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Protein-induced bilayer perturbations: Lipid ordering and hydrophobic coupling

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ABSTRACT

The host lipid bilayer is increasingly being recognized as an important non-specific regulator of membrane protein function. Despite considerable progress the interplay between hydrophobic coupling and lipid ordering is still elusive. We use electron spin resonance (ESR) to study the interaction between the model protein gramicidin and lipid bilayers of varying thickness. The free energy of the interaction is up to −6 kJ/mol; thus not strongly favored over lipid–lipid interactions. Incorporation of gramicidin results in increased order parameters with increased protein concentration and hydrophobic mismatch. Our findings also show that at high protein:lipid ratios the lipids are motionally restricted but not completely immobilized. Both exchange on and off rate values for the lipid ↔ gramicidin interaction are lowest at optimal hydrophobic matching. Hydrophobic mismatch of few Å results in up to 10-fold increased exchange rates as compared to the 'optimal' match situation pointing to the regulatory role of hydrophobic coupling in lipid–protein interactions.

Introduction

The amphipathic character of membrane spanning proteins and lipid molecules implies that in order to avoid the high energetic cost of exposing hydrophobic moieties to water [1], lipids and proteins self-assemble in order to minimize this energetic cost [2]. Understanding of this assembly process and its implications for protein function and stability is important not only in basic research on membrane proteins but also in the development of biomimetic membranes [3,4].

The interaction between lipids and membrane proteins is generally only slightly stronger than the interactions between lipids themselves [5,6] suggesting an important role for unspecific lipid–protein hydrophobic interactions. The hydrophobic lipid–protein coupling can to a first approximation be regarded as an energetic consequence of the adaptability of the (soft) lipid bilayer hydrophobic thickness to the hydrophobic length of (rigid) transmembrane proteins and has been formulated in the 'mattress model' for lipid–protein interactions [7]. A generalization of this picture implies that whenever protein function involves conformational changes in the proteins hydrophobic exterior, the lipid bilayer will deform to adapt to the protein hydrophobic length. Conversely a change in lipid bilayer material properties (e.g. hydrophobic thickness) will affect protein conformational equilibrium and hereby function [8–10]. As lipid–protein interactions can be described in energetic terms, it is important to understand the various energy components associated with the total free energy for the membrane protein conformational equilibrium [11–14].

A well-suited model system is gramicidin channels incorporated into lipid bilayers [15]. Gramicidin membrane spanning ion channels are formed by association of two β-helical 15 amino acid monomers each having four interfacial Trp residues. This arrangement results in a transmembrane protein with a hydrophobic length l of about 22 Å spanning the bilayer [15,16]. Whenever gramicidin channels are incorporated in bilayers having a hydrophobic thickness d_0 larger than the hydrophobic length of the gramicidin dimer channel there will be a hydrophobic mismatch and this mismatch will lead to local protein-induced bilayer deformation, i.e. lipid monolayer bending and compression [8–10] (see Fig. 1). The ensuing bilayer deformation energy can be quantified by measuring the stability of the gramicidin dimer using ion channel electrophysiological methods [11,15,17].

Energetic analysis reveals a significant dependence on the packing of lipids in the vicinity of the protein [9]. In particular for gramicidin induced deformation (i.e. whenever $d_0 \neq l$) continuum elastic models predict that less than 50–100 lipids (in addition to the 20 boundary lipids that can 'fit' around the gramicidin dimer channel) are involved in the deformation [9]. Depending on the assumptions one makes about how the boundary lipids are

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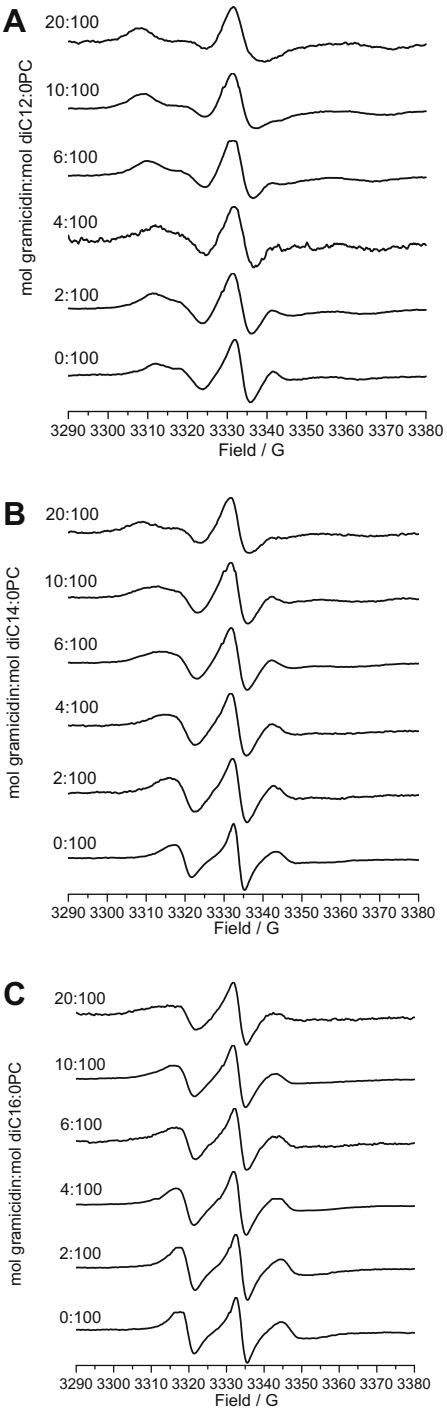

Fig. 1. ESR spectra of 10-doxyl PC in lipid (1:100) in gramicidin:lipid mixtures ranging from 0 to a lipid:protein ratio of 100:20 with excess water at 10 K above the main T_m for each lipid. (A) diC12:0PC at 281 K. (B) diC14:0PC at 307 K. (C) DiC16:0PC at 325 K.

organized the deformation energy can vary several kT, which is sufficient to affect protein stability and function [9,18]. It is therefore of interest to characterize the behavior of lipids in the vicinity of the inclusion.

Spin-label electron spin resonance (ESR) spectroscopy is a powerful tool for analyzing the dynamics of boundary and bulk (i.e. unperturbed by the inclusion) lipids [19,20]. Using ESR it has been shown that gramicidin alters the acyl-chain organization [21,22] and that it is possible to distinguish between boundary lipids from bulk lipids and to observe peptide aggregation and H_{II} phase formation [23,24]. The existence of boundary lipids has been linked to the broad components in ESR spectra of chain-labeled lipids in lipid–protein dispersions [25–30]. The fundamental view is that as the rotational diffusion rates approach the rigid limit the lipids are considered to be immobilized by the protein [29]. Despite experimental progress, discrepancies between the data from ESR and nuclear magnetic resonance (NMR) experiments show that it is still not entirely clear how lipid organization behavior is modulated by protein inclusions and vice versa [23,26,29,31,32].

Here we use ESR of spin-labeled lipids to investigate gramicidin–lipid interactions. In particular we investigate the dependence of the maximum outer hyperfine splitting, lipid exchange rates, and affinity on gramicidin:lipid molar ratios and hydrophobic mismatch. We used lipids of varying hydrophobic thickness d_0: dilauroylphosphatidtylcholine (diC14:0PC) with $d_0 = 20.9$ Å [33], dimyristoylphosphatidylcholine (diC14:0PC) with $d_0 = 25.4$ Å [33], and dipalmitoylphosphatidylcholine (diC16:0PC) with $d_0 = 28.6$ Å [34] doped with the same spin label: 1-palmitoyl-2-stearoyl-10-doxyl-PC. Although structurally closest to diC16:0PC, palmitoyl-stearoyl-PC ESR probes partition into the L_α phase of diC12:0PC [35] and accurately monitors the main phase transition temperature T_m in diC14:0PC [36]. We investigate all systems at 10 °C above T_m with the aim to characterize lipid ordering as a function of hydrophobic mismatch and gramicidin molar ratio.

Materials and methods

Materials. 1,2-Dilauryol-*sn*-glycero-3-phosphatidylcholine (diC12:0-PC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (diC14:0-PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl-choline (diC16:0-PC), and the spin-label 1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine-doxyl (10-doxyl PC) were all from Avanti Polar Lipids (Alabaster, AL, US). Lipid dispersions were prepared using standard protocols [37] to a final aqueous concentration of 10 mg/ml.

Data collection. Up to a few microliters of the vesicle suspensions were placed in 1.5–1.8 mm outer diameter vitrex capillary tube (Bie and Berntsen A–S) by capillary force. The tube was then sealed at both ends with an open flame and placed in the center of the spectrometer cavity. ESR spectra were recorded on a Bruker EMX spectrometer (Bruker, Billerica, MA, US) equipped with an X-band (9.35 GHz) microwave bridge with a modulation-amplitude of 1 G. To improve the signal-to-noise ratio, up to five scans were accumulated. A time constant of 2621 ms was chosen for a recording at 0.15 G/s. A Bruker Eurotherm B-VT2000 was used to control the sample temperature. Air was used as a temperature regulation medium for temperature above room temperature and nitrogen otherwise.

Analysis. Data were analyzed and displayed using OriginPro 8 (OriginLab Corporation, Northampton, MA). Spectral outer and inner hyperfine splittings were determined by fitting maxima and minima to Gaussians and calculating the field difference between extrema. Spectral analysis was done according to [38] and [39]. Apparent lipid acyl-chain order parameters were calculated as

$$S^{eff} = 0.5407(A_{\parallel} - A_{\perp})/a_0 \quad (1)$$

where $A_{\parallel} = A_{max}$ is determined from the maximal outer ^{14}N-hyperfine splitting in the spectra and $A_{\perp} = A_{min}$ is the inner hyperfine splitting in the spectra. The effective isotropic ^{14}N-hyperfine coupling constant is given as

$$a_0 = \frac{1}{3}(A_{\parallel} + 2A_{\perp}) \quad (2)$$

In order to calculate lipid exchange on and off rates and affinities to gramicidin initially, the fraction of motionally restricted lipids f was calculated from fitting the spectra with a two-compo-

nent summed spectra using component spectra for the spin-labeled lipid in a pure lipid bilayer (the fluid component) and for the spin-labeled lipid in a peptide/lipid complex with a peptide to lipid ratio of 20:100 (the motionally restricted component) [6]. The exchange on rate τ_f^{-1} was determined from the first derivative spectral linewidths δH as

$$\delta H = \left(\frac{\hbar}{\sqrt{3} g \mu_B}\right)\tau_f^{-1} + \delta H_G \quad (3)$$

where g is the nitroxide g value, μ_B the Bohr magneton, and δH_G is the first derivative Gaussian linewidth for the fluid component without exchange [6], The exchange off rate τ_b^{-1} was determined using the exchange equilibrium relation

$$\frac{\tau_b^{-1}}{\tau_f^{-1}} = \frac{1-f}{f} \quad (4)$$

The average relative association constant K_r^{av} was determined from

$$\frac{1-f}{f} = \frac{\tau_b^{-1}}{\tau_f^{-1}} = \frac{n_t}{N_l K_r^{av}} - \frac{1}{K_r^{av}} \quad (5)$$

where n_t is the total number of lipids per protein (the lipid–protein ratio) and $N_l = 10$ is the number of annular lipids immediately surrounding the gramicidin peptide [6,9]. The effective average association constant was determined as

$$K_{eff}^{av} = \frac{\tau_{diff}^{-1}}{\tau_b^{-1}} \quad (6)$$

where the lipid exchange rate due to lateral diffusion τ_{diff}^{-1} for spin-labeled phosphatidylcholine is $7.5 \times 10^7\ s^{-1}$ [6,40].

Results and discussion

Gramicidin induced lipid ordering

The effect of gramicidin on the lipid chain mobility in diC12:0PC, diC14:0PC and diC16:0PC all 10 °C above their respective main phase transition temperature T_m was determined from ESR spectra of 10-doxyl PC (see Fig. 1). For all lipids the degree of motional averaging decreases in the ESR spectra of 10-doxyl PC in gramicidin/PC complexes with increasing gramicidin to lipid ratio. The position of the low field outer wing is downshifted in field with increased n_t for all lipids consistent with the findings for gramicidin in diC16:0PC [22]. None of the spectra show the characteristic 'growing in' of a new spectral feature typically ascribed to completely immobilized annular lipids in the spectra [22]. The central peak of the spectrum of 10-doxyl PC broadens with increased protein concentration in diC14:0PC (Fig. 1B) and diC16:0PC (Fig. 1C). The observed increased outer peak separation could be due to a decrease in rotational diffusion rate or from an increase in ordering (where ordering implies decreased acyl-chain motion) or both [22].

In order to gain further insight, we analyzed the outer hyperfine splitting constant A_{max} and order parameter S^{eff} in the ESR spectra of 10-doxyl PC in gramicidin/diC12:0PC, gramicidin/diC14:0PC and gramicidin/diC16:0PC complexes as a function of protein concentration and this is shown in Fig. 2. The change in A_{max} with increased protein concentration is consistent with an ordering effect of gramicidin by decreasing the degree of motional averaging of the anisotropy (see Fig. 2A). A_{max} as a function of protein concentration could be described by exponential fits for all three lipids. The A_{max} values in the ESR spectra of 10-doxyl PC in gramicidin/diC12:0PC, gramicidin/diC14:0PC and gramicidin/diC16:0PC complexes at any given protein concentration decreases with increased lipid chain length: A_{max} (diC12:0PC) > A_{max} (diC14:0PC) > A_{max} (diC16:0PC). However with the possible exception of diC12:PC at high protein concentration no clear saturation at high protein concentrations values was observed irrespective of lipid chain length and thus hydrophobic mismatch. Thus there appears to be no clear gramicidin–lipid binding stoichiometry (i.e. based on the intercept between the initial phase of linear increase in A_{max} with the plateau value of A_{max}).

The ordering effect of gramicidin in the three lipid types is mirrored in the order parameter S^{eff} (see Fig. 2B). The effect of gramicidin on ordering follows S^{eff} (diC12:0PC) > S^{eff} (diC14:0PC) > S^{eff} (diC16:0PC). For both A_{max} and S^{eff} the highest values are obtained for diC12:0PC where the hydrophobic thickness (20.9 Å) matches the hydrophobic length of the bilayer spanning gramicidin dimer (21.5 Å) [41] with modest changes in S^{eff} values as n_t is increased going from 0.62 without protein to 0.75 at n_t = 100:20. The formation of a gramicidin dimer in diC12:0PC does not require much deformation due to hydrophobic mismatch of the bilayer. Consequently the bilayer can accommodate free gramicidin monomers (and dimers) with no significant compression of the lipid acyl-chain region between lipids in bulk and annular lipids surrounding the gramicidin monomers. As the hydrophobic mismatch between the length of the gramicidin dimer and the host lipid increases, the S^{eff} values are generally lower and the effect of adding gramicidin is now much more pronounced. For the longer-chain diC16:0PC, S^{eff} increases more than 2-fold from 0.21 without protein to 0.57 at n_t = 100:20.

In order to investigate how the gramicidin induced order depends on temperature, S^{eff} was determined for 10-doxyl PC in pure diC12:0PC, diC14:0PC and diC16:0PC bilayers and in the presence of gramicidin at n_t = 100:4 and this is shown in Fig. 3. Generally S^{eff} decreases with temperature and the main phase transition is clearly visible as a sharp decrease in S^{eff} for diC14:0PC around 298 K and diC16:0PC around 313 K. For all lipids investigated $S^{eff}(T)$ for 10-doxyl PC approaches the same values above T_m described by a linear trend with $\Delta S^{eff}/\Delta T = -6.5 \times 10^{-3}$/K. The addition of gramicidin in the fluid phase increases S^{eff} of 10-doxyl PC in all lipids. For diC12:0PC the effect is modest whereas the effect of gramicidin addition to the longer acyl lipids is more pronounced. For diC14:0PC and diC16:0PC $S^{eff}(T)$ above T_m in the presence of gramicidin follows a similar linear trend as $S^{eff}(T)$ for the pure lipids with $\Delta S^{eff}/\Delta T = -7.7 \times 10^{-3}$/K. Thus the presence of gramicidin shifts the 'set point' for the $\Delta S^{eff}/\Delta T$ relation but preserves the temperature sensitivity for the motional restriction.

Gramicidin–lipid association

Both exchange on rate τ_f^{-1} and off rate τ_b^{-1} values (see Fig. 4 and Table 1) for the lipid ↔ gramicidin interaction are lower in diC12:PC as compared to diC14:0 and diC16:0PC consistent with optimal hydrophobic matching for gramicidin in diC12:0PC which will tend to maximize hydrophobic van der Waals interactions between lipids and gramicidin. τ_f^{-1} is maximal for gramicidin:diC14:0PC for all n_t values investigated and the values are ordered as: τ_b^{-1} (diC14 : 0PC) > τ_b^{-1} (diC16 : 0PC) > τ_b^{-1} (diC12 : 0PC) (see Fig. 4). τ_b^{-1} is maximal for gramicidin:diC14:0PC and gramicidin:diC16:0PC at low gramicidin concentration n_t < 4:100 (see Table 1). Thus hydrophobic mismatch leads to higher exchange rates at low protein concentration compared to the 'optimal' match situation consistent with the notion that the gramicidin induced bilayer deformation tend to destabilize the hydrophobic interaction between gramicidin and lipid.

The fraction of motionally restricted lipids varies between 24% and 60% corresponding to a range of restricted lipids per monomer between 10 and 14 (see Table 1). At low n_t this implies that the effective restriction only involves the first lipid annulus. This is

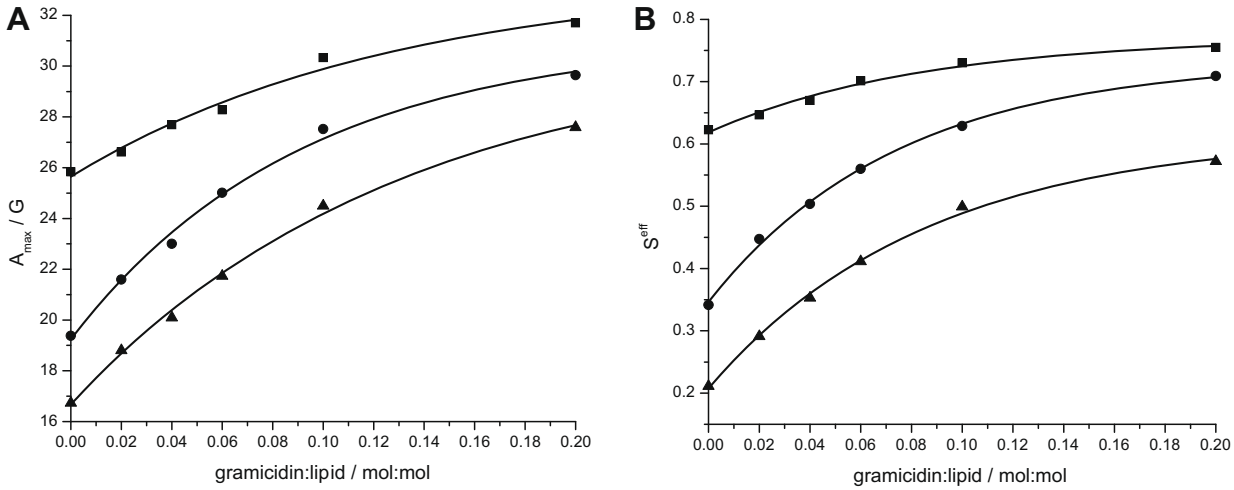

Fig. 2. (A) Outer hyperfine splitting constants A_{max} (Eq. (2)) in ESR spectra of 10-PC as function of n_t and lipid type: diC12:0PC (squares), diC14:0PC (circles), and diC16:0PC (triangles), all at T_m + 10 K. Lines represent exponential fits to the data points. (B) Effective order parameter (Eq. (1)) in ESR spectra of 10-PC as function of gramicidin:lipid ratio and lipid type all at T_m + 10 K. Lines represent exponential fits to the data points. Legend as in (A).

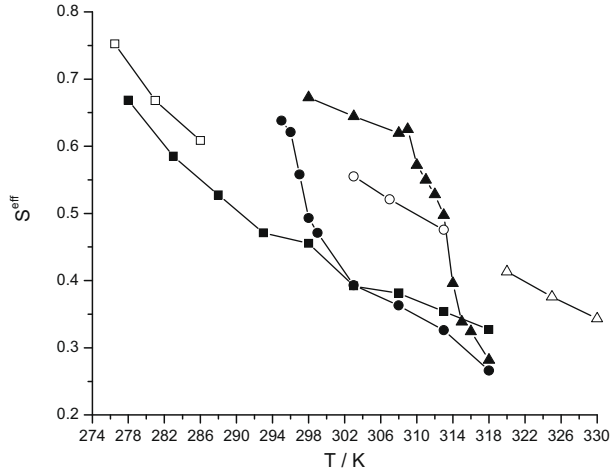

Fig. 3. Effective order parameter (Eq. (1)) in ESR spectra of 10-PC as function of temperature for n_t = 4:100 and lipid type: diC12:0PC (squares), diC14:0PC (circles), and diC16:0PC (triangles).

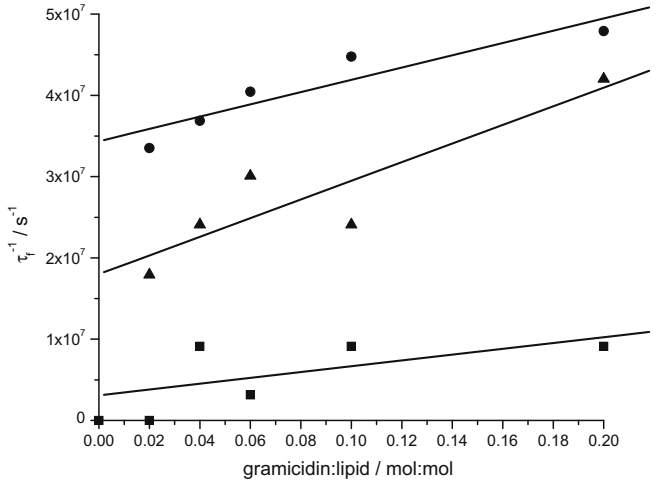

Fig. 4. Exchange on rate for 10-PC as a function of gramicidin:lipid ratio and lipid type: diC12:0PC (squares), diC14:0PC (circles), and diC16:0PC (triangles), all at T_m + 10 K, determined from Eq. (6). Lines represent linear fits to the data points.

Table 1
Exchange off rates τ_b^{-1}, fraction of motionally restricted lipids f and number of motionally restricted lipids per gramicidin monomer $f{\cdot}n_t$.

Lipid	n_t	τ_b^{-1}/s^{-1}	f	$f{\cdot}n_t$
diC12:0PC	100:2	n.d.	0.24	12
	100:4	9.9×10^6	0.48	12
	100:6	2.1×10^6	0.60	10
diC14:0PC	100:2	9.5×10^7	0.28	14
	100:4	3.4×10^7	0.56	14
	100:6	2.7×10^7	0.60	10
diC16:0PC	100:2	7.2×10^7	0.20	10
	100:4	3.6×10^7	0.40	10
	100:6	2.0×10^7	0.60	10

consistent with elastic continuum modeling showing that inclusion-induced deformation only involves lipids in the immediate vicinity of the protein [9]. At high gramicidin concentration the number of restricted lipids per gramicidin monomer saturates at 10 as there are vanishingly few bulk lipids to exchange with—virtually all lipids are annular (and motionally restricted) lipids.

For the average relative association constant K_r^{av} which is defined relative to the background lipid with which the spin label is competing for sites on the protein we generally find $K_r^{av} \geqslant 1$. Thus the gramicidin–lipid interaction tends to be stronger than the lipid–lipid interaction. K_r^{av} (diC14 : 0PC) $> K_r^{av}$ (diC12 : 0PC) $> K_r^{av}$ (diC16 : 0PC) for n_t = 100:2 and 100:4 but all $K_r^{av} < 2$ indicating that the gramicidin–lipid interaction is not strongly favored over lipid–lipid interaction. Both for diC14:0PC and diC16:0PC, $K_r^{av} > 1$, whereas in diC16:0PC, $K_r^{av} = 1$, for the three values of n_t investigated. This is consistent with the notion that sufficiently high hydrophobic mismatch will tend to destabilize the gramicidin–lipid interaction.

The effective average association constants K_{eff}^{av} (see Table 2) are between 0.8 and 7.6 for $n_t \leqslant$ 100:4 and this corresponds to a free energy of interaction of up to −6 kJ/mol [6]. The exception is for gramicidin in diC12:0PC at n_t = 100:6, where also S_{eff} may possibly begin to reach its asymptotic (saturated) level and possibly phase separation and/or lipid entrapment may take place.

The unusual gramicidin features, e.g. the β-helix fold and transmembrane dimerization may limit the extension of our findings. A more 'natural' model peptide could be single membrane spanning α-helices [42]. But we note that the bilayer induced

Table 2
Relative average association constants for the gramicidin–lipid interaction.

Lipid	K_r^{av}			K_{eff}^{av}		
n_t	100:2	100:4	100:6	100:2	100:4	100:6
diC12:0PC	1.3	1.4	1.0	n.d.	7.6	35.7
diC14:0PC	1.6	1.9	1.0	0.8	2.2	2.8
diC16:0PC	1.0	1.0	1.0	1.0	2.1	3.8

changes by helical peptides observed experimentally have been very small [43] or non-detectable [44]. This apparent adaptation may arise as a consequence of peptide tilt, proteins with larger cross-sectional areas (such as gramicidin and larger integral membrane proteins) do not have this positional freedom [13]. Indeed ESR studies on Ca^{2+} ATPase have revealed an optimal hydrophobic thickness for ATPase activity pointing to the importance of hydrophobic matching for protein function [45].

In conclusion we have demonstrated that gramicidin decreases lipid motional averaging (i.e. increases lipid order) where the effect increases with increased gramicidin–lipid hydrophobic mismatch and gramicidin concentration. In addition we have shown that the exchange on and off rates for the lipid–gramicidin association are correlated to the hydrophobic mismatch. The results suggest that even though the lipid binding appears rather non-specific, it can be regulated by bilayer material properties and this may have implications for understanding how the lipid bilayer regulate protein function.

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