). Further, even if the elastic properties were transferable, and the bilayer deformation were well described, the continuum elastic parameters measured in protein-free lipid systems may not be relevant for estimating ΔG_{cont} [9, 14, 35]. First, these parameters are measured during global deformations of lipid mono- or bilayers, that extend over ranges from several nm to several μm [25, 52–55], while the local bilayer perturbation surrounding an inclusion is likely to involve only a few rings of lipid molecules and extend less than 1 nm [50]. Second, the topology of the deformation may be different; for example the bending modulus, measured during bilayer bending, involves monolayer curvatures of opposite sign, whereas the monolayer curvatures imposed by a bilayer inclusion may be of the same sign (cf figure 1(A)). Given these uncertainties, and the fact that a change in bilayer molecular composition may have opposite effects on the different contributions to ΔG_{cont} (cf equation (6)), even the sign of the change in this value is generally difficult to predict [9, 14].

We have investigated the practical feasibility of the HCM using gramicidin (gA) channels in planar lipid bilayers as a model system. This system has a number of unique features, which allow for such studies. The structure of the gA channel is known at atomic resolution [77–80] and the channel function can be studied with single molecule resolution using a voltage clamp technique. Gramicidin channel formation involves a simple 'conformational change', which is associated with a deformation of the surrounding lipid bilayer [27, 30, 49–51]. A change in ΔG_{def} is directly reflected in the channel function. Gramicidin channels can thus be used to measure the effects of changes in bilayer molecular composition on ΔG_{def}.

The following section describes studies which have demonstrated the energetic feasibility of the HCM using gA channels in planar lipid bilayers. These studies have further shown that the gA channel can be used as a molecular force-transducer for *in situ* measurements of the bilayer elasticity experienced by an embedded protein. Section 3 describes studies showing that membrane function in living cells can be regulated by changes in bilayer elasticity, as measured using gA channels. The final sections discuss the possible implications of the HCM.

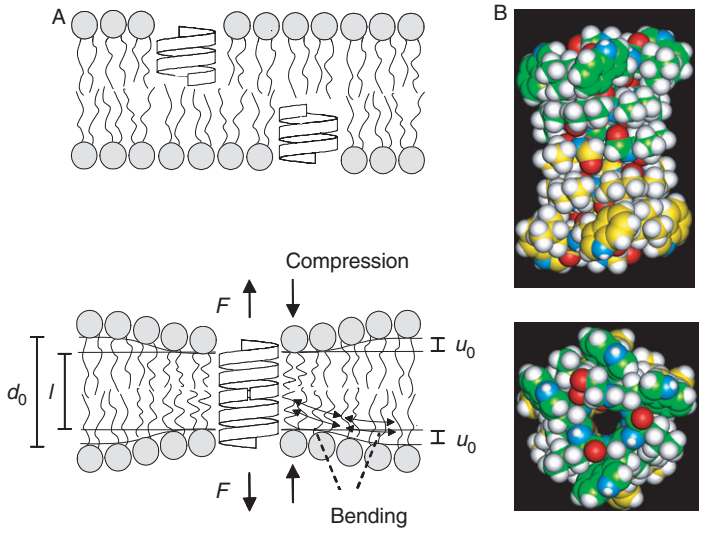

Figure 2. (A) Gramicidin channel formation by the trans-bilayer association of two monomers causes a local bilayer deformation. Modified from [14]. (B) Side and end views of a bilayer-spanning gramicidin channel. Energy minimized structure representing a composite of the structures determined using NMR [77–79].

2. Gramicidin channels as molecular force transducers

2.1. Gramicidin channels as a model system

Gramicidin A (gA) is a bacterial pentadecapeptide with the sequence formyl-L-Val-D-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine. The standard gA channel is a symmetrical cation-selective ion channel formed by formyl-NH-terminal to formyl-NH-terminal association of a right-handed $\beta^{6.3}$-helical monomer from each monolayer in a lipid bilayer (figure 2(A)) (for a recent review, see [81]). The monomers are joined by six intermolecular hydrogen bonds. Their hydrogen-bonded peptide backbones line the ion conducting pore, while the hydrophobic amino acid side chains face the surrounding lipid bilayer core. The atomic resolution structure of the gA channel has been determined by solution and solid-state NMR [77–79] (figure 2(B)). The radius of the channel is ∼1 nm, and the channel hydrophobic length is ∼2.2 nm [82]. Channel dissociation involves loss of conductance, when the monomers are separated by ∼0.16 nm [83, 84]. gA channels are particularly amenable to sequence modifications, and channels of different lengths and chirality can be produced using standard solid-state peptide chemical methods [85, 86].

The dimerization of two gA monomers (M), one from each monolayer in a lipid bilayer, to form a conducting channel dimer (D) may be described as

$$M + M \underset{k_d}{\overset{k_a}{\rightleftarrows}} D, \tag{9}$$

where k_a and k_d denote the monomer association and dissociation rate constants, respectively. The channel dimerization constant (K_D) is given by:

$$K_D = k_a/k_d = [D]/[M]^2, \tag{10}$$

where $[M]$ and $[D]$ are the bilayer concentrations of M and D [28].

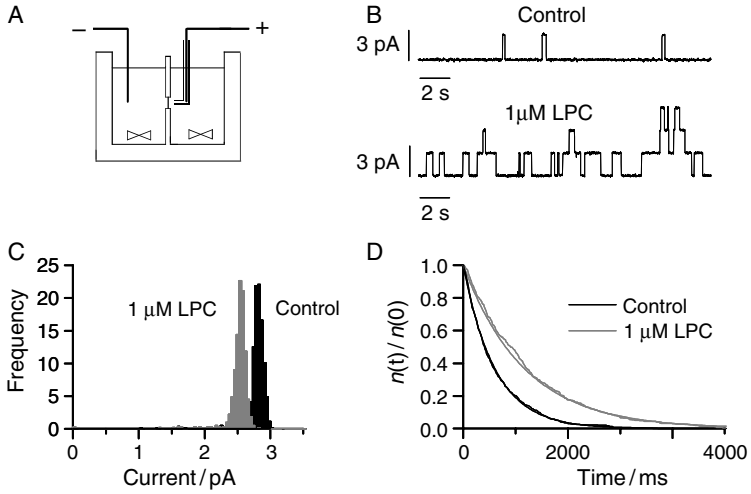

Figure 3. (A) Experimental setup used in gA channel experiments. (B) Effects of lysophosphatidylcholine (LPC) on gA channel behaviour in a DPhPC/n-decane bilayer. Single-channel current traces from the same large membrane before (top) and after (bottom) addition of 1 μM LPC to the electrolyte solution. Trans-bilayer potential 200 mV. Current traces filtered at 200 Hz. (C) Current transition amplitude histograms for gA channels in the control situation or in the presence of LPC. (D) Normalized survivor histograms for gA channels in the control situation or in the presence of LPC. The average channel lifetime, τ, is determined by fitting a single exponential distribution ($n(t)/n(0) = \exp\{-t/\tau\}$, where $n(0)$ and $n(t)$ denote the number of channels at time zero and t) to the results. Modified from [28].

gA channel function in planar lipid bilayers may be studied using the bilayer-punch method illustrated in figure 3(A) [87]. Briefly, a 'large' membrane is formed across a hole (diameter ~1.6 mm) in a Teflon partitioning separating two electrolyte solutions. gA (~1–10 pM) is added to the solutions and due to its hydrophobic character adsorbed to the bilayer. A small area of the bilayer is isolated using the tip of a glass pipette (diameter ~30 μm), and a trans-bilayer potential difference is applied. The appearance and disappearance of single gA channels are observed as distinct transitions in the current amplitude (figure 3(B)). The average channel lifetime (τ) is equal to $1/k_d$. In a bilayer where $[M] \gg [D]$, a change in $[D]$ will not significantly affect $[M]$ (cf equation (9)), and the channel appearance rate (f) and the time averaged number of conducting channels (channel activity, N) are proportional to k_a and K_D, respectively [28]. The absolute value of k_d, and changes in k_a and K_D, thus can be measured directly.

Formation of a gA channel in a symmetrical lipid bilayer with an unperturbed hydrophobic thickness, d_0, larger than the channel hydrophobic length, l, is associated with a hydrophobic mismatch of $d_0 - l$ (figure 2(A)). Principally, the energetic penalty due to such mismatch could have contributions from: (1) exposure of hydrophobic surface to the aqueous environment (hydrophobic slippage); (2) a change in l; (3) a local adjustment of bilayer hydrophobic thickness to match l.

Hydrophobic slippage is unlikely to occur, except in cases of very large hydrophobic mismatch [30]. Further, a gA channel is very rigid compared to a lipid bilayer, as shown by the fact that the channel helical pitch is not affected by changes in d_0 [88]. l therefore may be considered invariant[2]. gA channel formation is thus associated with an elastic bilayer

[2] The behaviour of gA channels in dierucoylphosphatidylcholine (di-$C_{22:1}$-PC)/n-decane bilayers, suggests that a very large channel–bilayer hydrophobic mismatch may affect the channel conformation [210].

deformation, in which the bilayer hydrophobic thickness locally adjusts to match the channel hydrophobic length. Due to this deformation the bilayer will impose a disjoining force, F, on the channel, which is directly reflected in f, τ, and N.

The energetic cost of gA channel formation, ΔG_{gA}, may be described as

$$\Delta G_{gA} = \Delta G_{gA,int} + \Delta G_{def} = \Delta G_{gA,int} + \Delta G_{packing} + \Delta G_{cont}, \tag{11}$$

where $\Delta G_{gA,int}$ is the intrinsic cost of channel formation, defined as all energetic contributions not included in ΔG_{def}.

The relative importance of the energetic contributions from compression–expansion, bending and interfacial tension to ΔG_{cont}, when a lipid bilayer locally adjusts to match an inclusion with the dimensions of a gA channel, has been analysed using the continuum theory of elastic liquid-crystal deformations [27, 49–51]. The contributions from interfacial tension and Gaussian curvature were found to be negligible, which means that ΔG_{cont} may be approximated as (cf equation (6))

$$\Delta G_{cont} = \pi \int_{r_0}^{\infty} \left(K_a \left(\frac{2u_0}{d_0} \right)^2 + K_c \left(\left(\frac{c_1 + c_2}{2} - c_0 \right)^2 - c_0^2 \right) \right) r \, dr. \tag{12}$$

Using a relaxed boundary condition (in which molecular detail, at the bilayer–channel interface, is ignored, cf [50, 51]), ΔG_{cont} should be increased by larger values of K_a and K_c, as well as by more negative values of c_0.

ΔG_{cont}, as described by equation (12), may be expressed by the relation:

$$\Delta G_{cont} = H_B \cdot (2u_0)^2 + H_X \cdot 2u_0 \cdot c_0 + H_C \cdot c_0^2, \tag{13}$$

where the coefficients H_B, H_X and H_C are determined by r_0, d_0, K_a, and K_c [14, 51]. The bilayer disjoining force on the channel, F, ($=d\Delta G_{cont}/d2u_0$) is given by [9]:

$$F = 2H_B \cdot (2u_0) + H_X \cdot c_0. \tag{14}$$

F will be increased by larger values of H_B and u_0, as well as by more negative values of c_0 and H_X (as H_X is always negative).

Because F is an increasing function of the bilayer–channel hydrophobic mismatch, τ is a decreasing function of d_0 [82, 89–94]. As will be shown below, the relation between τ and d_0 may be described using equation (13) [27, 30, 49]. gA channel function can thus be modulated by thickness-dependent changes in ΔG_{def}. In order to investigate the feasibility of the HCM, we have studied whether gA channel function could similarly be modulated by changes in ΔG_{def} induced by altered bilayer elasticity. The experimental studies have been based on, and tested the validity of, the theoretical continuum analysis described above.

2.2. Effects of micelle-forming amphiphiles and cholesterol

2.2.1. Lysophospholipids. Lysophospholipids (LPLs) regulate the function of a wide range of membrane proteins. Some of these effects occur at sub-micromolar concentrations and involve interactions with specific receptors. Lysophosphatidic acid and the LPL-like molecule, platelet activating factor (PAF), bind with nanomolar affinity to their cognate receptors [95, 96]. A high affinity receptor for lysophosphatidylcholine (LPC) has similarly been described [96] (although the identity of this receptor has been questioned [97]). At micromolar concentrations LPLs regulate a number of unrelated membrane proteins with little apparent specificity (e.g., K_{ATP} channels [98]; Na^+, K^+-ATPase [99]; nicotinic acetylcholine receptors [100]; TREK channels [101]; HERG channels [102] and voltage-dependent sodium channels [103]).

Adsorption of LPLs to a lipid bilayer will alter the bilayer physical properties. LPC decreases the apparent area expansion modulus [104] and bending modulus [105] of lipid

bilayers. LPLs due to their molecular shape, further, promote a positive c_0. A lipid, in which the cross-sectional area of the polar head group is similar to that of the acyl chains in the bilayer hydrophobic core, may be described as having an effective cylindrical shape (figure 1(B)) [106]. Such a lipid will form a bilayer with a c_0 of zero. LPL molecules have an inverted cone shape; the cross-sectional area of the polar head group region is larger than that of the single acyl chain (figure 1(B)) [106][3]. LPLs therefore are micelle-forming and adsorption of LPLs to a lipid bilayer promotes a positive c_0 [107, 108]. For comparison, polyunsaturated free fatty acids, in which the cross-sectional area at the bilayer/solution interface is smaller than that of the acyl chain in the bilayer core, promote the formation of inverted hexagonal phases (H_{II}-phases) with a negative c_0 (figure 1(B)) [109, 110]. Most bilayer-forming lipids have a more or less negative c_0, which means that the bilayers formed exist in a state of curvature stress, where the tendency of the monolayers to adopt a non-planar structure is opposed by the hydrophobic interactions between the monolayers, cf [40, 109, 111].

Could LPLs modulate membrane protein function by altering the elasticity of the host lipid bilayer? PAF, at micromolar concentrations, increases gA channel lifetime in planar lipid bilayers, suggesting that this may be the case [112]. We have investigated the question in a systematic study of the effects of different LPLs on gA channel behaviour in diphytanoylphosphatidylcholine (DPhPC)/n-decane bilayers [28]. The hydrophobic thickness of a DPhPC/n-decane bilayer is ~4.7 nm, which is about twice the hydrophobic length of the gA channel [28]. Given that LPLs promote a positive c_0, and decrease K_a and K_c, one would expect the bilayer disjoining force on the channel to decrease, and both f and τ to increase (cf equations (12)–(14)).

Figure 3(B) shows current traces from an experiment, where the effects of LPC on gA channel function are studied. Addition of 1 μM LPC to the electrolyte solution (1 M NaCl) causes an increase in f and τ, and thus in N. The channel conductance (g) is slightly decreased. The changes in τ and g are quantified in the corresponding current transition amplitude and lifetime distribution histograms (figures 3(C)–(D)). As shown in figure 4(A), LPLs with four different polar head groups (LPC; lysophosphatidylethanolamine (LPE); lysophosphatidylinositol (LPI); and lysophosphatidylserine (LPS)) all cause a concentration-dependent increase in τ. A bilayer-forming monoglyceride with an effective cylindrical shape (1-monooleoyl-rac-glycerol) does not increase τ.

As shown in figure 4(B) the four LPLs all increase gA channel activity in a concentration-dependent manner. The channel appearance rate f is similarly increased, and for all concentrations the increase in f is larger than in τ [28].

In summary the four LPLs all increase f, τ, and N. 2 μM LPC causes a 100-, 5- and 500-fold increase in f, τ, and N, respectively. The bilayer hydrophobic thickness and the surface tension of DPhPC monolayers are not affected by 2 μM LPC [28].

The fact that the LPLs increase both f and τ is in agreement with a regulatory mechanism based on a decrease in F. Such a mechanism, further, would be expected to cause a larger increase in f than in τ. Monomer association involves a local change in bilayer hydrophobic thickness from ~4.7 nm to $l + \delta \sim 2.36$ nm (the channel hydrophobic length plus the monomer separation when the channel starts to conduct). This is a change of ~2.3 nm. Monomer dissociation involves a change in bilayer hydrophobic thickness from l to $l + \delta$. This is a change of ~0.16 nm. Given that F may be approximated by equation (14), the bilayer elastic energy, stored when the monomers connect, is larger than the elastic energy released when the monomers separate to the distance where channel conductance is lost. Moreover, a decrease

[3] According to a less common convention some studies describe LPLs as having the shape of a cone rather than an inverted cone (e.g., [28, 101]).

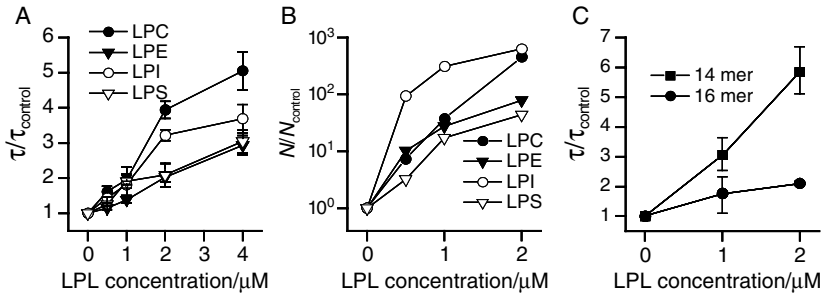

Figure 4. (A) Effects of lysophospholipids on gA channel lifetime (τ) normalized by the control value ($\tau_{control}$). (B) Effects of lysophospholipids on channel activity ($N/N_{control}$). (C) Effects of LPC on lifetime of [*des*-Val^{1}]gC channels formed by 14 amino acid monomers (14 mer), or [*endo*-Gly^{0a}]gC channels formed by 16 amino acid monomers (16 mer). Mean ± range, $n \geqslant 2$. Modified from [28].

in F, caused either by a decrease in H_B or by a more positive c_0, will cause a larger increase in the energy stored during monomer association than in that released during dissociation (cf equations (13), (14)). The change in f should thus be larger than in τ. This is what is observed.

If the LPLs decrease the elastic coefficient H_B one would further expect the increase in τ to be larger for a short channel than for a longer channel [9] (cf equations (13), (14)). This is what is found. Figure 4(C) shows the effects of LPC on channels formed by two analogues of gramicidin C (in which Trp^{11} in gA, is substituted for Tyr^{11}); [*des*-Val^{1}gC], which is 14 amino acids long, and [*endo*-Gly^{0a}gC], which is 16 amino acids long. This corresponds to a difference in hydrophobic length of ∼0.32 nm [83]. Both analogues form channels that are structurally similar to gA channels and shorter than the hydrophobic thickness of the bilayer. As shown in figure 4(C), 2 μM LPC causes a six- and two-fold increase in τ for the short and the long channel, respectively [28].

2.2.2. Synthetic micelle-forming amphiphiles. The fact that LPLs with four different head groups modulate gA channel function in a similar manner suggests that specific interactions between the head groups and gA are not involved. Could the effects be due to other properties specific for LPLs? This has been investigated using four structurally different synthetic micelle-forming amphiphiles: Triton X-100 (TX100), reduced Triton X-100 (rTX100), β-octyl-glucoside (βOG) and Genapol X-100 (GX100) [14]. TX100 promotes a positive c_0, and the other compounds should have the same effect [9]. TX100 modulates the function of a wide variety of membrane proteins in a reversible manner (e.g., voltage-dependent potassium channels [113]; voltage-dependent sodium channels [14]; N-type calcium channels [29]; K_{ATP} channels [114]; nicotinic acetylcholine receptors [115]; $GABA_A$ receptors [14]; bacteriorhodopsin [116]; Na^+, K^+-ATPase [117]; Ca^{2+}-ATPase [118]), suggesting that changes in bilayer physical properties could be involved.

Figure 5 shows results from experiments probing the effects of TX100, βOG, rTX100, and GX100 on gA channel behaviour in dioleoylphosphatidylcholine (DOPC)/*n*-decane bilayers. Figure 5(A) shows current traces from the same bilayer recorded before and after the addition of 300 μM βOG to the electrolyte solution. βOG causes a large increase in f, τ and N. TX100, rTX100 and GX100 have the same effects. The bilayer concentrations of these compounds, as described below, depend on their critical micellar concentration (CMC). Figure 5(B) shows the relation between τ and the concentrations of TX100, βOG, rTX100 or GX100, normalized by their individual CMC (300, 25 000, 250 and 150 μM for TX100; βOG; rTX100 and GX100,

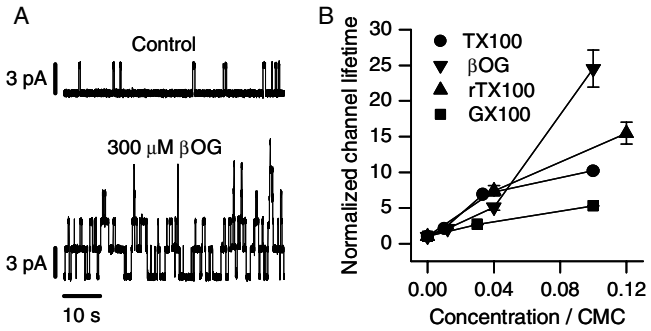

Figure 5. (A) Single-channel current traces recorded from the same DOPC/n-decane bilayer before (top) and after (bottom) the addition of 300 μM βOG. (B) Concentration-dependent increase in gA channel lifetime in a DOPC/n-decane bilayer, induced by βOG, GX100, TX100, or rTX100. The results are given as $\tau/\tau_{\text{contrl}}$ versus the amphiphile concentration normalized by the CMC. Mean $\pm$ standard error of mean (SE), $n \geqslant 3$. Modified from [14].

respectively [119]). All the amphiphiles increase τ in a concentration-dependent manner. The bilayer hydrophobic thickness ($\sim$5.0 nm) is not affected by TX100 or βOG [29]. Similar effects of micelle-forming amphiphiles on gA channel function have been obtained in other studies [29, 120].

2.2.3. Cholesterol. Cholesterol increases K_a and K_c for phospholipid bilayers [121, 122] (see also [41, 123]), and in lipid mixtures may promote H_{II}-phases with a negative c_0 [109, 124]. Cholesterol, further, decreases the hydrophobic thickness of hydrocarbon-containing phospholipid bilayers [29, 125] (while the thickness of hydrocarbon-free bilayers is actually increased [126]). Given that the micelle-forming amphiphiles affect gA channel function by increasing the bilayer elasticity, one would expect cholesterol to have the opposite effects. Cholesterol, in accordance, *decreases* τ and N for gA channels in DOPC/n-decane bilayers [14, 29].

2.2.4. Lipid bilayer concentrations of micelle-forming amphiphiles. The effects of micelle-forming amphiphiles on gA channel function depend on the concentration in the bilayer, which is not known. Amphiphiles adsorb to all hydrophobic surfaces involved in the experiments and their aqueous concentration (C_A) may be well below the nominal concentration. At low C_A the bilayer mole-fraction would be expected to vary as C_A/CMC [120, 127]. The CMC for LPC, LPE and LPS is $\sim$4–6 μM; cf [28]. The changes in gA channel function thus occur at nominal concentrations from $\sim$0.1 CMC, and at similar concentrations in the bilayer [28]. The effects of TX100, βOG, rTX100 and GX100 occur at $\sim$0.01–0.1 CMC [14].

2.2.5. Bilayer damage is not involved. Micelle-forming amphiphiles, at concentrations near their CMC, may cause breakdown of lipid bilayers [128]. The changes in gA channel behaviour do not depend on such effects. The bilayer conductance not related to gA channels is unaltered, the bilayers are stable for hours, and the lowest concentrations affecting gA channel function are well below the CMC [14, 28, 29].

Micelle-forming amphiphiles, in early studies, were observed to induce heterogeneous gA channel behaviour [120]. Such compounds also may cause 'channel-like' conductance changes in lipid bilayers [129]. In the experiments described here, such effects were not

observed [14, 28, 29]. Only a single channel type was observed in each experimental situation, as may be seen for LPC in figures 3(C) and (D).

2.2.6. Effects not due to specific interactions. The amphiphile-induced changes in gA channel behaviour are not due to specific interactions with the channel. First, a number of structurally very different micelle-forming amphiphiles have remarkably similar effects on channel function; both f and τ are increased and there is a larger increase in f than in τ. Second, the effects do not depend on the detailed channel structure; τ is increased also in sequence-modified channels [28].

2.2.7. Expectations to a regulatory mechanism based on changes in ΔG_{def}. Given that the effects of the micelle-forming amphiphiles on f and τ are not due to specific interactions, we may compare these to the expectations of a mechanism based on altered bilayer elasticity. The effects conform to the expectations of such a mechanism in that there are the same effects of a number of structurally different micelle-forming amphiphiles; the same effects on sequence modified channels; the same effects in bilayers of different composition; the same direction of changes in f and τ; and a larger increase in f than in τ. We thus conclude that the micelle-forming compounds regulate gA channel function by changing (increasing) the bilayer elasticity [14, 28, 29].

2.2.8. Role of changes in ΔG_{cont}. How do the changes in the gA channel function relate to the expected changes in ΔG_{cont}? Generally, adsorption of water-soluble amphiphiles to a lipid bilayer will tend to decrease the area compression and bending moduli [104, 105, 130–137]. LPC, in accordance, decreases the apparent area expansion modulus [104] and bending modulus [105] of phospholipid bilayers. The decrease in the elastic moduli occurs, at least in part, because the reversible adsorption of water soluble amphiphiles varies as a function of the bilayer tension [104, 132] and curvature [107][4]. A decrease in the elastic moduli should reduce the disjoining force on the gA channel and therefore increase f and τ [14, 28, 29]. A more positive c_0, induced by the micelle-forming amphiphiles, similarly should decrease F (cf equation (14)). In summary, the effects of the micelle-forming compounds on gA channel function agree with a mechanism based on a decrease in the bilayer disjoining force on the channel, and thus in ΔG_{cont}.

2.2.9. Hydrocarbon-containing versus hydrocarbon-free lipid bilayers. The theoretical analysis of the bilayer deformation energy associated with gA channel formation (equations (12)–(14)) refers to a hydrocarbon-free lipid bilayer. Most gA channel experiments to date have been done in planar bilayers containing *n*-decane. How does this affect the comparison of the experimental results with the theoretical analysis? The thickness of *n*-decane-containing lipid bilayers is considerably larger than that of nominally hydrocarbon-free bilayers [138, 139]. During the bilayer deformation associated with gA channel formation *n*-decane is likely to be 'squeezed' out of the bilayer adjacent to the channel. The acyl chain packing in close proximity to the channel should thus approximate that in a hydrocarbon-free bilayer [40]. More importantly, the experimental results do not provide evidence for

[4] In apparent contrast with the decrease in the apparent area expansion and bending moduli [104, 105], LPC does not affect the monolayer bending modulus measured using osmotic stress [108]. However, the former measurements were done using micropipette methods on lipid vesicles in excess aqueous solution [122, 211]. The latter was done in a system where the lipid/aqueous phase volume ratio is ∼1. A decrease in the bending modulus, based on reversible adsorption, may thus not be observed in this system.

qualitatively different effects of the micelle-forming amphiphiles in hydrocarbon-containing and hydrocarbon-free bilayers. First, the experiments in planar lipid bilayers conform to the predictions of the theoretical analysis. Second, TX100 and βOG also increase gA channel activity when gA is incorporated into the plasma membrane of living cells [14]. Further, the qualitative relation between τ and bilayer thickness is the same in hydrocarbon-containing bilayers and in bilayers made using squalene in the bilayer-forming solution, considered to be virtually hydrocarbon-free [30, 140]. Nevertheless, studies of gA channels in *n*-decane-containing bilayers, of course, also become a test of whether the theoretical analysis applies to a hydrocarbon-containing bilayer. Qualitatively different effects of altering the bilayer molecular composition on gA channels in hydrocarbon-containing and hydrocarbon-free bilayers have not yet been found.

2.3. Effects of changes in bilayer electrostatic energy

The studies using micelle-forming amphiphiles show that gA channel function can be modulated by changes in bilayer elasticity. Does this regulatory mechanism depend on reversible adsorption of water soluble amphiphiles to the bilayer? The feasibility of the HCM has been investigated using gA channels in dioleoylphosphatidylserine (DOPS)/*n*-decane bilayers, a system where such effects can be excluded [40]. The monolayer spontaneous curvature of DOPS bilayers can be modulated without altering the molecular composition. In an aqueous solution, at neutral pH, the head group of DOPS carries a net negative charge. The steric volume of the head group is rather small, but due to electrostatic repulsion among the head groups, the cross-sectional area of the head group region in DOPS bilayers is comparable to that of the acyl chains in the bilayer core. Cations, that bind to and screen the head group charge, decrease the electrostatic repulsion and thus the 'effective' head group size, which promotes a negative c_0 [58, 141, 142]. The 'effective' molecular shape and monolayer spontaneous curvature of DOPS thus can be modulated by altering the electrolyte composition of the aqueous solution. We have investigated the feasibility of the HCM by studying the effects of Ca^{2+} on the gA channel function in DOPS/*n*-decane bilayers [40]. Based on the changes in c_0, one would expect Ca^{2+} to decrease both f and τ (the effects of Ca^{2+} on the bilayer elastic moduli are not known).

Ca^{2+} causes a concentration-dependent decrease in f, τ and N for gA channels in DOPS/*n*-decane bilayers (in 1 M NaCl, pH 5). Figure 6(A) shows the effects on τ and the single channel conductance, g. Increasing $[Ca^{2+}]$ from (nominally) 0 to 100 μM causes a 20-, 3- and 60-fold decease in f, τ and N, respectively. The single channel conductance is decreased by a factor of $\sim$2. The bilayer hydrophobic thickness ($\sim$4.9 nm) is not affected by Ca^{2+} [40].

Figure 6(B) shows results from an experiment, where the reversibility of the Ca^{2+}-induced effects are evaluated from the time-dependent changes in the conductance of a 'large' membrane. Initially the conductance of the membrane is measured in an electrolyte solution where $[Ca^{2+}]$ is (nominally) zero (1 M NaCl, 100 μM ETDA, pH 5). When gA is added, the conductance increases $\sim$1000-fold to a new stable level, corresponding to the presence of $\sim$1000 conducting channels in the membrane. Increasing $[Ca^{2+}]$ to 100 μM (by addition of 200 μM $CaCl_2$) causes a $\sim$130-fold decrease in conductance. Taking the lower single channel conductance, in the presence of Ca^{2+}, into account, this corresponds to a 60-fold decrease in N. When Ca^{2+} is chelated using excess EDTA the bilayer conductance reverts to the prior level. Thus, the Ca^{2+}-induced effects are fully reversible.

Although Ca^{2+} causes a substantial decrease in the conductance of gA channels, the changes in f, τ and N are not due to specific interactions with the channel. Other cations,

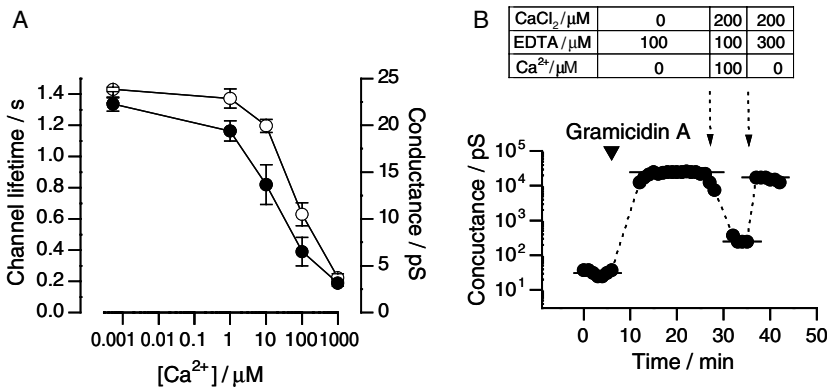

Figure 6. (A) Effects of $[Ca^{2+}]$ on (●) gA channel lifetime and (○) single channel conductance in DOPS/n-decane bilayers. (B) Reversible effects of 100 μM Ca^{2+} on the conductance of a DOPS/n-decane membrane in the presence of gA. First arrow: gA (∼1 pM) is added. Second arrow: 200 μM $CaCl_2$ is added ($[Ca^{2+}] = 100$ μM). Third arrow: 200 μM EDTA is added ($[Ca^{2+}] = 0$ μM). Modified from [40]. Mean ± SE, $n \geqslant 3$.

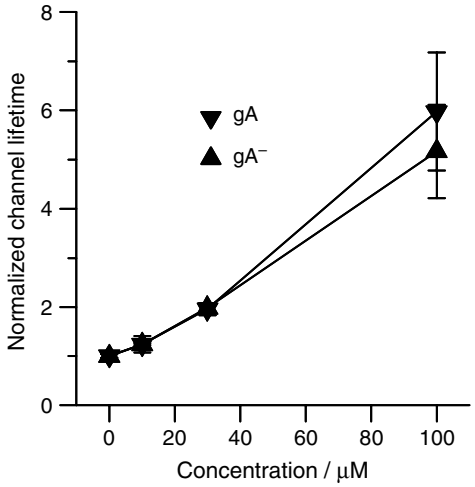

Figure 7. Effects of capsaicin on the lifetime of channels formed by gA or gA^-. Mean ± SE, $n \geqslant 2$. Modified from [9].

such as Na^+, Mg^{2+} and H^+, have similar effects. Further, Ca^{2+} does not affect τ for gA channels in DOPC/n-decane bilayers, at pH 7, where DOPC carries no net charge [40].

The Ca^{2+}-induced changes in gA channel behaviour conform to a mechanism based on an increase in F, as described by equation (14) in that both f and τ are decreased, and that the decrease in f is larger than in τ. We conclude that the effects are due to altered (decreased) bilayer elasticity and conform to those expected from inducing a more negative c_0 [40]. Regulation of gA channel function by the HCM thus does not depend on reversible adsorption of amphiphiles to the bilayer.

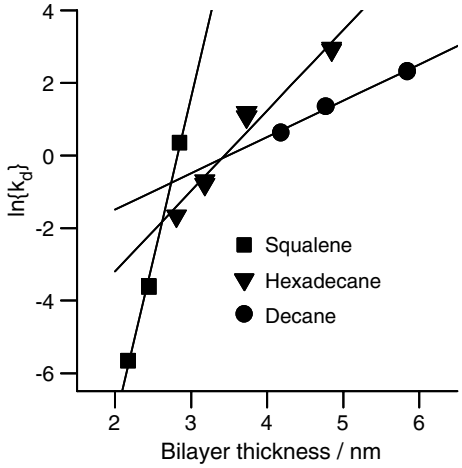

Figure 8. Relation between $\ln\{k_d\}$ and the hydrophobic thickness of monoacylglyceride bilayers. Results obtained by Elliot *et al* [82] using squalene in the bilayer forming solution (in 0.5 M KCl); or by Kolb and Bamberg [92] using *n*-hexadecane (in 1 M NaCl) or *n*-decane (in 1 M NaCl or 1 M CsCl). The bilayer hydrophobic thickness was determined from capacitance measurements [82, 138]. Figure modified from [30].

2.4. Effects of capsaicin—going beyond monolayer curvature

Capsaicin, the pungent ingredient in pepper, is an example of one of the many amphiphiles that regulate the function of a wide range of membrane proteins [9]. At sub-micromolar concentrations, capsaicin specifically activates the TRPV1 receptor involved in pain sensation [143]. At micro- to millimolar concentrations, often used in pharmacological and clinical research, capsaicin modulates a plethora of unrelated membrane proteins, many of which are similarly affected by capsazepine, an antagonist at the TRPV1 receptor (for a list of examples, see [9]). Could capsaicin regulate membrane protein function by altering the bilayer elasticity? This has been investigated using gA channels [9].

Capsaicin has profound effects on gA channel behaviour in DPhPC/*n*-decane bilayers. 30 μM capsaicin causes a 5-, 2- and 10-fold increase in f, τ, and N, respectively. Figure 7 shows the concentration-dependent increase in τ. The effects are not due to specific interactions with gA. Channels formed by the enantiomer gA^-, which are of opposite chirality (left- as opposed to right-handed), are similarly affected (figure 7). The capsaicin-induced changes in gA channel behaviour conform to the expectations of a mechanism based on an increase in bilayer elasticity in that both f and τ are increased; the effects on f are larger than on τ; the effects on τ are larger for a short channel than a longer channel. Capsazepine has qualitatively the same effects on gA channel function.

How do the capsaicin-induced changes in gA channel function relate to the changes in the continuum elastic properties of lipid bilayers? Capsaicin promotes a negative c_0 [9, 144]. Based on this effect one would expect the bilayer disjoining force on the gA channel to increase—and thus f and τ to decrease (cf equation (14)). The opposite is observed! Similar observations have been made for other H_{II}-phase-forming amphiphiles. As mentioned, polyunsaturated fatty acids (PUFAs) due to their molecular shape form H_{II}-phases with a negative c_0 (cf figure 1(B)) [109, 110]. Nevertheless, PUFAs, such as docosahexanoic acid (DHA), increase the bilayer elasticity measured using gA channels [145]. The effects of capsaicin and PUFAs on gA channel function thus are not primarily due to changes in c_0. The observed increase

in bilayer elasticity is most likely due to a decrease in the bilayer elastic moduli caused by reversible adsorption of these amphiphiles to the bilayer [9, 14].

2.5. The linear spring model—measurement of the bilayer spring constant

Results of early investigations, where the relation between bilayer thickness and gA channel lifetime was studied, have provided the experimental basis for a quantitative description of the bilayer deformation energy associated with gA channel formation. Figure 8 shows $\ln\{k_d\}(=-\ln\{\tau\})$ as a function of the hydrophobic thickness, d_0, of monoacylglyceride bilayers with different acyl chains lengths (results from [30, 82, 92]). $\ln\{k_d\}$ is a linearly increasing function of d_0. The slope of the relation depends on the hydrocarbon (squalene, hexadecane or decane) used in the bilayer-forming solution. The linear relation between $\ln\{k_d\}$ and d_0 is predicted by the continuum theory of elastic liquid-crystal deformations [27, 30, 49]. Hexadecane and decane will be present in bilayers formed using these hydrocarbons, whereas bilayers formed using squalene are virtually hydrocarbon-free [140]. Thus, the presence of hydrocarbon does not affect the qualitative relation between $\ln\{k_d\}$ and d_0. As the energetic cost of 'squeezing' hydrocarbon out of the bilayer adjacent to the gA channel is relatively low, the $\ln\{k_d\}$ versus d_0 relation is less steep in the hydrocarbon-containing bilayers than in bilayers formed using squalene [30]. We have shown that the linear relation between $\ln\{k_d\}$ and d_0 implies that ΔG_{def} can be approximated by a linear spring model, where the bilayer elastic properties are described by a phenomenological spring constant, which can be measured using gA channels [30]. This model will be described in the following.

The activation energy for gA channel dissociation, ΔG_d^*, is given by:

$$\ln\{k_d\} = -\ln\{\tau\} = -\Delta G_d^*/RT - \ln\{\tau_0\}, \tag{15}$$

where τ_0 is a frequency factor describing the reaction. ΔG_d^* may be expressed as:

$$\Delta G_d^* = \Delta G_{int,d}^* + \Delta G_{def,d} = \Delta G_{int,d}^* + \Delta G_{packing,d} + \Delta G_{cont,d}, \tag{16}$$

where $\Delta G_{int,d}^*$ is the internal activation energy for channel dissociation. $\Delta G_{def,d}$, $\Delta G_{packing,d}$ and $\Delta G_{cont,d}$ are the differences in ΔG_{def}, $\Delta G_{packing}$ and ΔG_{cont} respectively, for bilayer deformations of $2u_0$ (corresponding to the channel hydrophobic length, l) and $2u_0$-δ (corresponding to a separation of the monomers by a distance, δ when the channel conductance is lost) [30].

Using equation (13), $\Delta G_{cont,d}$ is given by:

$$\Delta G_{cont,d} = H_B((2u_0 - \delta)^2 - (2u_0)^2) + H_X c_0((2u_0 - \delta) - (2u_0)) = H_B\delta(\delta - 4u_0) - H_X c_0\delta. \tag{17}$$

If the change in $\ln\{k_d\}$, observed when the bilayer thickness is altered, is due to a change in $\Delta G_{cont,d}$, and neither $\Delta G_{int,d}^*$ nor $\Delta G_{packing,d}$ are altered, the thickness-dependent change in $\ln\{k_d\}$ (using equations (15)–(17)) will be given by:

$$\mathrm{d}\ln\{k_d\}/\mathrm{d}u_0 = (-\Delta\, G_{cont,d}/\mathrm{d}u_0)/RT = 4H_B\delta/RT, \tag{18}$$

that is, $\ln\{k_d\}$ should be a linear function of u_0. This is in agreement with the experimental observations. When the bilayer thickness is varied, the bilayer properties affecting the relation between $\ln\{k_d\}$ and u_0 thus may be approximated by a linear spring model, where H_B is a phenomenological spring constant summarizing the bilayer elastic properties [30].

Using the results shown in figure 8, H_B for bilayers made from a squalene-containing solution is 69 ± 6 kJ mol^{-1} nm^{-2} (mean $\pm$ SE) [30]. This may be compared to the value of H_B for monoolein bilayers calculated from experimentally determined values of K_a, K_c and d_0. Using a constrained boundary condition (where the energetic penalty due to lipid packing close

to the gA channel is large), H_B should be 70–90 kJ mol^{-1} nm^{-2}. Using a relaxed boundary condition (where the energetic penalty due to lipid packing close to the gA channel is ignored), H_B should be 20–30 kJ mol^{-1} nm^{-2} [30]. The experimental estimate of H_B, obtained using gA channels, is thus in good agreement with predictions based on the constrained boundary condition, and is two to three times larger than predictions based on the relaxed boundary condition [30].

In summary, the relation between τ and d_0 may be approximated by a linear spring model, in which the bilayer elastic properties are summarized by a single phenomenological spring constant which can be measured using gA channels. The use of gA channels as molecular force transducers thus provide a quantitative description of the bilayer elasticity experienced by a bilayer-embedded protein.

2.6. Quantitative description of changes in bilayer elasticity

The basis for the quantitative description of lipid bilayer elasticity, provided by the relation between τ and d_0, is that the activation energy for gA channel dissociation is measured using bilayer deformations of different magnitudes [30]. A similar description of *changes* in lipid bilayer elasticity, induced by altered molecular composition, may be obtained by studying the effect on gA channel functional transitions involving bilayer deformations of different magnitude. In the following the effects on the activation energy for monomer association (ΔG_a^*), the activation energy for monomer dissociation (ΔG_d^*), and the energetic cost of channel formation (ΔG_{gA}) will be studied. As mentioned, monomer association involves a change in the magnitude of the bilayer deformation from zero to $2u_0 - \delta$ (a change in local bilayer thickness from d_0 to $l + \delta$), while monomer dissociation involves a change from $2u_0$ to $2u_0 - \delta$. Adjusting the bilayer thickness to match the channel length, l, involves a change from zero to $2u_0$ (cf figure 2(A)). We study the effects of LPLs, capsaicin, capsazepine, genistein, daidzein and phloretin[5] using DPhPC/n-decane bilayers, as well as of Ca^{2+} and Na^+ using DOPS/n-decane bilayers. As the hydrophobic thickness of these bilayers is ∼4.7 nm and ∼4.9 nm, respectively [28, 40], the channel–bilayer hydrophobic mismatch will be similar.

Using equation (15), the relation between a change in the activation energy for channel dissociation ($\Delta\Delta G_d^*$) and $\ln\{k_d\}$ is given by:

$$\Delta\Delta G_d^*/RT = (\Delta G_{d,mod}^* - \Delta G_{d,cntrl}^*)/RT = -\ln\{k_{d,mod}/k_{d,cntrl}\}, \tag{19}$$

where the subscripts 'cntrl' and 'mod' denote the control situation and the modified bilayer, respectively.

Similarly, a change in the energetic cost of channel formation ($\Delta\Delta G_{gA}$) is reflected in the channel dimerization constant, K_D [28]:

$$\Delta\Delta G_{gA}/RT = (\Delta G_{gA,mod} - \Delta G_{gA,cntrl})/RT = -\ln\{K_{D,mod}/K_{D,cntrl}\}. \tag{20}$$

Given that the number of conducting channels, N, is proportional to K_D, $K_{D,mod}/K_{D,cntrl} = N_{mod}/N_{cntrl}$ [28].

The relation between $\Delta\Delta G_{gA}$ and $\Delta\Delta G_d^*$ may thus be studied by plotting $\ln\{K_{D,mod}/K_{D,cntrl}\}$ as a function of $\ln\{k_{d,mod}/k_{d,cntrl}\}$, using the observed changes in τ and N. Figure 9(A) shows such a comparison. The effects of LPC, LPE, LPS, capsaicin and genistein on gA channel behaviour in DPhPC/n-decane bilayers (results from [9, 28, 146]), as well as of Ca^{2+} or Na^+ on channel behaviour in DOPS/n-decane bilayers (results from [40]), may all be described by a single linear relation:

$$\ln\{K_{a,mod}/K_{a,cntrl}\} = a \cdot \ln\{k_{d,mod}/k_{d,cntrl}\}, \tag{21}$$

[5] Genistein, daidzein and phloretin, are other amphiphiles that increase lipid bilayer elasticity measured using gA [146].

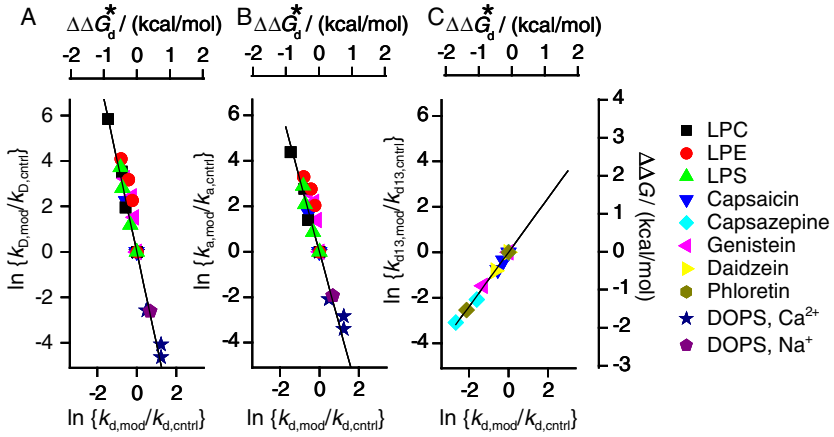

Figure 9. Relation between $\ln\{K_{D,mod}/K_{D,cntrl}\}$ and $\ln\{k_{d,mod}/k_{d,cntrl}\}$ obtained from the changes in N and τ. The effects on N were determined from either: (a) changes in the conductance of a gA-containing 'large membrane' as described above (results for LPLs [28], Na^+ and Ca^{2+} [40]); (b) changes in f and τ, using the relation $N_{mod}/N_{cntrl} = (f_{mod}/f_{cntrl}) \cdot (\tau_{mod}/\tau_{cntrl})$ [28] (results for capsaicin [9] and genistein [146]); or (c) changes in the concentration of gA needed to obtain a similar channel activity in a modified bilayer and in the control situation (given that N is proportional to the square of the gA concentration in the aqueous solution [206–208], results for Ca^{2+} and Na^{2+} [40]). (B) Relation between $\ln\{k_{a,mod}/k_{a,cntrl}\}$ and $\ln\{k_{d,mod}/k_{d,cntrl}\}$ obtained from the changes in f and τ. The effects on f were measured directly (results for capsaicin and genistein [9, 146]), or obtained from the changes in τ and N (results for LPLs [28], Ca^{2+} and Na^+ [40]) (C) Relation between $\ln\{k_{d,13,mod}/k_{d,cntrl}\}$ and $\ln\{k_{d,mod}/k_{d,cntrl}\}$ based on the changes τ for gA channels and channels formed by monomers of 13 amino acids (results for capsaicin, capsazepine, genestein, daidzein and phloretin [9, 146]).

where a is -4.20 ± 0.21 (slope $\pm$ standard error of mean (SE), $r = 0.98$).[6] Thus, $\Delta\Delta G_{gA} = -4.20 \cdot \Delta\Delta G^*_d$. Despite the very different methods used to alter the bilayer elasticity, the ratio between the changes in the energetic cost of channel formation and the changes in the activation energy for channel dissociation is constant!

In analogy with equation (19), the relation between a change in the activation energy for monomer association ($\Delta\Delta G^*_a$) and $\ln\{k_a\}$ is given by:

$$\Delta\Delta G^*_a/RT = (\Delta G^*_{a,mod} - \Delta G^*_{a,cntrl})/RT = -\ln\{k_{a,mod}/k_{a,cntrl}\}. \quad (22)$$

As f is proportional to k_a, $k_{a,mod}/k_{a,cntrl} = f_{mod}/f_{cntrl}$ [28].

The relation between $\Delta\Delta G^*_a$ and $\Delta\Delta G^*_d$ may thus be studied by plotting $\ln\{k_{a,mod}/k_{a,cntrl}\}$ as a function of $\ln\{k_{d,mod}/k_{d,cntrl}\}$, using the changes in τ and f (figure 9(B)). The effects of LPC, LPE, LPS, capsaicin and genistein (using DPhPC/n-decane bilayers [9, 28, 146]), and of Ca^{2+} and Na^+ (using DOPS/n-decane bilayers [40]), may be described by a single linear relation:

$$\ln\{k_{a,mod}/k_{a,cntrl}\} = b \cdot \ln\{k_{d,mod}/k_{d,cntrl}\}, \quad (23)$$

where b is -3.20 ± 0.21 (slope $\pm$ SE, $r = 0.96$).

Finally, the relation between $\Delta\Delta G^*_d$, and the change in the activation energy for dissociation of a [des-Val^1-Gly^{-2}]gA^- channel formed by monomers of 13 amino acids ($\Delta\Delta G^*_{d,13}$), may be studied by plotting $\ln\{k_{d,13,mod}/k_{d,13,cntrl}\}$ as a function of $\ln\{k_{d,mod}/k_{d,cntrl}\}$. Figure 9(C) shows the effects of capsaicin, capsazepine, genistein, daidzein and phloretin

[6] Results obtained using LPI deviate significantly from this relation and are not included in the analysis.

(using DPhPC/n-decane bilayers [9, 146]). All the results may be described by a single linear relation:

$$\ln\{k_{\mathrm{d,13,mod}}/k_{\mathrm{d,13,cntrl}}\} = c \cdot \ln\{k_{\mathrm{d,mod}}/k_{\mathrm{d,cntrl}}\}, \tag{24}$$

where c is 1.19 ± 0.02 (slope $\pm$ SE, $r > 0.99$).

Using equations (20)–(24), we find that

$$\Delta\Delta G^{*}_{\mathrm{d}} = \frac{\Delta\Delta G^{*}_{\mathrm{d,13}}}{1.19} = \frac{\Delta\Delta G^{*}_{\mathrm{a}}}{-3.20} = \frac{\Delta\Delta G_{\mathrm{gA}}}{-4.20}. \tag{25}$$

That is, the changes in: (1) the activation energy for monomer association, (2) the activation energy for monomer dissociation, (3) the energetic cost of gA channel formation, and (4) the activation energy for dissociation of a shorter channel are all linearly related. The fact that the effects of all the methods used to manipulate the bilayer properties are described by the same linear relations strongly supports the notion that a common mechanism of action is involved.

The energetic cost of gA channel formation may be described as the sum of $\Delta G_{\mathrm{gA,int}}$ and ΔG_{def} (cf equation (11)). Similarly $\Delta G^{*}_{\mathrm{a}}$ and $\Delta G^{*}_{\mathrm{d}}$ may described as the sum of $\Delta G_{\mathrm{int,a}}$ and $\Delta G^{*}_{\mathrm{def,a}}$, and of $\Delta G_{\mathrm{int,d}}$ and $\Delta G^{*}_{\mathrm{def,d}}$, respectively. If the manipulations used to alter the bilayer elasticity also affected $\Delta G_{\mathrm{gA,int}}$, $\Delta G_{\mathrm{int,a}}$ or $\Delta G_{\mathrm{int,d}}$, it is very unlikely that the changes in these values would be the same for all the different methods used. Given the constant relation between $\Delta\Delta G^{*}_{\mathrm{a}}$, $\Delta\Delta G^{*}_{\mathrm{d}}$, $\Delta\Delta G_{\mathrm{gA}}$ and $\Delta\Delta G^{*}_{\mathrm{d,13}}$, we conclude that the intrinsic energetic contribution from gA is not significantly altered. The observed changes in channel function are due to altered contributions from ΔG_{def}. This means that $\Delta\Delta G^{*}_{\mathrm{a}}$, $\Delta\Delta G^{*}_{\mathrm{d}}$, $\Delta\Delta G_{\mathrm{gA}}$ and $\Delta\Delta G^{*}_{\mathrm{d,13}}$ provide quantitative measures of changes in the bilayer deformation energy—and thus of changes in bilayer elasticity.

In conclusion, by comparing the energetics of gA channel functional transitions involving bilayer deformations of different magnitude, one can obtain a *quantitative* description of changes in bilayer elasticity as experienced by an embedded protein. It is further possible to quantitatively compare the changes in the bilayer elastic response to protein functional transitions involving bilayer deformations of different magnitude. The results shown in figure 9 represent the first example of such a comparison for a bilayer embedded protein. It is further the first example of a protein where the effects of altered bilayer physics on one functional transition (e.g., gA monomer association) predict the effects on another (e.g., gA monomer dissociation).

2.7. Hydrophobic coupling—sufficient to provide for a regulatory mechanism?

The studies using gA channels show that the function of a bilayer-embedded protein can be regulated by the hydrophobic coupling between protein conformation and lipid bilayer elasticity. The changes in bilayer elasticity, induced by the manipulations described above, cause a several hundred-fold change in the number of conducting gA channels. The effects shown in figures 9(A) correspond to changes in the energetic cost of gA channel formation of ± 3 kcal mol^{-1}. Analysis using the continuum theory of elastic liquid-crystal deformations predicts that ΔG_{cont} should scale as a linear function of the radius of a bilayer inclusion [50, 51]. Thus for a nicotinic acetylcholine receptor with a radius of $\sim$3 nm (e.g., [147]), the corresponding values should be ± 9 kcal mol^{-1}. For comparison, the strength of a hydrogen bond is usually assumed to be 3 kcal mol^{-1}, and the energy released by hydrolysis of one molecule of ATP to ADP is 9 kcal mol^{-1} [148]. The changes in bilayer elasticity should thus be sufficient to be important for protein function.

2.8. Lipid bilayer stiffness and other measures of bilayer physical properties

Using the continuum theory of liquid crystal deformations, the relation between ΔG_{cont} and u_0 is given by the elastic coefficients H_B, H_X, and H_C, as well as by c_0 (cf equation (13)). When the bilayer hydrophobic thickness is varied by altering the acyl chain length, the relation between ΔG_{cont} and u_0 is given simply by H_B (cf equation (18)). By varying the bilayer thickness, gA channels can be used to measure H_B. By studying gA channel functional transitions involving bilayer deformations of different magnitude, a quantitative description of *changes* in bilayer elasticity further can be obtained.

More generally, a change in bilayer elasticity that affects the bilayer disjoining force on a gA channel is operationally defined as a change in bilayer stiffness [9, 14]. A decrease in bilayer stiffness decreases F and thus increases f and τ, and vice versa. In the following the term bilayer 'elasticity' will be used broadly to describe the deformability of lipid bilayers. The term bilayer stiffness, in contrast, specifically refers to measurements using gA channels.

As mentioned, regulation of membrane protein function by the bilayer physical properties has been described using a number of different descriptors, such as changes in bilayer coupling, bilayer compression energy, curvature frustration energy; acyl chain packing, bilayer deformation energy, bilayer stiffness, lateral pressure profile across the bilayer, lipid packing stress, bilayer free volume, or bilayer fluidity [2, 9, 14, 23–34]. Though couched in different terms, these descriptions all represent approaches to parameterize the lateral interactions among the bilayer-forming lipids and between the lipids and embedded membrane proteins [14]. Generally, a change in any of the descriptors will also be reflected in the others [9, 14]. However, the similar effects of micelle-forming compounds and capsaicin on gA channel function (and on membrane protein function in living cells, see below) demonstrate the limitations of unimodal attempts to relate changes in the bilayer properties to changes in membrane protein function. The advantage of gA-based measurements of changes in bilayer physics is that they reflect *net* changes in bilayer elasticity as experienced by a single bilayer-embedded protein. For recent reviews discussing the relation between gA channel function and bilayer physics observed in the studies described above, see [1, 2, 4–7, 33, 81, 149–151].

The relation the between amphiphile-induced changes in the gA channel function and bilayer fluidity should be specifically mentioned. Micelle-forming amphiphiles decrease the acyl chain order of lipid bilayers and increase the fluorescent polarization of bilayer-embedded diphenylhexatriene, which has been interpreted to signify that the bilayer fluidity is increased (e.g., [152, 153]). Capsaicin has similar effects [154], while cholesterol decreases the bilayer fluidity [155, 156]. Nevertheless, the changes in gA channel behaviour cannot be explained in terms of altered fluidity. The causal relation between protein function and bilayer fluidity remains unclear, except for the conclusion that changes in 'fluidity', *per se*, cannot affect membrane protein conformational or chemical equilibria [157]. The changes in gA channel activity, which reflect a shift in the equilibrium between gA monomers and dimers, thus cannot be due to altered bilayer fluidity. Moreover, if the increase in f, induced by the micelle-forming amphiphiles or capsaicin, was argued to be due to higher bilayer 'fluidity', τ by the same argument would be expected to decrease. The opposite is observed.

3. Membrane protein function and lipid bilayer elasticity

3.1. Voltage-dependent sodium channels and N-type calcium channels

The studies using gA channels have demonstrated the practical feasibility of the HCM in a simple model system. Could complex membrane proteins in the heterogeneous lipid bilayer of a cell membrane be similarly regulated? This has been investigated by studying the effects

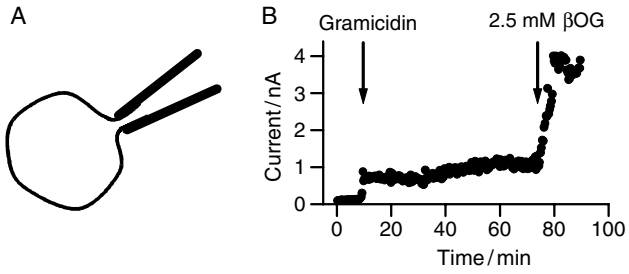

Figure 10. (A) Using the voltage clamp technique, electrical connection to the interior of an HEK293 cell is obtained with a glass pipette. A feedback amplifier controls the membrane potential and measures the resulting current. (B) Effects of 2.5 mM βOG on the current across the plasma membrane of an HEK293 cell in the presence of gA. After establishing the whole cell configuration, 10 μM gA is added to the extra cellular solution (first arrow). When βOG is applied the current increases to a new steady level. The current is measured during 5 s pulses to +40 mV from a holding potential of −70 mV.

of amphiphile-induced changes in bilayer elasticity on voltage-dependent sodium channels (VDSCs) and N-type calcium channels in living cells [9, 14, 29]. VDSCs are regulated by a number of amphiphiles known to affect the physical properties of lipid bilayers (e.g., LPC [103]; PUFAs [158]; genistein [159]; barbiturates [160]; benzodiazepines [161]; and chlorpromazine [162]).

The effects of amphiphiles, shown to affect lipid bilayer stiffness measured using gA channels, on VDSCs heterologously expressed in human embryonic kidney (HEK293) cells, have been studied using the whole cell voltage clamp technique [9, 14]. Briefly, a glass pipette is used to establish electrical connection to the interior of the cell under investigation (figure 10(A)). A feedback amplifier controls the membrane potential (potential difference across the plasma membrane) and measures the resulting current. The cell is placed in a continuously flowing extracellular solution, through which the tested amphiphiles are applied.

Initially the effects of βOG and TX100 on the bilayer stiffness of HEK293 cell plasma membranes were studied using gA channels [14]. Figure 10(B) shows results from an experiment studying the effects of βOG. Using the whole cell voltage clamp configuration, the current across the plasma membrane of an HEK293 cell is measured during 5 s test pulses to +40 mV from a membrane holding potential of −70 mV. Initially the current is ∼100 pA. When gA is added to the extracellular solution, the current increases ∼10-fold, as conducting gA channels are formed in the membrane (cf figure 10(B)). Once the current has reached a steady level, 2.5 mM βOG is added, and the current increases to a new steady level. βOG does not affect the single channel conductance of gA channels in the plasma membrane, or the membrane conductance in the absence of gA. The increase in the current therefore is due to a larger gA channel activity in the presence of βOG. Addition of 10 μM TX100 has the same effects. Thus βOG and TX100 decrease the bilayer stiffness of the plasma membrane in HEK293 cells [14].

The VDSC function involves voltage-dependent transitions between different functional states, which may be summarized as

$$\begin{array}{ccc} \textit{closed} & \leftrightarrow & \textit{open} \\ & \searrow \quad \swarrow & \\ & \textit{inactivated} & \end{array}$$

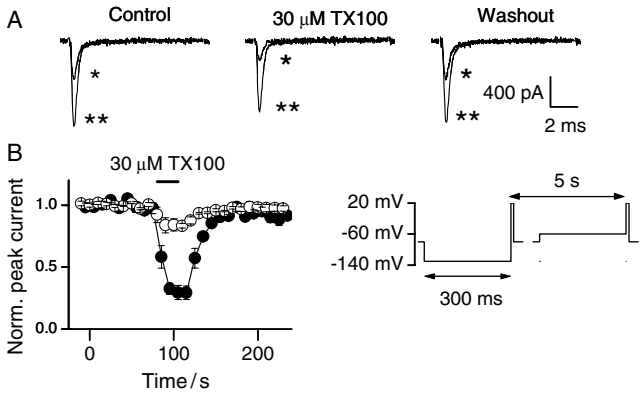

Figure 11. (A) Current traces showing the reversible inhibition of VDSC currents by TX100. Every 5 s the cells were depolarized to +20 mV (for 10 ms) from a 300 ms prepulse to either −60 mV (∗) or −130 mV (∗∗). Holding potential: −80 mV. The inset illustrates the voltage protocol. (B) The time course of peak current inhibition induced by a 25 s application of 30 μM TX100. (●)—60 mV prepulse, (○)—130 mV prepulse. Experimental conditions as in figure 11(A). Mean ± SE ($n = 3$). Modified from [14].

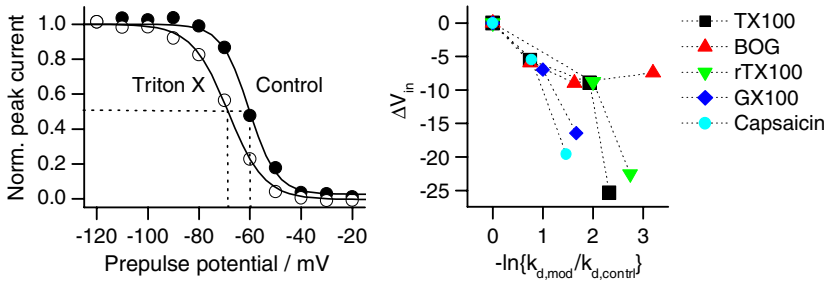

Figure 12. (A) Effects of 10 μM TX100 on VDSC peak current versus prepulse potential relation. Results from a single experiment. The voltage dependence of inactivation is described by a two-state Boltzmann distribution: $I(V)/I(-130) = 1/(1 + \exp\{(V - V_{in})/S_{in}\})$, where $I(V)$ and $I(-130)$ are the peak current using prepulses of V and −130 mV, respectively. V_{in} is the voltage of half maximal inactivation and S_{in} is a slope factor. Every 5 s the cells are depolarized to +20 mV from 300 ms prepulses to potentials varying from −130 to +50 mV. Holding potential −80 mV. (B) ΔV_{in} versus $-\ln\{k_{d,mod}/k_{d,cntrl}\}$. Modified from [14].

The channels are described as being in one of three functional states: a conducting 'open' (active) state, and two non-conducting states: 'closed' (but activatable), or 'inactivated' (not activatable). Each of these states may represent a number of underlying molecular and functional states (cf [14]).

Figure 11(A) shows current traces from an experiment studying the effects of TX100 on VDSC function in HEK293 cells. The left panel in figure 11(A) illustrates the control situation. The inset shows the voltage protocol used. At the membrane holding potential of −80 mV the channels are in the closed or the inactivated state, and no current is observed. A 300 ms pulse to −130 or −60 mV shifts the channel population towards the closed or the inactivated state, respectively, but does not activate the channels. A subsequent test pulse to +20 mV induces a transient current, reflecting the fast activation (opening) and slower inactivation of the channels. Because more channels are inactivated during the −60 mV prepulse than during the −130 mV

prepulse, the peak current amplitude is smaller using the former prepulse. When 30 μM TX100 is applied, the peak currents are decreased using both prepulse potentials. However, the relative decrease is largest following the −60 mV prepulse, showing that channel inactivation is promoted by TX100. After washout of TX100 the peak currents revert to their initial amplitude. Figure 11(B) shows the time-dependent changes in the peak current amplitude, when the channels are repetitively activated using prepulses of either −130 or −60 mV (for each prepulse the current amplitude is normalized by the average control value). TX100 causes a much larger decrease in the peak currents following the −60 mV prepulse than following the −130 mV. After washout of TX100 the currents revert to their initial amplitude.

Figure 12(A) shows the effects of 10 μM TX100 on the voltage dependence of channel inactivation, determined from the relation between the prepulse potential and the amplitude of the peak current induced by a fixed test potential. In both the absence and presence of TX100, the peak current versus prepulse relation may be described by a two-state Boltzmann distribution (see legend to figure 12). TX100 induces a reversible negative shift in the potential causing 50% channel inactivation (V_{in}). That is, channel inactivation is promoted in a reversible manner. Three other micelle-forming amphiphiles, which decrease bilayer stiffness (rTX100, βOG and GX100), promote channel inactivation in a similar manner.

A quantitative comparison of the effects of the amphiphiles on VDSC function and bilayer stiffness measured using gA channels may be obtained by expressing the shift in V_{in} (ΔV_{in}) as a function of $-\ln\{k_{d,mod}/k_{d,cntrl}\}$ ($=\Delta\Delta G^*_d/RT$). At low concentrations of the amphiphiles (up to 0.1 CMC), the relation between the effects on ΔV_{in} and on $-\ln\{k_{d,mod}/k_{d,cntrl}\}$ is remarkably similar. The compounds thus promote VDSC inactivation in quantitative correlation with the effects on bilayer stiffness measured using gA channels. At higher concentrations this correlation breaks down (figure 12(B)).

If the micelle-forming amphiphiles promote VDSC inactivation by decreasing the bilayer stiffness, one would expect capsaicin and capsazepine to have similar effects. This is indeed the case. Both compounds cause a concentration-dependent reversible negative shift in V_{in}. When used at a concentration of 30 μM, the effect of capsaicin on ΔV_{in} conforms to the quantitative correlation with $-\ln\{k_{d,mod}/k_{d,cntrl}\}$ observed for the micelle-forming amphiphiles (figure 12(B)) [9].

The capsaicin-induced changes in VDSC function are not due to interactions with the TRPV1 receptor. First, this receptor has not been described in HEK293 cells. Second, capsaicin does not affect the membrane conductance not related to the VDSC. Third, capsazepine modulates VDSC function in a similar manner [9].

If capsaicin and the micelle-forming amphiphiles promote VDSC inactivation by decreasing the bilayer stiffness, one would expect cholesterol to have the opposite effect. This is the case. Cellular cholesterol depletion, by exposure to methyl-β-dextrin, causes a reversible negative shift in V_{in} [14]. Raising the cellular cholesterol content above the normal level, however, does not affect V_{in}, suggesting a saturation of the effects of cholesterol [14].

A qualitative whole cell voltage clamp study of N-type calcium channels in IMR32 cells has shown that these channels are regulated in a manner similar to VDSC. TX100 and βOG promote channel inactivation, whereas cholesterol has the opposite effect [29].

In summary, several structurally different amphiphiles, which decrease the bilayer stiffness measured using gA channels, regulate the function of VDSC and N-type calcium channels in a remarkably similar manner. The effects on VDSC function are quantitatively correlated with the changes in the bilayer stiffness measured using gA channels. Cholesterol, which increases bilayer stiffness, in both channel types, has the opposite effect. Based on these findings we conclude that VDSC and N-type calcium channels are regulated by the elasticity of the host lipid bilayer [9, 14, 29].

3.2. $GABA_A$ receptors

$GABA_A$ (γ-amino butyric acid)$_A$ receptors are ligand-gated ion channels, structurally unrelated to voltage-dependent ion channels. $GABA_A$ receptors are also regulated by a number of structurally different amphiphiles that affect the physical properties of lipid bilayers (cf [42]). Studies using the substituted cysteine accessibility method show that $GABA_A$ receptor function involves conformational changes at the trans-membrane domain, cf [74]. To further investigate whether membrane protein function in living cells could be regulated by amphiphile-induced changes in bilayer elasticity, we have studied the effects of TX100, βOG, capsaicin and DHA on $GABA_A$ receptor function in HEK293 cells [42]. The four amphiphiles all promote $GABA_A$ receptor high-affinity binding of the agonist muscimol. A high affinity state of the receptor is promoted without altering the affinity of this state. A semi-quantitative analysis shows a very similar relation between the effects on muscimol binding and on $\ln\{k_d/k_{d,control}\}$ for gA channels. Cholesterol, in contrast, inhibits high affinity muscimol binding. In parallel whole cell voltage clamp experiments TX100, βOG, capsaicin and DHA all increase the rate of receptor desensitization (the rate of transition towards the desensitized state with the highest affinity for agonists) [42].

If the effects of TX100, βOG, capsaicin, DHA and cholesterol on $GABA_A$ receptor function were due to changes in bilayer elasticity, one would expect structurally related proteins to be similarly affected. $GABA_A$ receptors belong to the Cys-loop superfamily of ligand-gated ion channels, considered to posses a common quaternary structure (cf [42]). Desensitization of the nicotinic acetylcholine receptor, another member of this superfamily, involves a conformational change at the hydrophobic exterior of the trans-membrane domain, which suggests that the hydrophobic length is altered (decreased) [60]. TX100 causes a time-dependent block of the nicotinic acetylcholine receptor, suggesting that receptor desensitization is promoted [163]. Solubilization in Triton X-100 or octyl-β-glucoside promotes a receptor structure similar to the desensitized state [164]. Further long-chain fatty acids decrease the single-channel open time [165] and capsaicin inhibits acetylcholine-induced currents [166]. Cholesterol, in contrast, promotes a resting (non-desensitized) state of the this receptor [167]. Thus $GABA_A$ receptors and nicotinic acetylcholine receptors are similarly regulated by amphiphiles that alter bilayer stiffness.

In summary, despite their structural dissimilarity, TX100, βOG, DHA and capsaicin regulate $GABA_A$ receptor function in a similar manner. Cholesterol has the opposite effects. Comparable results have been obtained in the nicotinic acetylcholine receptor. We conclude that $GABA_A$ receptor function is regulated by lipid bilayer elasticity [42].

4. Hydrophobic coupling—a novel regulatory mechanism

The feasibility of the HCM has been demonstrated using gA channels in planar lipid bilayers as a model system. Changes in bilayer molecular composition can alter the bilayer elasticity sufficiently to substantially alter the function of an embedded protein. The studies using VDSC, N-type calcium channels and $GABA_A$ receptors show that membrane proteins in living cells can be similarly regulated. The lipid bilayer thus becomes an allosteric modulator of protein function [9].

The correlation between amphiphile-induced changes in bilayer stiffness measured using gA channels in planar lipid bilayers, and in membrane protein function in living cells, is surprisingly simple. This correlation does not imply similar conformational changes in gA channels and these proteins. A change in the physical properties of a lipid bilayer, which is detected as altered bilayer stiffness using gA channels, should affect also the energetic cost

of bilayer deformations associated with more complex conformational changes in membrane proteins [14].

A regulatory mechanism based on hydrophobic coupling between membrane protein conformation and bilayer elasticity would be expected to affect membrane protein function generally. Table 1 shows the effects of the amphiphiles described above on six types of ion channels, altogether representing three protein superfamilies. One cannot exclude that specific mechanisms may be involved in some of the examples listed, but they all involve amphiphile concentrations that affect the lipid bilayer stiffness measured using gA channels. As may be seen from table 1, the correlation between the effects of the amphiphiles extends over proteins from several superfamilies. Membrane proteins may thus generally be regulated by the elastic properties of the host lipid bilayer.

5. Membrane protein sorting and bilayer elasticity

It has been shown that the interactions between a membrane protein and the host lipid bilayer allow the bilayer elasticity to regulate protein function by controlling the distribution among protein conformational states. Could the bilayer elasticity similarly regulate membrane protein function by controlling protein sorting between different cellular membrane compartments? We have investigated this question using the continuum theory of elastic liquid-crystal deformations [41].

Cellular membrane proteins, synthesized in the endoplasmatic reticulum, follow the secretory pathway to the Golgi complex. In the Golgi complex the proteins are laterally sorted, such that plasma membrane proteins enter transport vesicles destined for the plasma membrane, while Golgi resident proteins are retained in the Golgi membranes (cf [41]). A specific Golgi retention signal has not been identified, and retention of Golgi proteins cannot be saturated by overexpression [168–170]. The underlying mechanism(s) are poorly understood [41].

Several lines of evidence show that protein sorting between the Golgi complex and the plasma membrane, at least in part, is determined by the length of the protein trans-membrane domains (TMDs). First, Golgi proteins have shorter TMDs (∼15 amino acids) than plasma membrane proteins (∼20 amino acids) [171, 172]. Second, a protein normally retained in the Golgi complex is targeted to the plasma membrane if the length of the TMD is increased [172–174], but is minimally affected if the TMD is replaced by a leucine segment of the same hydrophobic length [173]. Third, proteins normally trafficked to the plasma membrane are retained in the Golgi complex, if the hydrophobic length of the TMD is shortened [175].

Bretscher and Munro (1993) [171] proposed a mechanism, whereby membrane protein sorting in the Golgi complex could depend on the preferential association of plasma membrane proteins with cholesterol-enriched membrane domains. The cholesterol content of cellular membranes increases along the secretory pathway. Cholesterol thus constitutes ∼20% and ∼50% of the lipids in the Golgi complex and the plasma membrane, respectively [176–178]. As cholesterol increases the hydrophobic thickness of lipid bilayers [126], it was proposed that plasma membrane proteins with long TMDs, in the Golgi complex, would be allowed to enter cholesterol-enriched thicker membrane compartments from which transport vesicles are formed, while Golgi proteins with shorter TMD would be excluded (figure 13(A)). General support for such a sorting mechanism has been obtained in studies of the insertion of hydrophobic α-helices into synthetic lipid bilayers, which correlates with bilayer thickness and cholesterol content [179, 180].

The genesis and maintenance of the Golgi complex remains unresolved, cf [181], but all mechanisms of membrane protein sorting eventually involve a lateral segregation of proteins between different membrane domains. Because of the role of TMD length in protein sorting,

Table 1. Amphiphile modulation of ion channel function. Gramicidin channels (gA), voltage-dependent sodium channels (VDCS), N-type calcium channels (N-type Ca^{2+}), calcium-activated potassium channels (BK_{Ca}), nicotinic acetylcholine receptors (nAChR) and $GABA_A$ receptors.

Superfamily	Prokaryotic channel		Voltage-dependent channels					Cys-loop receptors		
Channel	Gramicidin		VDSC		N-type Ca^{2+}		BK_{Ca}	nAChR	$GABA_A$	
Function	k_a	k_d	Activation	Inactivation	Activation	Inactivation	Activation	Desensitization	Desensitization	Musc. binding
Triton X-100	↑ [14, 29]	↓ [14, 29]	0 [14]	↑ [14]	0 [29]	↑ [29]		↑ [163, 164]	↑ [42]	↑ [42, 199]
βOG	↑ [14, 29]	↓ [14, 29]	0 [14]	↑ [14]	0 [29]	↑ [29]		↑ [164]	↑ [42]	↑ [42, 199]
Capsaicin	↑ [9]	↓ [9]	0 [9]	↑ [9]			↑ [200]		↑ [42]	↑ [42, 199]
DHA	↑ [145]	↓ [145]	0 [158]	↑ [158]			↑ [201]	↑ [202]	↑ [42, 203]	↑ [42, 204]
Cholesterol depl.	↑ [14, 29]	↓ [14, 29]	0 [14]	↑ [14]						↑ [42]
Cholesterol	↓ [14, 29]	↑ [14, 29]	↓ [14]	↓ [14]	0 [29]	↓ [29]	↓ [205]	↓ [167]		↓ [42]

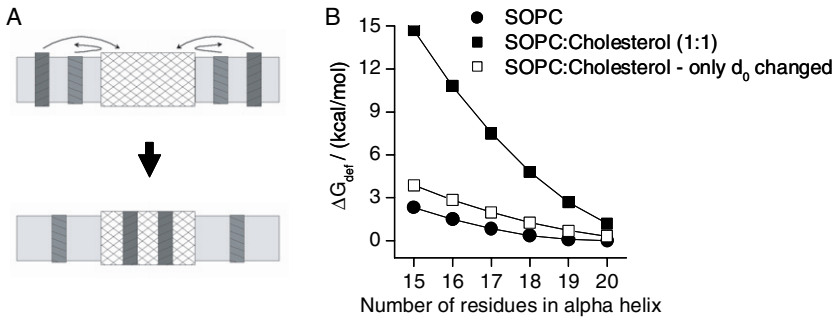

Figure 13. (A) Lateral sorting of membrane proteins (dark-hatched) between thin, cholesterol-poor bilayer domains (light grey) and thicker, cholesterol-enriched bilayer domains (cross-hatched). The proteins will tend towards the domain in which the protein length matches the bilayer thickness. (B) ΔG_{cont} for α-helices of 15–20 amino acids in (●) SOPC, (■) SOPC:cholesterol (1:1) bilayer, or a bilayer with a thickness as SOPC:cholesterol (1:1), but with elastic properties as SOPC (□). Modified from [41].

and because cellular cholesterol depletion leads to mistargeting of plasma membrane proteins, the association of membrane proteins with cholesterol-enriched membrane domains is currently viewed as a potential sorting mechanism [182–184].

The mechanism proposed by Bretscher and Munro is based on the energetic penalty due to hydrophobic mismatch. It does not consider the energetic contribution from the bilayer deformation associated with hydrophobic mismatch. Further, cholesterol not only increases the lipid bilayer thickness, it also decreases the bilayer elasticity. We have evaluated the energetic feasibility of a cholesterol-induced sorting process by estimating the effects of cholesterol on the energetic cost of adjusting the thickness a lipid bilayer to the hydrophobic length of an embedded α-helix [41].

ΔG_{cont} for the bilayer deformation induced by a bilayer-spanning hydrophobic α-helix, with a hydrophobic length, l, shorter than the bilayer hydrophobic thickness, d_0, was calculated using equation (13). The calculations were done for $c_0 = 0$, which means that ΔG_{cont} is given only by H_{B} and u_0. As cholesterol may promote a negative c_0 [109, 124], the cholesterol-induced changes in ΔG_{cont} will be lower estimates.

H_{B} for α-helices with a radius of 0.65 nm in 1-stearoyl-2-oleoyl-phosphatidylcholine (SOPC) or SOPC:cholesterol (1:1) was calculated using experimentally determined values of K_{A}, K_{c}, and d_0, and the scaling relation derived by Nielsen and Andersen (2000) [51]. K_{A} and K_{c} are four- and three-fold higher in SOPC:cholesterol (1:1) than in SOPC [121, 122]. d_0 for SOPC (∼3 nm [185]) further should be increased ∼10% by cholesterol [126]. Using these values H_{B} becomes ∼3-fold higher in SOPC:cholesterol than in SOPC [41].

Figure 13(B) shows ΔG_{cont} for α-helices of 15–20 amino acids in SOPC or SOPC:cholesterol bilayers. The length of a 20 amino acid helix matches the thickness of SOPC. ΔG_{cont} therefore is zero. This value is increased by only 1 kcal mol^{-1} in SOPC:cholesterol. For α-helices of 15 and 17 amino acids, in contrast, ΔG_{cont} is increased from 2 and 1 kcal mol^{-1} in SOPC, to 14 and 7 kcal mol^{-1} in SOPC:cholesterol. To compare the relative importance of the cholesterol-induced changes in the bilayer thickness versus changes in the elastic moduli, ΔG_{cont} was also calculated assuming that cholesterol altered only d_0 (figure 13(B)). In this situation the effects of cholesterol would be much weaker. The increase in ΔG_{cont} for helices of 15, 17 and 20 amino acids would thus be eight-, six- and four-fold lower than when both the thickness and the elastic moduli are affected (figure 13(B)).

Based on the cholesterol-induced changes in ΔG_{cont}, the distribution of α-helices between bilayer domains of SOPC and SOPC:cholesterol may be calculated [41]. If helices of 20 amino acids were allowed to distribute freely between such domains, the ratio between the number of helices in the two types of domains would be 10^{-1}. However, for a 17 amino acid helix this ratio would be 10^{-5}, or four orders of magnitudes lower. If cholesterol altered only d_0, the ratio for a 20 amino acid helix and a 17 amino acid helix would be 0.6 and 0.15, respectively. That is, only four-fold lower for the shorter helix. The cholesterol-induced changes in the elastic moduli thus contribute significantly to the sorting process.

Given that ΔG_{cont} scales as a linear function of the radius of a lipid bilayer inclusion [50, 51], the effects of cholesterol will be higher for a membrane protein, such as the nicotinic acetylcholine receptor, where the hydrophobic length and the radius are both ~3 nm (e.g., [147]). Using this radius and values of l equal to 3, 2.85, or 2.7 nm, the cholesterol-induced increase changes in ΔG_{cont} would be 5, 13 and 23 kcal mol^{-1}, respectively. A difference in protein hydrophobic length of 0.3 nm (corresponding to two amino acids in an α-helix) would thus lead to an energy difference corresponding to the hydrolysis of several ATP molecules [41, 148].

We conclude that a sorting mechanism based on cholesterol-induced changes in the physical properties of lipid bilayers would be energetically feasible. The increase in bilayer thickness, *per se*, only modestly affects sorting. The major effect arises because cholesterol decreases the bilayer elasticity, which augments the bilayer deformation energy associated with the protein–bilayer hydrophobic mismatch. The notion that membrane protein sorting could be regulated by bilayer elasticity is supported by studies showing that the adsorption of amphipathic peptides to a lipid bilayer varies as a function of the area compression modulus [186]. For recent reviews discussing experimental studies which have tested and confirmed the predictions of the theoretical analysis described above, see [187–189].

Cellular membranes have been proposed to contain membrane domains enriched in cholesterol and sphingolipids, so-called lipid rafts (although the 'raft-hypothesis' is still controversial, cf [190–193]). As compared to lipid/cholesterol mixtures, the presence of sphingolipids in such rafts would even further increase the energetic cost of the bilayer deformation associated with protein–bilayer hydrophobic mismatch [189]. Membrane protein sorting into lipid rafts may thus be regulated by the bilayer elastic properties [41].

6. Physiological importance of lipid bilayer elasticity

We have shown that the hydrophobic interactions between a membrane protein and the surrounding lipid bilayer provide a coupling mechanism whereby protein function can be regulated by the bilayer elasticity. The practical feasibility of the HCM has been demonstrated using gA channels in planar lipid bilayers as a model system. gA channels can further be used as molecular force transducers for *in situ* measurements of the bilayer elasticity experienced by an embedded protein. The correlation between the effects of changes in bilayer molecular composition on membrane protein function, and bilayer stiffness measured using gA channels, is surprisingly simple. Using gA channels as molecular force transducers, a theoretical and technological framework to study the regulation of membrane protein function by bilayer elasticity has been established.

Does the feasibility of the HCM imply that changes in bilayer elasticity represent a physiological mechanism regulating cell function? The conclusion of the studies summarized in the present review seems to be: either cell membranes maintain a constant bilayer elasticity, or changes in bilayer elasticity represent a general mechanism regulating cell function, just as the membrane potential, $[Ca^{2+}]$ and pH.

The use of gA channels as molecular force transducers provides a novel tool to investigate the role of changes in lipid bilayer physics in diseases where the lipid composition of cellular membranes is altered. The current obesity epidemic has led to a dramatic rise in the number of people affected by the metabolic syndrome and related diseases such as cardiovascular diseases and type II diabetes [18, 19, 194]. Altered body lipid composition is considered to be key factor in these diseases, but the underlying mechanisms are poorly understood. In cardiovascular diseases, PUFAs decrease the risk of 'sudden cardiac death' by up to 50%, most likely by preventing malignant arrhythmias, cf [18, 195, 196]. PUFAs have acute anti-arrhythmic effects, which cannot be ascribed to long-term changes in metabolism or gene expression [18, 195]. PUFAs may prevent cardiac arrhythmia by promoting VDSC inactivation, which in cultured cardiac myocytes increases the refractory period and decrease the contraction rate [18]. However, TX100 and βOG, in this system, have the same effect! Based on these findings, Leaf *et al* (2003) [18] have suggested that PUFAs might prevent cardiac arrhythmia by affecting the elasticity of cellular membranes, as measured using gA channels. In type II diabetes, increased production of free fatty acids is considered to be a key factor [19]. Insulin resistance in humans correlates with cell membrane 'fluidity' [197], and insulin receptor function is regulated by the physical properties of the host lipid bilayer [198]. Nevertheless, in both cardiovascular diseases and type II diabetes the role of changes in bilayer physics is unknown, partly because of the complex changes in cell membrane lipid composition. gA-based *in situ* measurements of the *net* changes in cell membrane elasticity provide a nanotechnological tool to investigate whether altered bilayer elasticity is causally involved.

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