 REVIEW

Lipid bilayer regulation of membrane protein function: gramicidin channels as molecular force probes

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Membrane protein function is regulated by the host lipid bilayer composition. This regulation may depend on specific chemical interactions between proteins and individual molecules in the bilayer, as well as on non-specific interactions between proteins and the bilayer behaving as a physical entity with collective physical properties (e.g. thickness, intrinsic monolayer curvature or elastic moduli). Studies in physico-chemical model systems have demonstrated that changes in bilayer physical properties can regulate membrane protein function by altering the energetic cost of the bilayer deformation associated with a protein conformational change. This type of regulation is well characterized, and its mechanistic elucidation is an interdisciplinary field bordering on physics, chemistry and biology. Changes in lipid composition that alter bilayer physical properties (including cholesterol, polyunsaturated fatty acids, other lipid metabolites and amphiphiles) regulate a wide range of membrane proteins in a seemingly non-specific manner. The commonality of the changes in protein function suggests an underlying physical mechanism, and recent studies show that at least some of the changes are caused by altered bilayer physical properties. This advance is because of the introduction of new tools for studying lipid bilayer regulation of protein function. The present review provides an introduction to the regulation of membrane protein function by the bilayer physical properties. We further describe the use of gramicidin channels as molecular force probes for studying this mechanism, with a unique ability to discriminate between consequences of changes in monolayer curvature and bilayer elastic moduli.

Keywords: hydrophobic coupling; lipid bilayer elasticity; gramicidin channels

1. INTRODUCTION

Cell membranes are dynamically regulated composites of many different types of lipids and proteins, with the overall membrane organization being a lipid bilayer ‘backbone’ plus membrane-spanning and peripherally adsorbed proteins. The importance of having a variety of membrane proteins can be understood with reference to the number of functions that any given membrane supports. The importance of having a variety of membrane lipids similarly reflects their roles in many biological functions. A key lipid bilayer function, being a barrier to unregulated solute movement, can be provided by just one, or a few, lipid species. Cell membranes, however, are composed of more than 200 different lipid species (Myher et al. 1989), and many cell and membrane protein functions are regulated by changes in membrane lipid composition (Spector & Yorek 1985).

The regulation of membrane protein function by membrane lipids may occur through mechanisms ranging from specific chemical interactions between proteins and individual lipid molecules, to non-specific interactions between proteins and the bilayer behaving as a macrostructure with collective physical properties (e.g. thickness, intrinsic monolayer curvature or elastic moduli; Lee 2005; Lundbæk 2006, 2008; Andersen & Koepe 2007; Marsh 2008). Specific regulation may arise from phospholipid head group interactions with specific proteins—as in the case of the polyphosphoinositides (Hilgemann et al. 2001; McLaughlin & Murray 2005; Suh & Hille 2008)—or because lipids are structural elements in membrane proteins (e.g. Lee 2003). Specific regulation also arises when membrane lipids are precursors for cell signalling.
molecules—such as arachidonic acid in prostaglandin biosynthesis (Schmitz & Ecker 2008) or the hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-triphosphate and diacylglycerol (Su & Hille 2008). Such specific mechanisms can be investigated using well-known concepts from ligand–receptor interactions; they will not be discussed further here.

The focus of this review is the non-specific regulation of membrane protein function by the host bilayer, which arises from the hydrophobic coupling between the protein and the bilayer. Membrane proteins perturb the surrounding lipids (Seelig et al. 1981; Jähnig et al. 1982; Nezil & Bloom 1992; Marsh 2008), and this perturbation will incur an energetic cost (Marcolja 1976; Kirk et al. 1984; Mouritsen & Bloom 1984; Huang 1986; Fattal & Ben-Shaul 1993). Protein conformational changes that involve the protein–bilayer boundary will alter the packing and dynamics of the adjacent lipids to locally deform the bilayer—and the ensuing bilayer deformation energy contributes to the free energy difference between the protein conformations (Huang 1986; Nielsen et al. 1998).

The bilayer deformation energy varies as a function of bilayer physical properties, such as thickness, intrinsic curvature and the bilayer compression and bending moduli (Huang 1986; Nielsen et al. 1998; Nielsen & Andersen 2000), meaning that changes in bilayer physical properties can alter membrane protein conformational distribution and function (Sackmann 1984). Physico-chemical studies on peptide–bilayer interactions have provided mechanistic insight into how bilayer physical properties can be determinants of membrane protein function. Studies on the bilayer regulation of membrane protein function in systems with defined lipid composition have shown that changes in bilayer properties, such as bilayer thickness or intrinsic lipid curvature, for example, lead to changes in protein function (Andersen & Köpppe 2007). The interpretation of such experiments is not straightforward, however, because experimental manoeuvres designed to alter a desired bilayer property, such as intrinsic curvature, are likely to alter other bilayer properties also, such as thickness (Rostovtseva et al. 2008).

That is, though the general principles are well understood, the relative importance of the different contributions to the bilayer deformation energy may be difficult to assess, and it becomes important to have methods to determine the overall energetic consequences of changes in bilayer composition.

Lipids and lipid metabolites that alter bilayer physical properties (such as lysophospholipids, cholesterol and polyunsaturated fatty acids; Lundbaek & Andersen 1994; Lundbaek et al. 1996, 2004; Bruno et al. 2007) regulate membrane proteins in a seemingly non-specific manner (see references in Lundbaek & Andersen 1994; Lundbaek et al. 1996, 2004; Bruno et al. 2007), but it is not clear to what extent the altered bilayer physical properties play a causal role in these effects. Similarly, many drugs that regulate membrane protein function are amphiphiles that adsorb to lipid bilayers and alter bilayer physical properties at concentrations used in cell physiological studies, e.g. genistein, capsaicin or curcumin (Hwang et al. 2003; Lundbaek et al. 2005; Ingolfsson et al. 2007; Lundbaek 2008). Again, the contributions from altered bilayer properties have, with a few exceptions (e.g. Lundbaek et al. 2004, 2005), not been established—and are often not even considered.

The aim of this review is to provide an introduction to bilayer mechanics and the energetics of protein-induced bilayer deformations, and to introduce the use of gramicidin channels as molecular force probes to examine how changes in bilayer composition, or the adsorption of amphiphiles, alter the bilayer deformation energy. Section 2 describes the core concepts, the basis for the regulation of membrane function by bilayer physical properties and the use of gramicidin channels as probes for changes in these properties. Section 3 provides a more quantitative analysis of the underlying principles, with emphasis on the gramicidin channel-based approach, which allows for discrimination between the consequences of changes in monolayer curvature versus bilayer elastic moduli, as experienced by bilayer-spanning proteins.

2. LIPID BILAYER REGULATION OF PROTEIN FUNCTION

2.1. The hydrophobic coupling mechanism

The organization of a cell membrane reflects the need to optimize hydrophobic interactions among membrane lipids and proteins (Singer & Nicolson 1972; Mouritsen & Andersen 1998). The energetic penalty for exposing hydrophobic surfaces to water, approximately 5 kcal (mol nm$^2$)$^{-1}$ (Sharp et al. 1991), causes lipid molecules to be organized as a bilayer, in which the hydrophilic lipid head groups are exposed to the aqueous solution and the acyl chains are sequestered in the bilayer hydrophobic core. In proteins, correspondingly, the side chains that are exposed to the aqueous solution are hydrophilic, whereas those at the exterior surface of the proteins’ trans-membrane domains (TMDs) are hydrophobic.

A mismatch between a TMD’s hydrophobic length (length of the protein exterior facing the bilayer core) and the bilayer’s hydrophobic thickness (thickness of the acyl chain core) would—unless the bilayer and protein adapt to each other—incur a considerable energetic cost owing to increased contact between hydrophobic and polar residues (including water). For a protein with a 2 nm radius, the energetic penalty for a 0.15 nm mismatch, corresponding to one amino acid in a transmembrane α-helix, would be approximately 9 kcal mol$^{-1}$. (For comparison, the energy released by covalent bond breaking during hydrolysis of ATP to ADP is approx. 13 kcal mol$^{-1}$ (Veech et al. 1979).) Therefore, a protein-bilayer mismatch will cause a local change in bilayer thickness as well as a change in protein length (Mouritsen & Bloom 1984), whether it is by minor adjustments of the amino acid side chains or by a shift in the balance between different protein conformations. Focusing on the bilayer, a local thickness change has an associated energetic cost, the bilayer deformation energy ($\Delta G_{def}$) (Mouritsen & Bloom 1984; Huang 1986). The function of membrane proteins whose conformational changes involve the boundary between the TMD and the bilayer core thus will be influenced by changes in $\Delta G_{def}$. 
Figure 1 shows examples of membrane proteins for which there is structural information about more than one conformation: the KcsA channel in a closed and open state; the mechano-sensitive MscS channel in an open and closed state; the maltose transporter MalFGK2 in an outward- and an inward-facing state; and the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase in a Ca\(^{2+}\)-bound E1 state and a Ca\(^{2+}\)-free E2 state. In all four cases, the protein conformational changes involve the TMD/bilayer boundary. Table 1 lists other membrane proteins for which there is structural information about more than one conformation. Except in two cases (the \(\beta\)-barrel outer-membrane proteins (OmpG; Yildiz \textit{et al.} 2006) and the colicin I receptor (Buchanan \textit{et al.} 2007)), the structural transitions involve the TMD/bilayer boundary. Similar conclusions have been drawn for other proteins based on chemical cross-linking, spectroscopic and cysteine accessibility studies, summarized in Lundbæk (2006).

Because bilayer deformations incur an energetic cost (Huang 1986), the difference in bilayer deformation energy associated with two protein conformations \(A\) and \(B\) (\(\Delta G_{\text{bilayer}}^{A-B}\)) will contribute to the total energetic cost of the protein conformational change (\(\Delta G_{\text{tot}}^{A-B}\)):

\[
\Delta G_{\text{tot}}^{A-B} = \Delta G_{\text{prot}}^{A-B} + \Delta G_{\text{bilayer}}^{A-B},
\]

where \(\Delta G_{\text{prot}}^{A-B}\) denotes energetic contributions due to the protein (contributions other than those due to the bilayer deformation \textit{per se}). The equilibrium distribution between the protein conformations (in the case of an ion channel, figure 2, between open and closed states) is given by

\[
\frac{n_B}{n_A} = \exp\left\{-\frac{\Delta G_{\text{prot}}^{A-B} + \Delta G_{\text{bilayer}}^{A-B}}{k_B T}\right\},
\]

where \(n_A\) and \(n_B\) denote the number of proteins (per unit area) in each of the two states, and \(k_B\) and \(T\) are Boltzmann’s constant and temperature in kelvin. The protein–bilayer hydrophobic interactions thus provide...
Table 1. Membrane proteins with structural information about more than one conformation.

<table>
<thead>
<tr>
<th>Protein</th>
<th>PDB ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KcsA</td>
<td>1BL8,</td>
<td>Doyle et al. (1998); Perozo et al. (1999)</td>
</tr>
<tr>
<td>NaK channel</td>
<td>2AHY,</td>
<td>Shi et al. (2006); Alam &amp; Jiang (2009)</td>
</tr>
<tr>
<td>M2 proton channel</td>
<td>2RLF</td>
<td>Schnell &amp; Chou (2008)</td>
</tr>
<tr>
<td>mecano-sensitive MscS channel</td>
<td>2OAU,</td>
<td>Bass et al. (2002); Wang et al. (2008)</td>
</tr>
<tr>
<td>mecano-sensitive MscL channel</td>
<td>2V5</td>
<td>Chang et al. (1998); Perozo et al. (2002)</td>
</tr>
<tr>
<td>nicotinic acetylcholine receptor</td>
<td></td>
<td>Toyoshima &amp; Unwin (1998)</td>
</tr>
<tr>
<td>prokaryotic pentameric ligand-gated ion channel pLGIC</td>
<td>2VL0, 2EHZ, 3EAM</td>
<td>Hilf &amp; Dutzler (2008), Okada et al. (2004), Bocquet et al. (2009)</td>
</tr>
<tr>
<td>gap junction channel</td>
<td>2ZW3</td>
<td>Unwin &amp; Ennis (1984); Maeda et al. (2009)</td>
</tr>
<tr>
<td>OmpG</td>
<td>2IWV,</td>
<td>Yildiz et al. (2006)</td>
</tr>
<tr>
<td>colicin I receptor</td>
<td>2HDF,</td>
<td>Buchanan et al. (2007)</td>
</tr>
<tr>
<td>rhodopsin</td>
<td>3CAP,</td>
<td>Park et al. (2008); Park et al. (2004)</td>
</tr>
<tr>
<td>bacteriorhodopsin</td>
<td>1CS8,</td>
<td>Luecke et al. (1999); Vondk (2000)</td>
</tr>
<tr>
<td>intramembrane protease, GlpG</td>
<td>2IC8,</td>
<td>Wang et al. (2006); Wang &amp; Ha (2007)</td>
</tr>
<tr>
<td>Ca(^{2+})-ATPase</td>
<td>1SU4,</td>
<td>Toyoshima et al. (2000); Toyoshima &amp; Nomura (2002)</td>
</tr>
<tr>
<td>maltose transporter, MalFG2</td>
<td>2R6G,</td>
<td>Oldham et al. (2007); Khare et al. (2009)</td>
</tr>
<tr>
<td>multidrug transporter</td>
<td>2DHH,</td>
<td>Murakami et al. (2006)</td>
</tr>
<tr>
<td>LeuT leucine transporter</td>
<td>2A65,</td>
<td>Yamashita et al. (2005); Singh et al. (2008)</td>
</tr>
<tr>
<td>NhaA Na(^{+})/H(^{+}) antiporter</td>
<td>1ZCD,</td>
<td>Hunte et al. (2005); Appel et al. (2009)</td>
</tr>
<tr>
<td>BtuCD ABC transporter</td>
<td>2QB9</td>
<td>Hvorup et al. (2007); Gerber et al. (2008)</td>
</tr>
<tr>
<td>ModBC ABC transporter</td>
<td>3D31</td>
<td>Gerber et al. (2008)</td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>3G5U,</td>
<td>Aller et al. (2009)</td>
</tr>
<tr>
<td>DsbB–Fab complex</td>
<td>2UQ</td>
<td>Inaba et al. (2009)</td>
</tr>
<tr>
<td>Mhp1 benzyl-hydantoin transporter</td>
<td>2JLN</td>
<td>Weyand et al. (2008)</td>
</tr>
</tbody>
</table>

Figure 2. Hydrophobic coupling between a bilayer-spanning membrane protein (ion channel) and host lipid bilayer. A protein conformational change causes a local bilayer deformation; for simplicity, there is no hydrophobic mismatch in the closed state. Modified from Andersen & Kopepe (2007).

for an energetic coupling between the protein conformational preference and the cost of deforming the bilayer. This hydrophobic coupling provides a means by which changes in bilayer composition can regulate membrane protein function (Lundbæk et al. 2004).

It is non-refutable that protein function, in principle, could be regulated by the hydrophobic coupling mechanism, as described by equations (2.1) and (2.2). For this mechanism to be an important regulator of protein function, however, changes in bilayer composition should alter \( \Delta G_{A}^{\text{bilayer}} \), the bilayer contribution to \( \Delta G_{A}^{\text{A-B}} \), by more than \( k_{B} T \). It thus becomes important to understand what determines the magnitude of \( \Delta G_{A}^{\text{A-B}} \), and how \( \Delta G_{A}^{\text{bilayer}} \) is altered by changes in bilayer composition.

2.2. Theory of elastic bilayer deformations

The energetic cost of adapting the bilayer hydrophobic thickness to match the hydrophobic length of an embedded protein has been analysed using continuum theories of elastic bilayer deformations. We follow Huang (1986) and later studies (Helfrich & Jakobsson 1990; Dan & Safran 1998; Nielsen et al. 1998; Nielsen & Andersen 2000; Partenskii & Jordan 2002), in which the bilayer deformation is decomposed as the sum of two major contributions (cf. figures 2 and 3): bilayer compression (originally proposed by Mouritsen & Bloom (1984)); and monolayer bending (originally proposed by Gruner (1985)); the energetic consequences related to the bending of the bilayer per se have been considered by Phillips et al. (2009). Energetic contributions related to Gaussian curvature and bilayer interfacial tension should be negligible (Nielsen et al. 1998) and will not be considered here.

Lipid bilayer compression/expansion is associated with a compression energy density (CED):

\[
CED = \frac{1}{2} K_A \cdot \left(\frac{d - d_0}{d_0}\right)^2.
\]
where $K_a$ is the bilayer compression modulus (figure 3a), $d_0$ the equilibrium thickness of the unperturbed bilayer and $d$ the thickness of the perturbed bilayer. (Lipid bilayers are relatively soft, and the bilayer–solution interface undergoes thermal fluctuations (Wiener & White 1992), which involve local movement of individual phospholipid molecules and more global bilayer undulations and peristaltic motions (Wiener & White 1992; Lindahl & Edholm 2000). The bilayer–solution interface is thus ‘fuzzy’; but the average bilayer thickness, which is relevant here, is a well-defined parameter.)

Monolayer bending is similarly associated with a bending energy density (BED), which can be approximated as

$$\text{BED} = \frac{1}{2} K_{c,m} \left( \frac{c_1 + c_2}{2} - c_0 \right)^2,$$

(2.4)

where $K_{c,m}$ is the monolayer bending modulus, $c_1$ and $c_2$ are the principal curvatures and $c_0$ the intrinsic monolayer curvature (figure 3b).

The intrinsic curvature of a lipid monolayer is determined by the variation of intermolecular interactions across the monolayer (Helfrich 1981; Seddon 1990; Szleifer et al. 1990; Petrov 1999), which is often expressed in terms of the effective ‘shape’ of the lipids in the monolayer (Israelachvili 1977; Cullis & de Kruijff 1979). Cylindrical molecules will form planar and cone-shaped molecules will form non-planar monolayers (figure 3c). Though the intrinsic monolayer curvature may differ from zero, a bilayer formed by the apposition of two such monolayer leaflets of equal composition will be planar—and the associated change in the average molecular shape, from cone-shaped to cylindrical, causes a stress with an energy density $(K_{c,m}/2) \cdot c_0^2$ in each monolayer.

The energetic cost of the bilayer deformation needed to match the local thickness of a lipid bilayer to the length of a cylindrical bilayer-spanning protein of radius $r_0$ thus becomes (cf. equations (2.3) and (2.4))

$$\Delta G_{\text{def}} = \int_{r_0}^{r_0^*} \left\{ \frac{K_a}{2} \left( \frac{d - d_0}{d_0} \right)^2 + \frac{K_c}{2} \left( \frac{c_1 + c_2}{2} - c_0 \right)^2 \right\} r \, dr
+ \int_{r_0}^{r_0^*} \frac{K_{c,m}}{2} c_0^2 2\pi r \, dr,$$

(2.5)

where $d$ denotes the bilayer thickness as a function of $r$ ($d = l$, the protein hydrophobic length, at $r_0$), and $K_c$ the bilayer bending modulus ($=2K_{c,m}$). The second integral denotes the curvature frustration energy of the unperturbed (flat) bilayer.

Equation (2.5) can be solved analytically (Goulian et al. 1998; Nielsen et al. 1998; Nielsen & Andersen 2000) and can be expressed as a biquadratic function of the channel–bilayer mismatch ($l - d_0$) and $c_0$ (cf. Nielsen & Andersen 2000; Lundbæk et al. 2004; note that we have changed the convention for the...
hydrophobic mismatch, from \(d_0 - l\) to \(l - d_0\), such that \(H_X\) and \(H_C\) are positive: }

\[
\Delta G_{\text{def}} = H_B (l - d_0)^2 + H_X (l - d_0)c_0 - H_C c_0^2, \tag{2.6}
\]

where \(H_B\), \(H_X\) and \(H_C\) are elastic coefficients that are functions of \(K_a\), \(K_b\) and \(r_0\). \(H_B\), \(H_X\) and \(H_C\) increase with increasing elastic moduli (and vice versa). Quantitative estimates of the coefficients can be obtained as described in Nielsen & Andersen (2000).

In the above treatment, equation (2.6) is derived from equation (2.5), and the evaluation of \(H\) coefficients depends on a physical model similar to that underlying equation (2.5). The biquadratic relation between \(\Delta G_{\text{def}}\) and \((l - d_0)\) and \(c_0\) should apply quite generally, however, because equation (2.6) can be derived as a serial expansion of \(\Delta G_{\text{def}}\) in terms of \((l - d_0)\) and \(c_0\) (Andersen & Koepppe 2007). That is, though one may question how well a continuum description applies at molecular length scales, the simplicity of equation (2.6) should be a general feature of models of elastic bilayer deformations in which the deformation varies as a function of bilayer thickness and intrinsic lipid curvature.

The energetic cost of the hydrophobic adaptation between a bilayer-spanning protein and the host bilayer varies as a function of protein–bilayer mismatch, intrinsic monolayer curvature and the bilayer elastic moduli (equations (2.5) and (2.6)). Table 2 summarizes the direction of changes in \(\Delta G_{\text{def}}\) as a function of thickness, curvature and the elastic moduli.

The effects of changes in bilayer composition on \(d_0\), \(c_0\), \(K_a\) and \(K_b\) can be measured in protein-free systems (Helfrich 1973; Evans & Hochmuth 1978; Schneider et al. 1984; Gruner 1985; Rand & Parsegian 1997; Nagle & Tristram-Nagle 2000; Rawicz et al. 2000; Liu & Nagle 2004). So, in principle, one should be able to use equations (2.5) and (2.6) to predict the effects of changes in bilayer composition on the bilayer contribution, \(\Delta G_{\text{biayer}} = \Delta G_{\text{biayer}}^{A-B} = \Delta G_{\text{biayer}}^{A} - \Delta G_{\text{biayer}}^{B}\), to the free energy difference between two protein conformations, A and B, that differ in hydrophobic length, \(l\). In practice, this is not yet possible.

### 2.3. Determining \(\Delta G_{\text{def}}\)

Protein conformational changes at the TMD/bilayer boundary are likely to be complex (cf. figure 1) and the associated bilayer deformations remain largely unknown. Even if a protein conformational change involved a simple change in \(l\)—associated with a local adjustment of bilayer thickness—the effects of altered bilayer composition on \(\Delta G_{\text{def}}\) (and therefore the bilayer contribution to the free energy difference for the conformation change) may be difficult to estimate. This difficulty arises, in part, because \(d_0\), \(c_0\), \(K_a\) and \(K_b\) are treated as separable parameters in theories of elastic bilayer deformations (Rawicz et al. 2000), though they all are determined by the profile of intermolecular interactions across the bilayer (Helfrich 1981; Seddon 1990; Széler et al. 1990; Petrov 1999). In practice, therefore, these parameters are inter-related: \(K_a\) and \(K_b\) are related through scaling relations (Evans 1974); and the bilayer thickness varies with changes in phospholipid head group structure (Nagle & Wiener 1988; Kučerka et al. 2005; Rostovtseva et al. 2008) or acyl chain unsaturation (Lewis & Engelman 1983; Sepanovic & Gawrisch 1996), manipulations that are used to alter \(c_0\).

Changes in bilayer composition thus are likely to alter more than one of the parameters that determine \(\Delta G_{\text{def}}\). In addition, the local elastic properties adjacent to a protein may differ from the bulk properties (Partenskii & Jordan 2002), though the structure of equation (2.6) remains valid—and it remains unclear to what extent global bilayer properties determined in (protein-free) lipid vesicles apply to biological membranes with their diversity of lipid species and approximately 20 per cent of the membrane area being occupied by proteins (Dupuy & Engelman 2008).

The consequences of these ‘uncertainties’ can be considerable. Even the sign of \(\Delta G_{\text{def}}\) and thus \(\Delta G_{\text{biayer}}^{A-B} = \Delta G_{\text{biayer}}^{A} - \Delta G_{\text{biayer}}^{B}\) for a membrane protein conformational change, may be affected. In Lundbæk et al. (2005) and Bruno et al. (2007) for example, changes in \(c_0\) did not cause the predicted changes in \(\Delta G_{\text{biayer}}^{A-B}\) because of concomitant changes in the bilayer elasticity. That the net change in \(\Delta G_{\text{def}}\) may be difficult to estimate becomes a particular problem when the changes in different parameters, in isolation, should have opposite effects on \(\Delta G_{\text{def}}\). For example, in a bilayer–protein system with \(l < d_0\) addition of capsaicin (Lundbæk et al. 2005) or docosahexanoic acid (Bruno et al. 2007) would be expected to increase \(\Delta G_{\text{def}}\) by causing a negative-going change in \(c_0\) and to decrease \(\Delta G_{\text{def}}\) by decreasing \(K_a\) and \(K_b\) as both compounds are reversibly adsorbing amphiphiles (cf. Evans et al. 1995; Zhelev 1998). The quantitative consequences of an altered bilayer composition, in terms of \(\Delta G_{\text{def}}\), may be more complex than suggested in table 2.

To explore the bilayer–protein energetic coupling, one needs systems where the consequences of changes in bilayer composition on \(\Delta G_{\text{def}}\) can be measured directly—and where specific lipid–protein interactions should be of little importance (Gruner 1991). Moreover, the reporter protein should preferably be an ion-conducting channel or receptor (Andersen et al. 1998) where one can examine the equilibrium distribution between different conformations and

### Table 2. Direction of changes in \(\Delta G_{\text{def}}\) as a function changes in bilayer physical properties.

<table>
<thead>
<tr>
<th>bilayer physical property that is varied</th>
<th>long protein ((l &gt; d_0))</th>
<th>short protein ((l &lt; d_0))</th>
</tr>
</thead>
<tbody>
<tr>
<td>increasing bilayer thickness ((d_0))</td>
<td>(\Delta G_{\text{def}} \downarrow)</td>
<td>(\Delta G_{\text{def}} \uparrow)</td>
</tr>
<tr>
<td>more negative intrinsic curvature ((c_0))</td>
<td>(\Delta G_{\text{def}} \downarrow)</td>
<td>(\Delta G_{\text{def}} \uparrow)</td>
</tr>
<tr>
<td>decreasing elastic moduli ((K_a) and (K_b))</td>
<td>(\Delta G_{\text{def}} \downarrow)</td>
<td>(\Delta G_{\text{def}} \downarrow)</td>
</tr>
</tbody>
</table>
Channels formed by the linear gramicidins represent such a system. Gramicidin A (gA) is a mini protein that forms cation-conducting channels, and has been extensively used to explore how changes in bilayer properties alter protein function. Moreover, a methodology using gA channels as molecular force probes for *in situ* measurements of changes in $\Delta G_{\text{def}}$ has been developed (Lundbæk 2006; Andersen et al. 2007a), which can be used to predict the effects of changes in bilayer molecular composition on membrane protein function in living cells (Lundbæk et al. 2005).

### 2.4. Gramicidin channels as molecular force probes

Cation-selective gA channels are mini proteins formed by trans-bilayer association (O’Connell et al. 1990) of $\beta^{6,3}$-helical subunits from each leaflet (monolayer) in a lipid bilayer (figure 4a).

The sequence of the native [Val$^1$]gA is (Sarges & Witkop 1965):

```
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-ethanolamide.
```

Bilayer-incorporated gA dimers are ion-conducting channels (Bamburg & Läuger 1973; Veatch et al. 1975), and gA channel function can be studied at single-molecule resolution using electrophysiological methods; for a recent review see Andersen et al. (2007b). The channel structure, a single-stranded, right-handed $\beta^{6,3}$-helical dimer, is known at atomic resolution (Arseniev et al. 1986; Ketch et al. 1997; Townsley et al. 2001; Allen et al. 2003; figure 4b). The structure is remarkably invariant with respect to changes in the bilayer environment; minor differences between the structure in sodium dodecyl sulphate micelles (Arseniev et al. 1986; Townsley et al. 2001) and in dimyristoylphosphatidylcholine lipid bilayers (Ketch et al. 1997) can be reconciled by molecular dynamics simulations (Allen et al. 2003). In contrast to the situation in lipid bilayers, gA is conformationally polymorphic in organic solvents, where it exists in various double-stranded structures that are quite different from the single-stranded channel structure in bilayers or bilayer-nimetic environments (Bystrov & Arseniev 1988; Abdul-Manan & Hinton 1994); see also Andersen et al. (2007b).

For not too extreme changes in phospholipid acyl chain length (10 $\leq$ $n_C$ $\leq$ 20, where $n_C$ denotes the number of carbon atoms in the acyl chain), the channel structure is insensitive to the channel—bilayer hydrophobic mismatch (Wallace et al. 1981; Greathouse et al. 1994; Galbraith & Wallace 1998). Nor does the channel’s single-channel conductance (Mobashery et al. 1997; Bruno et al. 2007) or helical pitch (Katsaras et al. 1992) vary as a function of the hydrophobic mismatch, indicating that the average orientation of interfacial tryptophan residues varies little with changes in hydrophobic mismatch. Moreover, the channel function does not depend on the chirality of the channel-forming subunits or the bilayer-forming lipids (Providence et al. 1995), indicating that specific channel–phospholipid interactions are likely to be unimportant. Though the structure of the bilayer-embedded, non-conducting monomers has not been determined, it appears to be similar to that of the subunits in the conducting dimer (He et al. 1994).

The gA channels’ structural and functional features make the channels well suited to probe changes in lipid bilayer properties—as sensed by a bilayer-spanning channel. Channel formation in a lipid bilayer with a hydrophobic thickness, $d_h$ (typically 3–5 nm), that exceeds the channel hydrophobic length, $l$ (approx. 2.2 nm: Elliott et al. 1983; Huang 1986), involves a local decrease in bilayer thickness to match the channel (Harroun et al. 1999) as illustrated in figure 4 (cf. figure 2). The ensuing bilayer deformation energy contributes to the energetic cost of channel formation (Huang 1986).

Changes in $\Delta G_{\text{def}}$ (cf. figure 2) can be reconciled by $\Delta G_{\text{def}}$, $\Delta G_{\text{d}}$, and $\Delta G_{\text{M}}$ denote the bilayer deformation energies for the monomer and dimer states, respectively) are reflected in gA channel behaviour. An increase in $\Delta G_{\text{def}}$ is reported as

- decreased channel appearance rate ($f$),
- decreased channel lifetime ($\tau$), and
- decreased channel activity (time-averaged number of conducting channels, $n = f\tau$).

A decrease in $\Delta G_{\text{def}}$ has opposite effects. gA channels thus provide for *in situ* measurements of the bilayer response to a conformational change in an embedded protein.

### 2.5. Gramicidin single-channel experiments

Figure 5a shows an experimental setup used in gA single-channel experiments (see also Ingólfsson et al. 2008; Kapoor et al. 2008).

A lipid bilayer is formed across a hole (diameter approx. 1 mm) in a Teflon partition separating two electrolyte solutions in a Teflon chamber. gA (1–100 pM, depending on gA analogue and bilayer composition) is added to the electrolyte solutions on both sides of the bilayer (making sure to stir the solution). The hydrophobic gA subunits adsorb to the bilayer, where they fold into channel-forming $\beta^{6,3}$-helices. To observe single channels, it is necessary to have a good signal/noise ratio, which can be accomplished by isolating a small bilayer area using a glass pipette (diameter approx. 30 $\mu$m; Andersen 1983). The formation and disappearance of conducting gA channels are observed as rectangular current transitions when a trans-bilayer potential difference is applied; figure 5b shows a current trace from an experiment on the effects of the lipid metabolite lysophosphatidylcholine (LPC) on gA channels in a planar lipid bilayer (discussed further below). During subsequent computer-assisted analysis, the single channel conductance ($g$) is calculated from current-transition amplitude histograms (figure 5c), whereas $\tau$ is determined from single-channel lifetime distributions (figure 5d). The channel appearance rate, $f$, is determined by manual or computer-assisted assignment from the number of current transitions per unit time.

The effects of changes in lipid bilayer composition on gA channel function can be understood using the
framework provided by the continuum theory of elastic bilayer deformations described above (equations (2.5) and (2.6)). This approach has been validated by examining the relation between changes in gA channel lifetime and bilayer thickness (Huang 1986; Lundbæk & Andersen 1999) and bilayer tension (Goulian et al. 1998), where changes in tension (σ) alter gA channel function because of the associated changes in bilayer thickness (Evans & Needham 1987; Goulian et al. 1998).

Because gA channels form (and disappear) by trans-bilayer association/dissociation (O’Connell et al. 1990), it becomes useful to introduce the so-called disjoining force (Fdis) that acts on the bilayer-spanning channel, tending to break it apart. Fdis is obtained by differentiating ΔGdef (equation (2.6)) with respect to (l − d0) (Lundbæk et al. 2005; Andersen & Koeppe 2007):

\[ F_{\text{dis}} = \frac{d(\Delta G_{\text{def}})}{d(l - d_0)} = -2H_B(l - d_0) - H_X c_0 \]

\[ = 2H_B(d_0 - l) - H_X c_0. \]  

(2.7)

As is the case for ΔGdef (cf. table 2), Fdis is increased and f, τ and n are decreased by

— increasing bilayer–channel mismatch (d0 − l),
— more negative c0, and
— increasing bilayer elastic moduli.

These predictions have been validated experimentally, as will be described in the following.

2.6. Channel–bilayer mismatch

Figure 6a shows the relation between gA single-channel lifetime (τ) and the thickness of monoacylglyceride/squalene bilayers (Elliott et al. 1983; from Lundbæk & Andersen 1999). (Bilayers formed using squalene in the bilayer-forming solution are virtually hydrocarbon-free (Simon et al. 1977; White 1978), and squalene seems to be a universal component of living cells, even red blood cells (Liu et al. 1976).)

When the bilayer thickness, d0, is altered by changing the lipid acyl chain length, ln{τ} decreases as a linear function of d0. This decrease is expected because a larger channel–bilayer mismatch should increase the magnitude of Fdis (cf. equation (2.7)) and thereby lower the activation energy for channel dissociation. The linearity of the ln{τ}–d0 relation conforms to the continuum theory of elastic bilayer deformations: Fdis varies as a linear function of the channel–bilayer hydrophobic mismatch, d0 − l, meaning that ΔGdef should vary as (d0 − l)² (cf. equation (2.6)). Moreover, the slope of the ln{τ}–d0 relation conforms to predictions based on the continuum theory using experimentally determined elastic moduli (Lundbæk & Andersen 1999; see also §3). (The agreement between observed and predicted slopes of the ln{τ}–d0 relations is gratifying, but should not be overinterpreted. It is most likely another reflection of the surprising ability of continuum theories to provide reasonable predictions at the molecular level, as evident in the use of the
Stokes–Einstein to estimate ionic radii (e.g. Robinson & Stokes 1965, pp. 43–44), or the Born model to estimate ionic solvation energies (e.g. Bockris & Reddy 1970, pp. 48–72).

The relation between channel lifetime and channel–bilayer mismatch has also been studied using n-decane-containing phospholipid bilayers. Figure 6b shows results obtained when the hydrophobic mismatch was varied by altering either the lipid acyl chain length (bilayer thickness) or the channel length. In either case, \( \ln(\tau) \) decreases as an approximately linear function of \( (d_0 - l) \) and, though the single-channel lifetime does depend on the specific sequence at the subunit interface (Mattice et al. 1995), the slope of the relation is similar whether \( (d_0 - l) \) is altered by changing \( d_0 \) or \( l \). n-Decane increases the bilayer thickness (Benz et al. 1975) compared with nominally hydrocarbon-free bilayers, and makes the bilayers softer (Helfrich & Jakobsson 1990; Lundbæk & Andersen 1999), but the linear relation between \( \ln(\tau) \) and \( (d_0 - l) \) conforms to the continuum theory of elastic bilayer deformations as embodied in equations (2.6) and (2.7) (Lundbæk & Andersen 1999).

### 2.7. Intrinsic monolayer curvature

The relation between gA channel function and the intrinsic monolayer curvature, \( c_0 \), has been studied by varying the ‘shape’ of the bilayer-forming lipids (Lundbæk et al. 1997; Maer et al. submitted). A decrease in lipid head group size promotes a more negative \( c_0 \) (cf. figure 3c), as well as an increase in bilayer thickness (Nagle & Wiener 1988; Kučerka et al. 2005; Rostovtseva et al. 2008). Figure 7 shows results from experiments in which \( c_0 \) of dioleoylphosphatidylserine/n-decane bilayers was varied by altering the electrostatic repulsion among the charged serine head groups and thus the ‘effective’ head group size (Lundbæk et al. 1997). (Because the experiments were performed in n-decane-containing bilayers, the bilayer thickness did not vary as the head group interactions were altered by the addition of Ca\(^{2+}\) (Lundbæk et al. 1997) and the results can be interpreted in terms of changes in \( c_0 \).)

When the electrostatic repulsion is reduced, by increasing the aqueous [Ca\(^{2+}\)], the more negative \( c_0 \) is associated with a decrease in \( f, \tau \) and therefore \( n \). This is expected because a negative-going \( c_0 \) should increase the magnitude of \( F_{\text{dis}} \) (cf. equation (2.7)). Similar results were obtained when \( c_0 \) was altered by varying the ratios of dioleoylphosphatidylcholine (DOPC, with a relatively large head group) and dioleoylphosphatidylethanolamine (DOPE, with a relatively small head group) in \( n \)-decane-containing bilayers (Maer et al. submitted). As noted by Rostovtseva et al. (2008), increasing the mole-fraction of DOPE also will increase bilayer thickness, \( d_0 \), and it may be difficult to separate the consequences of the changes in \( c_0 \) and \( d_0 \).

This could be done in the experiments by Maer et al. (submitted) because the thickness changes are small in \( n \)-decane-containing bilayers.
It is instructive to compare the phospholipid head group-dependent changes in gA channel function, summarized above, with the changes in alamethicin channel function in bilayers of similar composition (Keller et al. 1993; Bezrukov et al. 1998). Alamethicin channels are formed by bilayer-spanning α-helices (He et al. 1996) that are organized in a barrel-stave arrangement (Qian et al. 2008). The channel structure has not been determined at molecular resolution, but it is believed that the channels can exist in different oligomeric assemblies that correspond to different conductance levels in the multistate single-channel behaviour (Woolley & Wallace 1992). Like gA channels, alamethicin channels also sense changes in lipid bilayer properties (Bezrukov 2000), and the changes in alamethicin and gA channel are related: manoeuvres that shift the gA monomer ↔ dimer equilibrium towards the dimeric state reduce the probability of observing the higher conductance states in gA channels (Lundbæk et al. 1997; Bezrukov 2000). That is, though the two channels form by different mechanisms, they have correlated responses to changes in bilayer physical properties—suggestive of a common regulatory mechanism.

2.8. Water-soluble amphiphiles

As noted above, changes in lipid bilayer composition often alter the bilayer physical properties in a manner that cannot be described by a change in just one of the parameters that determine \( \Delta G_{\text{tot}} \). Water-soluble amphiphiles, for example, may not only alter \( c_0 \) though their effects are often ascribed to changes in \( c_0 \). By reversibly adsorbing to lipid bilayers, they also tend to decrease the bilayer elastic moduli (Evans et al. 1995; Zhelev 1998; Ly & Longo 2004; Lundbæk et al. 2005; Zhou & Raphael 2005; Bruno et al. 2007) leading to a bilayer softening. In such cases, the net effect on \( F_{\text{dis}} \) becomes difficult to predict. Micelle-forming amphiphiles, owing to their molecular cone ‘shape’, tend to promote a positive-going change in \( c_0 \) (cf. figure 3c). Both the more positive \( c_0 \) and the decrease in elastic moduli will tend to decrease \( \Delta G_{\text{tot}} \) and \( F_{\text{dis}} \)—and micelle-forming amphiphiles increase \( f \), \( \tau \) and \( n \) (Lundbæk & Andersen 1994).

Figure 7 shows the concentration-dependent effects of micelle-forming LPC using an experimental setup as in figure 5. (The actual aqueous amphiphile concentration may be orders of magnitude less than the nominal concentration owing to partitioning into the non-polar phase (e.g. Bruno et al. 2007; Ingólfsson

**Figure 6.** gA channel lifetime (\( \tau \)) as function of the channel–bilayer hydrophobic mismatch. (a) Relation between \( \tau \) and hydrophobic thickness (\( d_0 \)) of monounsaturated gA analogues (endo-Gly-D-Ala-gA) in monounsaturated phosphatidylcholine/n-decane bilayers. Based on these results, the slope of the ln(\( \tau \))–\( d_0 \) relation is \(-8.9 \text{ nm}^{-1} \) (\( r = 0.99 \)), corresponding to \( H_0 = 69 \text{ kJ mol}^{-1} \text{ nm}^{-2} \). After Lundbæk & Andersen (1999). Lifetime results from Elliott et al. (1983); bilayer hydrophobic thickness determined from bilayer capacitance measurements (Benz et al. 1975). (b) Relation between channel lifetime and channel–bilayer hydrophobic mismatch for phosphatidylcholine/n-decane bilayers. The mismatch was altered using either a sequence-extended 17 amino acid long gA analogue (endo-Gly-D-Ala-gA) in monounsaturated phosphatidylethanolamine/n-decane bilayers having varying acyl chain length (13, 15 and 17) in dioleoylphosphatidylcholine/n-decane bilayers (filled triangles); bilayer hydrophobic thickness determined from bilayer capacitance measurements (Benz et al. 1975). Based on these results, the slope of the relation between ln(\( \tau \)) and (\( d_0 - l \)) is \(-7.2 \text{ nm}^{-1} \) (\( r = 0.98 \)), corresponding to \( H_0 = 56 \text{ kJ mol}^{-1} \text{ nm}^{-2} \). Experimental results from Hwang et al. (2003).

**Figure 7.** Concentration-dependent effects of \( \text{Ca}^{2+} \) on gA channel appearance rate (\( f \); dashed line); lifetime (\( \tau \); solid line) and activity (the time-averaged number of conducting channels, \( n \); dashed line). By increasing \( [\text{Ca}^{2+}] \), \( c_0 \) is changed in the negative direction, and the gA channels are increasingly destabilized. The dashed lines connecting the results for the normalized changes in \( f \) and \( n \) are meant to guide the eye. Experimental results from Lundbæk et al. (1997).
At the concentrations tested, some compounds (e.g. dimethylsulphoxide) do not alter bilayer $\Delta G_{\text{def}}$ and $F_{\text{dis}}$. This does not imply that these compounds are truly inert, just that the changes in $\Delta G_{\text{def}}$ and $F_{\text{dis}}$ are too small to be measured. Though some compounds, such as long-chain alcohols, cause changes that conform to predictions based on changes in curvature (i.e. that $\tau$ is decreased), this seems to be an exception rather than a rule.

### 2.9. Regulation of gA channel function by the hydrophobic coupling mechanism

The conclusion of studies on gA channels is that a bilayer-embedded protein can be regulated by the hydrophobic coupling between membrane proteins and their host bilayer. The equilibrium distribution between non-conducting monomers and conducting dimers (the number of conducting channels) can be altered several hundredfold by manoeuvres that alter $c_0$ or $d_0$ (Lundbæk et al. 1997; Mobashery et al. 1997). Similarly, the monomer $\leftrightarrow$ dimer equilibrium can be altered by amphiphiles or other manoeuvres that change the bilayer elastic moduli, as demonstrated by the differential effects on gA channels of different lengths (Lundbæk & Andersen 1994; Lundbæk et al. 2004; Bruno et al. 2007; Ingólfsson et al. 2007). The changes in channel activity correspond to changes in $\Delta G_{\text{def}}$ of about $\pm 5 k_B T$ or $\pm 3 \text{kcal mol}^{-1}$ (see §3).

Because the energetic cost of the deformation induced by a bilayer-spanning inclusion scales as an approximately linear function of the channel radius (Nielsen & Andersen 2000), the corresponding changes in $\Delta G_{\text{def}}$ could be $\pm 6 \text{kcal mol}^{-1}$ for a typical membrane protein with a radius of 2 nm (rather than 1 nm for the gA channel; cf. Andersen & Koeppe 2007).

### 2.10. Going from model system to membrane proteins in living cells—bilayer stiffness

The results on gA channels illustrate how changes in bilayer composition can alter the conformational preferences of membrane proteins—and that changes in channel behaviour, in simple cases, conform to predictions based on the continuum theory of elastic bilayer deformations. They also demonstrate the difficulties that arise when the changes in each of the bilayer parameters, per se, have opposite effects on $\Delta G_{\text{def}}$—and illustrate the peril of focusing on just a single parameter, such as curvature. Though the $\Delta G_{\text{bilayer}}$ may contribute to $\Delta G_{\text{tot}}$ (equations (2.1) and (2.2)) may differ among proteins embedded in the same bilayer, with some proteins being more sensitive to changes in curvature, $c_0$, and others to changes in hydrophobic mismatch (1$-d_0$), it is those changes in $\Delta G_{\text{bilayer}}$ that are important for protein function.

How can one determine whether amphiphiles, for example, regulate membrane protein function in biological systems by altering bilayer physical properties? It is possible to do so by using gA channels as molecular force transducers to measure the net effects of amphiphiles on the bilayer elastic response to a change in
hydrophobic length of an embedded protein. Given the underlying complexities, it is useful to summarize such changes as changes in bilayer stiffness.

Operationally, we define a change in bilayer stiffness as a change in bilayer physical properties that, at constant bilayer thickness, alters \( F_{\text{dis}} \) (the bilayer disjoining force acting on a G protein channel; Lundbæk et al. 1996, 2005). A decrease in stiffness means that \( F_{\text{dis}} \) (and \( \Delta G_{\text{bilayer}} \)) is decreased. This is observed as increases in both \( f \) and \( \tau \) (provided other factors affecting the monomer ↔ dimer kinetics remain constant). Conversely, an increase in stiffness means that \( F_{\text{dis}} \) (and \( \Delta G_{\text{bilayer}} \)) is increased, which is observed as decreases in both \( f \) and \( \tau \).

The use of bilayer stiffness as a descriptor of the changes in bilayer properties that regulate protein function implies that the regulation is an energetic question that to a first approximation can be addressed using the continuum theory of elastic bilayer deformations with minimal chemical specificity (Andersen et al. 1998).
The situation is similar to that for electrified interfaces, where the Gouy–Chapman theory of the diffuse double layer serves as a major organizing principle (e.g. McLaughlin 1989), with the Debye length being a major descriptor of the electrostatic interactions in the system.

### 2.11. Bilayer stiffness and membrane protein function in living cells

The functions of N-type calcium channels (NCCs) and voltage-dependent sodium channels (VDSCs) in mammalian cells have been studied using whole-cell voltage clamp (Lundbæk et al. 1996, 2004, 2005). Amphiphiles that decrease bilayer stiffness also, reversibly, promote NCC and VDSC inactivation. This is observed as a hyperpolarizing shift in the membrane potential causing 50 per cent channel inactivation ($V_n$). Figure 9 shows the relation between changes in $V_n$ for VDSCs and gA channel lifetime (where $\ln\{\tau/\tau_{\text{ref}}\}$ is a measure of the change in $F_{\text{gA}}$) for five structurally different amphiphiles.

At low amphiphile concentrations, the $V_n - \ln\{\tau/\tau_{\text{ref}}\}$ relations for the different compounds are very similar, indicating that both membrane environments respond similarly to the adsorption of the amphiphiles. Whereas manoeuvres that decrease bilayer stiffness promote inactivation of both NCCs and VDSCs, cholesteral, which increases bilayer stiffness, inhibits NCC inactivation (Lundbæk et al. 1996)—and cholesterol removal promotes VDSC inactivation (Lundbæk et al. 2004). Similarly, amphiphiles that decrease bilayer stiffness promote GABA$_A$ receptor desensitization and muscimol binding, whereas cholesterol promotes muscimol binding (Søgaard et al. 2006).

The correlation between amphiphile-induced changes in bilayer stiffness and protein function can be extended to other membrane proteins. Table 4 shows results obtained with six different channel types. Morris & Juranka (2007) provide additional information about the modulation of VDSCs by bilayer-modifying agents. In all cases, the nominal amphiphile concentrations used in the gA-based bilayer stiffness measurements are equal to or lower than those that regulate the biological channels.

In the case of Triton X-100 and β-octyl glucoside, the changes in channel function occur at concentrations that are one to two orders of magnitude less than their critical micellar concentrations, indicating that the changes in function occur at amphiphile mole-fractions in the membrane of 0.01–0.1. (Amphiphile mole-fractions in lipid bilayers scale as the aqueous concentration relative to the critical micellar concentration (Heerklotz & Seelig 2000)). There is less information about membrane concentrations of the other compounds, and one cannot exclude that specific mechanisms, such as displacement of boundary lipids, may be involved in some cases. Indeed, the fatty acid effects on ligand-activated channels may reflect, in part, the adsorption (or accumulation) of fluid-phase fatty acids at the protein–bilayer boundary (Andreassen & McNamara 1980; Antollini & Barrantes 2002). The overall results, however, are unlikely to reflect solely specific interactions. It will be a challenge to determine the relative importance of specific versus non-specific interactions, and the separation is unlikely to be straightforward because a protein-induced bilayer deformation in its own right may alter the local amphiphile concentration near the protein (cf. Bruno et al. 2007).

#### 2.12. Bilayer stiffness, fluidity, monolayer curvature and lateral pressure profile

The correlation between amphiphile-induced changes in bilayer stiffness measured in planar lipid bilayers and membrane protein function in living cells is surprisingly simple—and likely to break down at some point. Nevertheless, the correlations observed in figure 9 and table 4 strongly indicate that bilayer stiffness measurements report properties of lipid bilayers that are of general importance for membrane protein function. It further suggests that gA-based measurements of bilayer stiffness provide reliable information about the net effect of changes in bilayer composition on the bilayer elastic response to membrane protein conformational changes.

Historically, a number of descriptors have been used to describe (and explain) non-specific regulation of membrane protein function by the lipid bilayer (table 5); for recent reviews, see Lundbæk (2006) and Andersen & Koepepe (2007).

The first such descriptor was ‘bilayer fluidity’ (Linden et al. 1973). The consequences of altered bilayer composition continue to be ascribed to changes in bilayer ‘fluidity’, as evaluated using electron spin resonance probes, such as TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl; Shimshick & McConnell 1973), or fluorescent probes, such as diphenylhexatriene.
Fatty acids decrease the single-channel lifetimes and shorten burst durations, it is not clear whether these changes reflect of lipid bilayers. In cases where protein functional involve manipulations that alter the elastic properties Studies on membrane protein function in cells may alter bilayer properties

Table 4. Effects of amphiphiles on bilayer stiffness and ion channel function. The effects of structurally different amphiphiles on bilayer stiffness measured using gA channels and on VDSCs; NCCs; Ca2+-activated potassium channels (BKCa); nicotinic acetylcholine receptors (nAChR); and GABA_A receptors. Modified from Søgaard et al. (2006).

<table>
<thead>
<tr>
<th>Amphiphile</th>
<th>Bilayer stiffness (using gA channels)</th>
<th>Voltage-dependent channels</th>
<th>Cys-loop receptors</th>
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<td></td>
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<td>VDSC</td>
<td>NCC</td>
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<td></td>
<td>activation</td>
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<td>Positive c_0</td>
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<td>Triton X-100</td>
<td>↓^b</td>
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<td>β-octyl glucoside</td>
<td>↓^b</td>
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<td>Pentobarbital</td>
<td>↓^g</td>
<td>0^g</td>
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<td>Negative c_0</td>
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<td>Capsaicin</td>
<td>↓^1</td>
<td>0^j</td>
<td>↑^j</td>
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<td>Docosahexaenoic acid</td>
<td>↓^n</td>
<td>0^k</td>
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<td>Arachidonic acid</td>
<td>↓^m</td>
<td>0^l</td>
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<td>Oleic acid</td>
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<td>Cholesterol</td>
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*a Fatty acids decrease the single-channel lifetimes and shorten burst durations, it is not clear whether these changes reflect enhanced desensitization.

(b) (DPH; Shinitzky & Barenholz 1978). But, though a liquid-crystalline bilayer organization is likely to be required for membrane protein function (to allow for protein conformational changes), the causal relation between bilayer ‘fluidity’ and protein function has never been clear—in part because probes such as DPH report a combination of acyl chain dynamics and order (Lentz 1993). Moreover, the equilibrium distribution between conformational states of a (bilayer-embedded) protein cannot be altered by a change in ‘fluidity’, per se (Schurr 1970; Lee 1991).

Changes in membrane protein function often correlate with changes in lipid intrinsic curvature, c_0 (cf. Andersen & Koeppe 2007). Lipid bilayer regulation of function therefore is often interpreted in terms of changes in c_0, or changes in bilayer lateral pressure profile (Cantor 1997, 1999), which have equivalent effects on the conformation of an embedded protein (Andersen & Koeppe 2007; Marsh 2007). Such unimodal interpretations, however, do not encompass the consequences of changes in bilayer molecular composition that also alter the bilayer elastic moduli. Nor do they fully describe the interplay between changes in hydrophobic mismatch and curvature, or the curvature-dependent changes in bilayer thickness. Neither the effects of amphiphiles on gA channels (figure 8), nor on a number of other ion channels (table 4), correlate with changes in just c_0.

2.13. Amphiphile-regulation of membrane proteins—detecting bilayer-mediated effects

Studies on membrane protein function in cells may involve manipulations that alter the elastic properties of lipid bilayers. In cases where protein functional changes are induced by high nanomolar to micromolar (and higher) amphiphile concentrations, for example, it becomes prudent to consider whether some (or all) of the effects could result from the hydrophobic coupling between bilayers and their embedded proteins. It is therefore important to have experimental tests to determine whether amphiphile-induced changes in membrane protein function could be bilayer-mediated. Several such tests have been developed.

— Can the changes in protein function be reproduced by structurally very different amphiphiles known to alter bilayer stiffness, e.g. capsaicin or Triton X-100 (Smith & Proks 1998; Lundbæk et al. 2004, 2005; Søgaard et al. 2006; Matta et al. 2007)?

— At the concentrations used, does the amphiphile regulate the function of other, structurally unrelated bilayer-embedded proteins (including gA channels), suggesting that they may alter bilayer properties

Table 5. Descriptors of bilayer regulation of membrane protein function.

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<td>Bilayer fluidity</td>
<td>Linden et al. 1973</td>
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<td>Bilayer coupling</td>
<td>Sheetz &amp; Singer 1974</td>
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<td>Hydrophobic mismatch/bilayer compression</td>
<td>Mouritsen &amp; Bloom 1984</td>
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<td>Intrinsic lipid curvature</td>
<td>Gruner 1985</td>
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<td>Bilayer deformation energy</td>
<td>Huang 1986</td>
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<td>Bilayer free volume</td>
<td>Mitchell et al. 1990</td>
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<tr>
<td>Acyl chain packing</td>
<td>Fattal &amp; Ben-Shaul 1993</td>
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<td>Bilayer stiffness</td>
<td>Lundbæk et al. 1996</td>
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<td>Curvature frustration</td>
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<td>Lateral pressure profile</td>
<td>Cantor 1997</td>
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<td>Lipid packing stress</td>
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J. R. Soc. Interface (2010)
3. GRAMICIDIN CHANNELS AS MOLECULAR FORCE PROBES—A QUANTITATIVE DESCRIPTION

To obtain a more thorough understanding of gA channels as tools to explore changes in bilayer properties, it is necessary to develop a more physical/mathematical description. In the following, we describe the relations between channel energetics, kinetics and function, and the consequences of changes in bilayer physical properties. For more complete descriptions, the reader should consult Lundbæk (2006) and Andersen et al. (2007a).

3.1. Channel energetics, kinetics and function

The kinetics of gA channel formation/dissociation can be described as

$$M + M \xrightarrow{k_1} D \quad \text{(scheme 1)},$$

where M and D denote gA monomers (one in each leaflet) and bilayer-spanning dimers, conducting channels (Bamberg & Läuger 1973; Veatch et al. 1975), and $k_1$ and $k_{-1}$ are the association and dissociation rate constants.

The channel dimerization constant ($K_D$) is given by

$$K_D = \frac{k_1}{k_{-1}} = \frac{[D]}{[M]^2} = \exp \left\{ -\frac{\Delta G_{\text{tot}}^{M-D}}{k_B T} \right\},$$

(3.1)

where the bilayer concentrations of M and D are in moles per unit area, and $\Delta G_{\text{tot}}^{M-D}$ is the free energy difference for channel formation. All gA dimers seem to be conducting channels (Veatch et al. 1975); therefore, changes in $n$, the time-averaged number of channels per unit membrane area (cf. equation (2.2)), can be determined from changes in the (time-averaged) membrane conductance.

The relation between $k_1$, $f$ and the activation energy for subunit association ($\Delta G_{\text{prot}}^{M-D}$) becomes

$$k_1 = \frac{f}{[M]^2} = f_0 \exp \left\{ -\frac{\Delta G_{\text{prot}}^{M-D}}{k_B T} \right\},$$

(3.2)

where $f_0$ is a frequency factor for the reaction. The corresponding relation between $k_{-1}$, $\tau$ and the activation energy for dimer dissociation ($\Delta G_{\text{halayer}}^{M-D}$) is given by

$$k_{-1} = \frac{1}{\tau} = f_0 \exp \left\{ -\frac{\Delta G_{\text{halayer}}^{M-D}}{k_B T} \right\}.$$

(3.3)

3.2. Channel energetics and bilayer deformation energy

The contribution from the bilayer deformation energy to the energetics and kinetics of channel formation can then be expressed using the relations (cf. equation (2.1))

$$\Delta G_{\text{tot}}^{M-D} = \Delta G_{\text{prot}}^{M-D} + \Delta G_{\text{halayer}}^{M-D}$$

(3.4)

and

$$\Delta G_{\text{tot}}^{M-D} = \Delta G_{\text{tot}}^{M-D} - \Delta G_{\text{tot}}^{D-M},$$

(3.5)

where

$$\Delta G_{\text{tot}}^{M-D} = \Delta G_{\text{prot}}^{M-D} + \Delta G_{\text{halayer}}^{M-D}$$

(3.6)

$$\Delta G_{\text{tot}}^{M-D} = \Delta G_{\text{prot}}^{M-D} + \Delta G_{\text{halayer}}^{M-D},$$

(3.7)

such that

$$\Delta G_{\text{tot}}^{M-D} = (\Delta G_{\text{prot}}^{M-D} - \Delta G_{\text{prot}}^{D-M}) + (\Delta G_{\text{halayer}}^{M-D} - \Delta G_{\text{halayer}}^{D-M}).$$

(3.8)

where $\Delta G_{\text{halayer}}^{M-D}$, $\Delta G_{\text{halayer}}^{M-D}$ and $\Delta G_{\text{halayer}}^{M-D}$ denote the changes in bilayer deformation energy associated with adjusting the bilayer thickness from $d_0$ (the unperturbed thickness) to $l$ (the channel length), from $d_0$ to $(l + \delta)$, and from $l$ to $(l + \delta)$, respectively, where $\delta$ (approx. 0.16 nm; Durkin et al. 1993; Lundbæk & Andersen 1999; Miloshevsky & Jordan 2004) is the subunit separation at the transition state for channel formation/dissociation.

3.3. Bilayer deformation energy and channel energetics, kinetics and function

Changes in bilayer physical properties that alter the bilayer contribution to $\Delta G_{\text{tot}}^{M-D}$ can be evaluated by combining equations (3.1) and (3.4) (assuming that the experimental manipulation does not alter $\Delta G_{\text{prot}}^{M-D}$):

$$\Delta \Delta G_{\text{halayer}}^{M-D} = -k_B T \ln \left\{ \frac{K_D}{K_D^{\text{ref}}} \right\}$$

$$= -k_B T \ln \left\{ \frac{[D]/[M]^2}{[D]^{\text{ref}}/[M]^2_{\text{ref}}} \right\}$$

$$\approx -k_B T \ln \left\{ \frac{[D]}{[D]_{\text{ref}}} \right\},$$

(3.9)

where the subscript ‘ref’ denotes the reference state. The last relation becomes exact in the limit when $[M]_{\text{ref}} = [M]$ (Lundbæk & Andersen 1994). It thus becomes possible to estimate $\Delta \Delta G_{\text{halayer}}^{M-D}$ from $[D]/[D]_{\text{ref}}$, which can be estimated from the relative changes in membrane conductance, or time-averaged channel activity ($n = fr$).
Similarly for $\Delta G_{\text{bilayer}}^{4\text{M} \rightarrow \text{D}}$ and $\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}}$:

$$\Delta G_{\text{bilayer}}^{4\text{M} \rightarrow \text{D}} = -k_0 T \ln \left\{ \frac{k_1}{k_{1\text{,ref}}} \right\} = -k_0 T \ln \left\{ \frac{f/M_1^2}{f_{\text{ref}}/M_1} \right\} \approx -k_0 T \ln \left\{ \frac{f}{f_{\text{ref}}} \right\},$$

(3.10)

$$\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}} = -k_0 T \ln \left\{ \frac{k_{1\text{,ref}}}{k_1} \right\} = k_0 T \ln \left\{ \frac{\tau}{\tau_{\text{ref}}} \right\},$$

(3.11)

and using equation (3.5):

$$\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}} = -k_0 T \ln \left\{ \frac{k_1}{k_{1\text{,ref}}} \right\} - \ln \left\{ \frac{k_{1\text{,ref}}}{k_1} \right\} \approx -k_0 T \ln \left\{ \frac{f \tau}{f_{\text{ref}} \tau_{\text{ref}}} \right\}. $$

(3.12)

One can thus estimate $\Delta G_{\text{bilayer}}^{4\text{M} \rightarrow \text{D}},$ $\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}},$ and $\Delta G_{\text{bilayer}}^{4\text{M} \rightarrow \text{D}}$ from changes in $\ln(f)$ and $\ln(\tau).$ Similar monitoring of the energetics of elastic bilayer deformations for well-described changes in a bilayer-spanning inclusion (protein) is, at present, not possible for other systems.

### 3.4. Channel function and channel–bilayer mismatch

We are now in a position where we can compare experimental relations between lipid bilayer physical properties and gA channel function with quantitative predictions based on the continuum theory of elastic bilayer deformations. We first address the relation between changes in bilayer physical properties and the monomer --- dimer equilibrium (scheme 1). The relation between $\Delta G_{\text{bilayer}}^{4\text{M} \rightarrow \text{D}}$ and the bilayer physical properties is given by (cf. equation (2.6))

$$\Delta G_{\text{bilayer}}^{4\text{M} \rightarrow \text{D}} = \Delta G_{\text{diss}}^{4\text{D}} - \Delta G_{\text{diss}}^{4\text{M}} = H_B(l - d_0)^2 + H_X(l - d_0) c_0,$$

(3.13)

where we use that $\Delta G_{\text{diss}}^{4\text{M}} = -H_B c_0, \Delta G_{\text{diss}}^{4\text{D}}$ thus is a function of $H_B$ and $H_X,$ as well as $(l - d_0)$ and $c_0.$ We would expect that effects of changes in bilayer elasticity (reflected in $H_B$ and $H_X$) or $c_0$---as reported by changes in $[D]$---will depend on the hydrophobic mismatch $(l - d_0),$ which can be manipulated in experiments using thicker bilayers (increasing $d_0$) or shorter channels (decreasing $l.$) That is, the change in $[D]$ should be larger for larger values of $|l - d_0|.$ This has been observed in most (Lundbaek et al. 2005; Artigas et al. 2006; Bruno et al. 2007), but not all (Ashrafuzzaman et al. 2006) systems investigated so far.

The relation between $\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}},$ as reported by $\tau,$ and $d_0$ (or rather $d_0 - l$) is then obtained by noting that gA channel dissociation involves the separation of the two subunits by a distance $\delta$ (approx. 0.16 nm; Durkin et al. 1993; Lundbaek & Andersen 1999; Miloshevsky & Jordan 2004). Using equation (2.6), we find that $\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}},$ the difference in bilayer deformation energy in the transition state relative to the conducting dimer state is

$$\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}} = H_B((l + \delta - d_0)^2 - (l - d_0)^2) + H_X((l + \delta - d_0) - (l - d_0)) c_0,$$

$$= 2H_B \delta(l - d_0) + \delta/2) + H_X c_0 \approx -\delta(2H_B(d_0 - l) - H_X c_0).$$

(3.14)

$\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}},$ and thus $\ln(\tau),$ therefore should be linear functions of $d_0 - l$ (cf. equations (3.7) and (3.11)), which is the case (Lundbaek & Andersen 1999; see also figure 6). Moreover, in nominally hydrocarbon-free bilayers, the slope of the $\ln(\tau) - d_0$ relation is remarkably close to predictions based on the theory of elastic bilayer deformations (equation (2.5)) using independently measured values of the bilayer elastic moduli (Lundbaek & Andersen 1999). Though one should question the range of validity of continuum models when analysing molecular events, the continuum description of elastic bilayer deformations seems to provide a useful foundation for analysing the energetic coupling between membrane proteins and their host bilayers.

### 3.5. Channel function and $c_0$

The relative clarity that pertains to the consequences of changing $d_0$ extends also to changes in $c_0,$ as long as $c_0$ is altered by changes in head group interactions of the bilayer-forming lipid. That is, a negative-going change in $c_0$ increases $F_{\text{diss}}$ and $\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}}$ (Lundbaek et al. 1997; Maer et al. submitted; see also Andersen & Koepe 2007). If the changes in $c_0$ arise from changes in the bilayer-forming lipids that involve the bilayer hydrophobic core, such as increasing acyl chain unsaturation, the situation becomes murky. In this case, the lifetime changes tend to be in a direction opposite to that predicted from the expected changes in $c_0$ (Girshman et al. 1997), maybe because increasing acyl unsaturation will tend to thin lipid bilayers (Lewis & Engelman 1983) and reduce the bilayer bending moduli (Rawicz et al. 2000)—changes that in their own right will tend to reduce $\Delta G_{\text{bilayer}}^{4\text{D} \rightarrow \text{M}},$ and that may dominate over the $c_0$-dependent increase.

If $c_0$ is altered by small amphiphiles that adsorb to lipid bilayers, rather than by changing the bilayer-forming lipid, the curvature-dependent effects also tend to be less important (Lundbaek et al. 2005; Bruno et al. 2007), as would be expected from the thermodynamics of solute adsorption to the bilayer–solution interface (Evans et al. 1995; Zhelev 1998). Compounds that cause either negative or positive changes in $c_0$ therefore may have similar effects on gA channel function, as well as on the function of integral membrane protein channels (table 4). As noted earlier, this is because reversibly adsorbing amphiphiles decrease $K_T$ and $K_c$ (and thereby $H_B$ and $H_X$; cf. Evans et al. 1995; Zhelev 1998; Ly &
Longo 2004; Zhou & Raphael 2005), which would tend to decrease $\Delta G_{\text{prot}}^{D-M}$ and $F_{\text{dis}}$. If the decrease in the $H_B(l - d_0)^2$ term in equation (3.13) dominates the curvature-dependent increase in $H_X(l - d_0)\xi_0$ term, the changes in (membrane protein) function will deviate from predictions based solely on changes in $c_0$.

3.6. Separating effects of changes in $c_0$ versus changes in elastic moduli

The regulation of membrane protein function by changes in lipid bilayer physical properties has been extensively investigated—usually in terms of isolated changes in $c_0$, thickness or, maybe, elastic moduli. As noted previously, these unimodal interpretations may be misleading; it is important to have methods that discriminate between consequences of changes in $c_0$ versus bilayer elastic moduli.

$gA$ channels can be used for this purpose because, when the bilayer physical properties are altered, the resulting changes in $\tau$ can be separated into a term that depends on $(d_0 - l)$ and one that depends on $c_0$. Using equation (3.14) (and assuming that $\Delta G_{\text{prot}}^{D-M}$ remains invariant):

$$
\frac{\tau}{\tau_{\text{ref}}} = \exp\left\{ \frac{82(H_B(l - d_0) - H_{B,\text{ref}}(d_{0,\text{ref}} - l))}{k_B T} \right\} \times \exp\left\{ -\frac{\delta(H_Xc_0 - H_{X,\text{ref}}c_{0,\text{ref}})}{k_B T} \right\}.
$$

Changes in $\tau$ that are owing to altered bilayer elasticity ($H_B$ and $H_X$) thus should vary as a function of $(d_0 - l)$, whereas changes owing to altered $c_0$ should be impervious to changes in $(d_0 - l)$ (Maer et al. submitted). This distinction can be observed experimentally by comparing the effects of altered bilayer composition on $\tau$ for $gA$ channels formed by analogues of different length (e.g. 13 and 15 amino acids; Maer et al. submitted). In cases where $H_B$ is decreased, $\tau/\tau_{\text{ref}}$ will be larger for the shorter channels relative to the longer channels (Lundbæk et al. 2005; Bruno et al. 2007; Ingólfsson et al. 2007; Maer et al. submitted). When only $c_0$ is altered, $\tau/\tau_{\text{ref}}$ should be the same for the longer and shorter channels (Artigas et al. 2006; Ashrafuzzaman et al. 2006; Maer et al. submitted).

The quantitative relation between difference in channel length and changes in $\tau$ and $H_B$ may be expressed as

$$
\frac{\tau_2}{\tau_{2,\text{ref}}} = \exp\left\{ \frac{82((H_B - H_{B,\text{ref}})(l_2 - l_1))}{k_B T} \right\},
$$

where the subscripts ‘1’ and ‘2’ refer to the two different $gA$ channels (Maer et al. submitted). It thus becomes possible to evaluate whether an experimental manoeuvre alters bilayer elasticity, as expressed through $H_B$, in experiments with $gA$ channels of different lengths. This approach thus provides a means for resolving some of the persisting uncertainties in the bilayer regulation of membrane protein function.

4. CONCLUSION

This review has focused on non-specific regulation of membrane protein function by bilayer physical properties. The chemical heterogeneity of biological membranes and the complexity of protein–bilayer boundaries continue to pose barriers to a mechanistic understanding, but the hydrophobic coupling mechanism provides an organizing principle for exploring the energetic coupling between membrane proteins and their host bilayer. Moreover, the continuum theory of elastic bilayer deformation provides a foundation for how to relate events at the molecular level to changes in bilayer physical properties.

In many cases the regulation of protein function also depends on more specific lipid–protein interactions (Lee 2005; Marsh 2008; Suh & Hille 2008), and it may be difficult to determine the relative importance of lipid–protein and bilayer–protein interactions. Even when the regulation is due to bilayer–protein interactions, the lipid composition near a protein may differ from the bulk composition owing to constraints on the lipid packing around the protein (Sperotto & Mouritsen 1993). The bilayer control of membrane protein function, however, is an energetic question that to a first approximation can be approached using the continuum theory of elastic bilayer deformations.

One must be wary of using continuum arguments at the molecular level, but they provide a surprising ability to predict the direction and even magnitude of changes in function. In this context, $gA$ channels have proven useful because they are sufficiently simple to allow for a quantitative analysis of the channel–bilayer coupling. $gA$ channels also have proven useful as probes for changes in bilayer properties, expressed as changes in bilayer stiffness. Reducing the complexity of the lipid bilayer, and the bilayer–protein interactions, to just one descriptor is a major simplification (a sort of ultimate ‘coarse graining’ in molecular modelling terms).

This simplicity is both a strength, because it is a robust measure, and a weakness, because of the limitations of the continuum description. To explore these limitations, it is important to push the $gA$ system to the point where these limitations become evident, as already has been done in the case of the reversibly adsorbing amphiphiles. It also will be important to develop systems with more complex functions, which allow for a similar level of quantitative analysis and thereby allow further explorations of the limitations of the continuum description. Yet, the intuitive simplicity provided by the theory of elastic bilayer deformations is likely to continue to serve as an organizing principle for understanding the energetic coupling between membrane proteins and their host bilayer.

This work was supported in part by the National Institutes of Health grant GM21342 (O.S.A.) and the Josiah Macy Jr Foundation (O.S.A.). We thank Kevin Lum, Radda Rusinova and an anonymous reviewer for incisive comments on the manuscript.
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