ELSEVIER

Biophysical Chemistry 87 (2000) 87–102

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Correlation between the effects of a cationic peptide on the hydration and fluidity of anionic lipid bilayers: a comparative study with sodium ions and cholesterol

Roberto M. Fernandez, M. Teresa Lamy-Freund*

Instituto de Física, Universidade de São Paulo, CP 66318, CEP 05315-970, São Paulo, SP, Brazil

Received 16 March 2000; accepted 26 April 2000

Abstract

The cationic tridecapeptide α-melanocyte stimulating hormone (α-MSH) is known to interact with anionic vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), partially penetrating the lipid membrane. In the lipid liquid crystal phase, phospholipid derivatives spin labeled at the different C-atoms along the acyl chain, show that the peptide increases the bilayer packing at all depths. Parallel to that, there is an increase in the probe's isotropic hyperfine splittings, indicating that the peptide significantly decreases the membrane hydrophobic barrier. Accordingly, it is suggested that the increase in membrane packing yielded by α-MSH is partly due to a greater level of interchain hydration. This result is compared to the increase in packing and decrease in polarity yielded by cholesterol, and the absence of structural or polar alterations with Na^+. The latter result shows that the peptide effect is not related to an increase of positive charges at the anionic vesicle surface. Alterations on the lipid bilayer polar profile measured by the nitroxide hyperfine splitting *z* component in frozen samples are shown to be different from those obtained at room temperature. However, it is shown here that a certain correlation can be drawn between the increase in polarity measured in frozen samples and the packing effect caused by the different molecules in the lipid gel phase.

Keywords: α-Melanocyte stimulating hormone (α-MSH); 1,2-Dimyristoyl-*sn*-glycero-3-phosphoglycerol (DPMG); Polarity profile; Bilayer fluidity; Spin label; Peptide–lipid interaction

* Corresponding author. Tel.: +55-11-818-6829; fax: +55-11-813-4334.
E-mail address: mtfreund@fge.if.usp.br (M. Teresa Lamy-Freund).

0301-4622/00/$ - see front matter
PII: S0301-4622(00)00169-1

1. Introduction

The linear tridecapeptide α-melanocyte stimulating hormone (α-MSH; Ac–Ser^{1}–Tyr^{2}–Ser^{3}–Met^{4}–Glu^{5}–His^{6}–Phe^{7}–Arg^{8}–Trp^{9}–Gly^{10}–Lys^{11}–Pro^{12}–Val^{13}–NH_2), derived from the hormone precursor pro-opiomelanocortin (POMC), is the physiological relevant hormone regulating skin darkening in most vertebrates [1]. This peptide is also involved in a variety of other physiological and neurological processes, such as fetal growth, thermoregulation, obesity, learning, memory and attention [2]. In the pH value used here (7.4), α-MSH is approximately monovalent, with the positive residue Arg localized in the minimal sequence required to stimulate melanocytes, Ac–His^{6}–Phe^{7}–Arg^{8}–Trp^{9}–NH_2 [3,4]. Due to the conservation of that core sequence in several species, it is generally referred to as the primary active site or 'message sequence'. Studies with fluorescent spectroscopy using the Trp^{9} residue as a probe, and electron spin resonance (ESR) of spin labels incorporated in lipid vesicles, have shown that the cationic peptide strongly interacts with anionic micelles and vesicles. That is certainly due, primarily, to their electrostatic attraction, but there is a subsequent partial penetration of the peptide into the hydrophobic core [5–8]. It was found that the Trp^{9} residue is positioned around the 7th C-atom of the acyl chain [6]. In general, when the membrane is in the fluid liquid crystalline state, α-MSH was found to increase the lipid packing at all depths of the vesicle bilayer. In the more packed gel state, the peptide–lipid interaction alters differently the distinct regions of the bilayer, as will be shown here.

The aim of this study was to investigate possible alterations in the membrane hydrophobic barrier caused by the peptide–lipid interaction. It is well known that water can readily cross biological membranes, and particularly, phospholipid bilayers [9]. However, the amount of water and the extension of its presence along the acyl chains is still a matter of controversy. Whereas neutron and X-ray diffraction, and proton nuclear magnetic resonance indicate that there is no substantial water to be found in the membrane beneath the carbonyl groups [10–12], fluorescent and paramagnetic probes incorporated in the membrane, can clearly measure water molecules deeper within the lipid bilayer, in both model and natural membranes [13–18].[1] These spectroscopic techniques have indicated that the membrane hydrophobic barrier is strongly dependent on the presence of double bonds and hydrophobic molecules, like cholesterol and carotenoids. An increase on the membrane core hydrophobicity was correlated to a decrease on the permeation of small polar molecules, and was suggested to be relevant for functional aspects of biological membranes.

To study the lipid bilayer hydration we used spin labels with the nitroxide moiety at various depths in the membrane. It has been shown that the magnitude of the nitrogen isotropic hyperfine splitting (a_o, one-third of the trace of the hyperfine tensor) depends on several factors which increase the unpaired electron spin density at the nitrogen nucleus, like solvent polarity, the presence of electric fields or electron transfer complex [13,19]. For labels inside a lipid bilayer, there are strong indications that an increase in a_o is mainly related to the nitroxide–water hydrogen bonding [13]. Therefore, the extension of water presence into the bilayer can be estimated by the magnitude of the isotropic nitrogen hyperfine splitting. Alternatively, the analysis of the ESR spectra of spin labels in different solvents has shown that there is a linear correlation between the isotropic hyperfine splitting value and the z component of the hyperfine tensor (A_{zz}), which can be directly measured in frozen samples [13]. Based on A_{zz} measurements, it has been shown that both cholesterol and polar carotenoids, which completely penetrate the lipid membrane, cause an increase in the bilayer polarity at regions close to the membrane surface, and dramatically increase the hydrophobic barrier at the bilayer center of saturated lipid membranes [16,20].

In the present work, the effect the melano-

[1] Though not discussed in the present work, the observed discrepancies are possibly related to the sensitivity of the different techniques and to both the amount and the lifetime of interchain water.

tropic peptide α-MSH causes on the fluidity of anionic lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) bilayers is correlated to the variations that occur on the membrane polarity profile. DMPG, used here as a model system of acidic lipid domains in natural cells, is a rather convenient lipid system, as its gel–liquid crystal transition temperature (T_m) is approximately 20°C [21]. Hence, in the temperature range 0–45°C, it is possible to study the peptide–membrane interaction in both lipid phases. The two phases would mimic acidic micro-regions of different fluidity present in biological membranes at physiological temperatures.

The local polarity values were estimated both at high temperatures, with the lipid in the liquid crystalline phase, by the isotropic hyperfine splitting a_o, and in frozen samples, by the measurement of the z component of the hyperfine tensor A_{zz}. The reliability of the isotropic hyperfine splitting measurements is discussed, considering the known difficulty in separating polarity from mobility effects at room temperature [13]. Due to the difficulty of properly evaluating the a_o values at all the bilayer depths, the z component of the hyperfine tensor, measured in frozen samples, have been largely used in the literature [13,16,20,22]. However, it is shown here that the peptide effects on the DMPG bilayer polarity profile are not identical in the fluid phase and in frozen samples. The differences found are discussed in the light of the different structural alterations the hormone causes on the lipid bilayer above and below its gel–liquid crystal transition temperature (T_m). It is shown that the results obtained with frozen samples can be somehow correlated with those obtained with the lipid in the gel phase.

Considering that the peptide is a macro cation, and that its effect on the membrane polarity could be mostly related to the presence of positive charges at the membrane surface, the alterations caused by α-MSH on the polarity and fluidity profiles of DMPG bilayers are compared here to those caused by the cation Na^+. Cholesterol is also used as a reference of a hydrophobic molecule that completely penetrates the bilayer core.

2. Materials and methods

2.1. Materials

α-Melanocyte stimulating hormone (α-MSH), cholesterol, the spin labels 5-, 12- and 16-SASL (5, 12, 16-doxyl stearic acid spin label) and Hepes [4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The sodium salt of the phospholipid DMPG (1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol, lot no. 140PG-115) was obtained from Avanti Polar Lipids (Birmingham, AL, USA). The spin labels *n*-PCSL [1-palmitoyl-2-(*n*-doxyl stearoyl)-*sn*-glycero-3-phosphocholine; $n = 5$, 9, 10, 12, 14 or 16] were either purchased from Avanti Polar Lipids or kindly donated by A. Watts, from the University of Oxford, UK.

2.2. Lipid dispersion preparation

A film was formed from a chloroform solution of DMPG and spin label (0.2 mol%, relative to the lipid, for the 16th labeled amphiphiles, and 0.6 mol% for the other labels, were found to be the maximum spin label concentrations to display no spin–spin interaction), dried under a stream of N_2 and left under reduced pressure for a minimum of 2 h, to remove all traces of the organic solvent. Liposomes were prepared by the addition of the desired buffer solution, with or without peptide, cholesterol or salt, followed by vortexing above the phase transition temperature. The DMPG final concentration was 10 mM. If not stated otherwise, the buffer system used was 10 mM Hepes + 2 mM NaCl, adjusted with NaOH to pH 7.4.

2.3. ESR spectroscopy

ESR measurements were performed with a Bruker EMX spectrometer. A field-modulation amplitude of 1 G and microwave power of 5 mW were used. This modulation amplitude broadened only the Gaussian component of the ESR line as expected [23]. The temperature was controlled to approximately 0.2°C with a Bruker BVT-2000 variable temperature device. The temperature was

monitored with a Fluke 51 K/J thermometer with the probe placed just above the cavity. The magnetic field was measured with a Bruker ER 035 NMR Gaussmeter, and when necessary, the WINEPR software (Bruker) was used. All data shown are means of the results from at least three experiments, and the uncertainties are the standard deviations. When not shown the uncertainties are smaller than the size of the symbols.

The parameters of the ESR spectra of the more mobile probes, labeled at the 16th C atom, at high temperatures, were found by fitting each line to a Gaussian–Lorentzian sum function [24] taking advantage of the fact that the sum function is an accurate representation of a Gaussian–Lorentzian convolution, the Voigt function [23]. The hyperfine splitting, a_o, was taken to be one-half the difference in the resonance fields of the high- and low-field lines.

For the highly anisotropic spectra yielded by 5-PCSL and 5-SASL, incorporated in the liquid crystal phase of DMPG vesicles, the isotropic hyperfine splitting was calculated from the expression [25,26]

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

where $A_{//}$ $(=A_{\max})$ is the maximum hyperfine splitting directly measured in the spectrum (see Fig. 1), and

$$A_{\perp} = A_{\min} + 1.4\left[1 - \frac{A_{//} - A_{\min}}{A_{zz} - (1/2)(A_{xx} + A_{yy})}\right] \quad (2)$$

where $2A_{\min}$ is the measured inner hyperfine splitting (see Fig. 1) and A_{xx}, A_{yy} and A_{zz} are the principal values of the hyperfine tensor for doxylpropane [27].

Effective order parameters, S_{eff}, were calculated from the expression

$$S_{\text{eff}} = \frac{A_{//} - A_{\perp}}{A_{zz} - (1/2)(A_{xx} + A_{yy})} \frac{a'_o}{a_o}, \text{ where}$$

$$a'_o = (1/3)(A_{xx} + A_{yy} + A_{zz}) \quad (3)$$

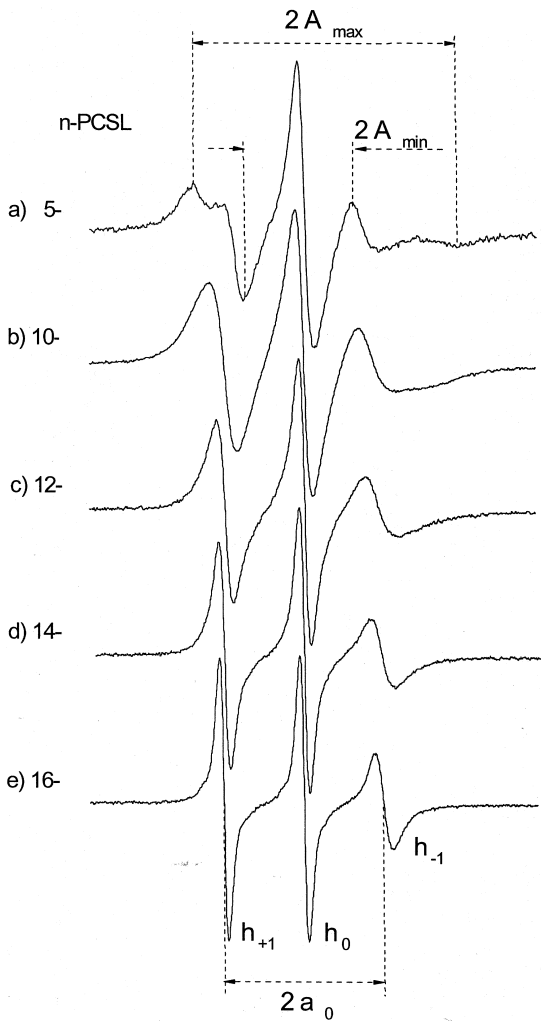

Fig. 1. X band (9 GHz) ESR spectra of *n*-PCSL in 10 mM DMPG dispersions at 35°C. Total spectra width 100 G. The outer and inner hyperfine splittings ($A_{\max}$ and $A_{\min}$), the isotropic hyperfine splitting (a_o) and the amplitudes of the three nitrogen hyperfine lines (h_{+1}, h_o and h_{-1} corresponding to $m_I = +1, 0, -1$, respectively) are indicated.

(Very similar values of order parameter were obtained by using the expression suggested by Marsh and Schorn [28]).

2.4. ESR spectral simulations

For least-squares analysis of ESR spectra we used the computer program NLSL developed by Freed and co-workers [29,30]. As the parameters

used have been extensively discussed in their papers, just the few most relevant points for performing the simulations will be commented on here. As discussed in the literature [31], the best yielded simulation of the spectrum of spin labels in dispersions (microscopic order and macroscopic disorder, MOMD) is strongly dependent on the initial parameters (seed values). Searches with different initial values will lead to distinct local minima. Hence, the least-squares analysis is not intended to be carried out fully automated, but requires the 'intuition' of the user. Considering that the variations on the g value with the label local polarity is rather small, the g-tensor components were kept as $g_{xx} = 2.0089$, $g_{yy} = 2.0058$ and $g_{zz} = 2.0021$ [31,32]. The hyperfine splitting tensor was considered to be axial, $A_{xx} = A_{yy} = A_{\perp}$, and the $A_{\perp}$ and A_{zz} values were allowed to vary within a reasonable interval [32]. We have not used fixed A_{zz} values, obtained from frozen samples, as we had indications that the hyperfine splitting obtained at this condition is not identical to that obtained at higher temperatures. Indeed, the amount of water inside the bilayer may be dependent on the lipid phase and temperature. The same Gaussian inhomogeneous broadening parameters were used for the simulations of the three spectra (gib0 = 0.82; gib2 = 0.16). Rotational correlation times ($\tau_{\perp}$ and $\tau_{//}$) were calculated based on the best results obtained for the principal values of an axially symmetric rotational diffusion tensor for the nitroxide moiety attached to the chain segment ($R_{\perp}$ and $R_{//}$; $\tau = 1/6R$; the $\tau_{//}$ values were found to be very small, almost out of the fitting sensibility, so they are not indicated in Table 1). For the diffusion tensor, the symmetry axis is the axis of the chain segment, z' [29] (the molecular frame). The angle between the molecular z' axis and the magnetic z''' axis (by convention, defined parallel to the $2p_z$ orbital of the N atom), the diffusion tilt angle β, was allowed to vary, but no better simulation could be obtained, so β was kept null (see discussions in [22,31]). The microscopic orientational ordering of the spin label is characterized here by the order parameter S_{20}, related to the amplitude of the rotational motion. It is a measure of the extent of alignment of the z' molecular axis with respect to the z'' local director frame, taken as the normal to the bilayer [29,31]. Another order parameter, representing the second term of the restoring potential [29], was found to be rather small, and is not listed in Table 1. As there was no clear indication of more than one component in the spectra, we chose to use simple fits with just one signal, though the best fittings were not perfect.

3. Results and discussion

3.1. Spin probes in pure DMPG bilayers: isotropic hyperfine splitting a_o

Fig. 1 shows the ESR signals of phospholipid derivatives (PCSL), incorporated in DMPG vesicles at 35°C, above the bilayer gel–liquid crystal transition temperature. The lipids shown are labeled at five different positions of the acyl chain, the 5th, 10th, 12th, 14th and 16th carbon atom. Their different spectra are illustrative of the average distinct nitroxide positions along the bilayer depth, making evident the well known flexibility gradient towards the membrane center [27]: the rate and amplitude of the segmental motion increase in the order 5-PCSL < 10-PCSL < 12-PCSL < 14-PCSL < 16-PCSL.[2] The nitrogen isotropic hyperfine splitting a_o can be relatively well determined in ESR spectra typical of probes displaying high order and fast movement, where the A_{max} and A_{min} values can be precisely measured (see Section 2) like that of 5-PCSL at high temperature (Fig. 1a). Otherwise, for relatively isotropic ESR signals, in the motional narrowing region [33], like the one obtained with 16-PCSL, a_o can be directly measured in the ESR spectrum, as indicated in Fig. 1e, or more accurately obtained by the fitting of the three hyperfine lines by Voigh functions (see Section 2). The difficulty in obtaining reliable a_o values from signals like

[2] Though the DMPG acyl chains have 14 C atoms, the 16-PCSL ESR spectrum is typical of a more mobile probe than that of 14-PCSL, evincing the greater mobility of the chain end.

those yielded by 10 and 12-PCSL (Fig. 1b,c) is evident, as the measurement of both A_{max} and A_{min} are rather inaccurate, and the signal is too anisotropic to allow a direct measurement of a_o. Although the 14-PCSL signal seems to be yielded by a probe in the motional narrowing regime, the a_o values directly measured in the spectrum were found to display a significant increase with temperature, which is not expected for the isotropic hyperfine parameter.[3] Moreover, the presence of both α-MSH and cholesterol decreases the lipid mobility, making the a_o measurement in the 14-PCSL spectra even less reliable. Therefore, in the DMPG fluid phase, it was only possible to directly measure the polarity of the regions monitored by the 5th and 16th labeled lipids. It is important to note that it is not possible to measure a_o values at low temperatures, with the lipid in the gel phase. Otherwise, the components of the hyperfine tensor of any ESR signal can be estimated from the computer simulation of the spectrum, as shown below.

3.2. Changes in the a_o values due to the presence of α-MSH: a comparison with Na^+ and cholesterol

In general, due to the similarity to the other bilayer lipids, phospholipid derivatives are the best spin labels to be used, having a polar head group well anchored at the membrane surface. However, considering that the stearic acid spin labels have been largely used in the literature in the measurement of lipid hydrophobic barriers [16,20], the results obtained with both phospholipid (PCSL) and stearic acid (SASL) spin label derivatives will be presented here. Moreover, it might be interesting to know if the spin label headgroup has any influence on the alterations caused by the different molecules on the bilayer structure and polarity. As discussed before [8], the stearic acid is mostly neutral (protonated) in the low ionic strength pH 7.4 DMPG dispersion, and penetrates deeper in the bilayer than the PCSL, in the lipid liquid crystal phase.

Fig. 2 shows the a_o values measured in the 5-PCSL (a) and 5-SASL (b) spectra, in DMPG at different temperatures above T_m, in the presence of 1 mM α-MSH (the total sample concentration is 10 mol% relative to DMPG), 100 mM NaCl and 3 mM cholesterol (30 mol% relative to DMPG, incorporated in the lipid vesicle). For comparison, the effects caused on the DMPG membrane fluidity, at the 5th C-atom position, are also shown (Fig. 2c,d). The effective order parameter, S_{eff}, (see Section 2) contains contributions from both order and rate of motion, although the principal contribution to S_{eff} is the amplitude of segmental motion of the acyl chain [34]. The effects yielded by α-MSH, NaCl and cholesterol, on both DMPG membrane polarity (a_o) and fluidity (h_{-1}/h_0), at the bilayer center, are shown in Fig. 3, based on the 16-PCSL and 16-SASL ESR spectra parameters. The parameter h_{-1}/h_0 is the ratio between the amplitudes of the lines corresponding to the nitrogen spin numbers $m_I = -1$ and $m_I = 0$ (see Fig. 1). The h_{-1}/h_o ratio, in general, gets closer to the unity as the label environment viscosity decreases.[4] As shown before, both α-MSH [7,8] and cholesterol [35] increase the packing of the liquid crystal lipid bilayer, both close to the surface (increase S_{eff} of the 5th labeled probe, Fig. 2c,d) and at the bilayer center (decrease the h_{-1}/h_0 ratio of the 16th labeled probe, Fig. 3c,d), whereas NaCl causes only minor effects on the membrane fluidity.

In pure DMPG dispersions, the known DMPG bilayer polarity profile is evident by comparing the data shown in Fig. 2a,b (■) with those in Fig. 3a,b (■): the 5th labeled amphiphilics display higher isotropic hyperfine splittings (approx. 14.9 G) than the 16th labeled C-atom probes (approx. 14.4 G). (The isotropic splitting for the water

[3] Properly measured a_o values are expected to be rather independent of temperature, as the variation of the isotropic hyperfine splitting of stearic acid labels in aqueous medium, where a_o can be very accurately evaluated, is less than 0.3 % between 20 and 50°C (data not shown). However, inside the lipid bilayer, a small increase of a_o with temperature could possibly indicate an actual increase in the number of water molecules as the chains get more mobile.

[4] Rotational correlation times [38] were not calculated because the spectra yielded in the presence of cholesterol and α-MSH could not be perfectly fit by the Voigt function [23].

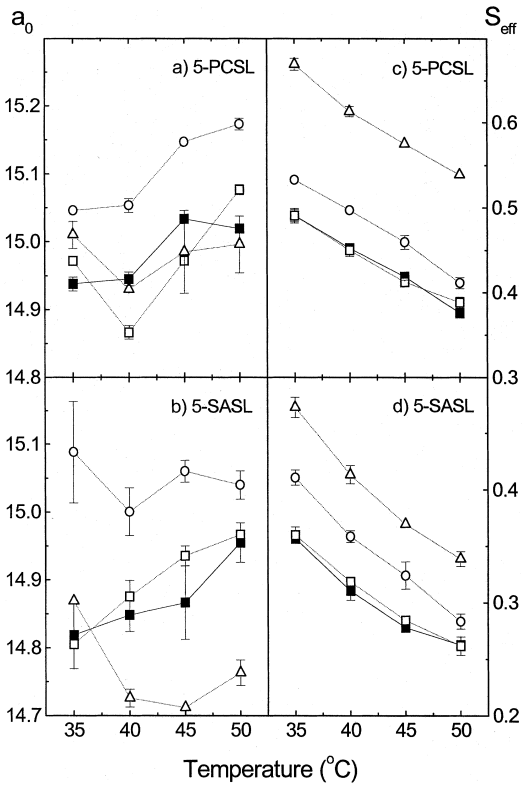

Fig. 2. Temperature dependence of the isotropic hyperfine splitting (a_o) (a and b) and effective order parameter (S_{eff}) (c and d) measured on ESR spectra of amphiphilics (PCSL and SASL) labeled at the 5th C-atom of the acyl chain, in pure 10 mM DMPG dispersion (■, solid line), and with the addition of 10 mol% α-MSH (○, dashed line), 30 mol% of cholesterol (△, dashed line) and 100 mM NaCl (□, dashed line). In all the figures the lines connecting the symbols are for helping visualization only.

soluble probes 5-, 12- and 16-SASL, in aqueous medium, was found to be 15.78 ± 0.01 G). As mentioned before, the ESR spectra of the 5th labeled probes clearly indicate that the phospholipid spin labels are localized in an average shallower position in the DMPG liquid crystalline bilayer than the stearic acid derivatives. This can be seen by the higher S_{eff} values yielded by 5-PCSL (■ in Fig. 2c) as compared to 5-SASL (■ in Fig. 2d). Accordingly, the a_o values obtained for 5-PCSL are slightly higher than those of 5-SASL (compare ■ in Fig. 2a,b). Possibly due to the large vertical fluctuation of the acyl chain end [36,37], the ESR parameters of the 16-PCSL and 16-SASL (a_o in Fig. 3a,b; h_{-1}/h_o in Fig. 3c,d) indicate that both labels, in average, monitor a rather similar vertical region in fluid DMPG bilayers.

As expected, the two parameters, S_{eff} and h_{-1}/h_o, which monitor the lipid fluidity close to the bilayer surface and at the center, respectively, display a considerable temperature dependence (Fig. 2c,d; Fig. 3c,d). It is interesting to note that, opposite to the a_o values, they present a smooth variation with temperature. The somewhat erratic data obtained for the a_o measurements, mainly with the five C-atom labeled probes, probably

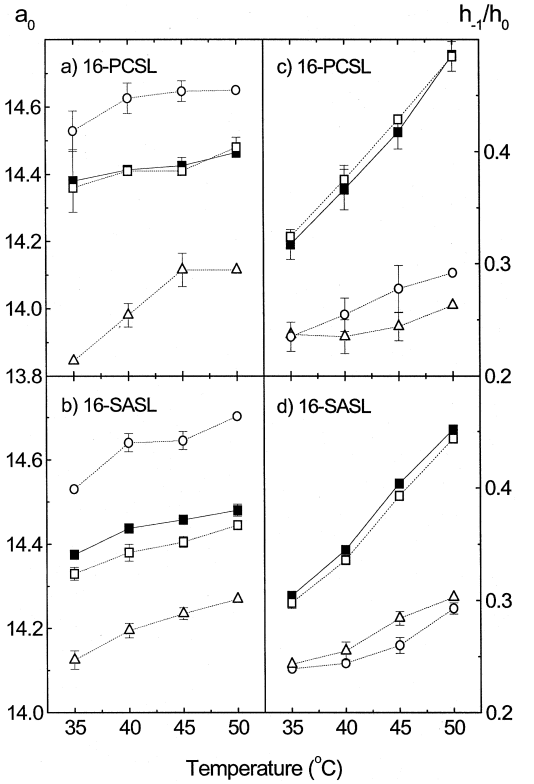

Fig. 3. Temperature dependence of the isotropic hyperfine splitting (a_o) (a and b) and the ratio between the amplitudes of the high and central field lines (h_{-1}/h_0) (c and d) measured on ESR spectra of amphiphilics (PCSL and SASL) labeled at the 16th C-atom of the acyl chain, in pure 10 mM DMPG dispersion (■, solid line), and with the addition of 10 mol% α-MSH, (○, dashed line), 30 mol% of cholesterol (△, dashed line) and 100 mM NaCl (□, dashed line).

indicate the difficulty of completely eliminating the contributions of the label order and mobility to the hyperfine measurements in fluid lipid bilayers (the average values shown in Table 1 were calculated between 40 and 50°C). Even though, a few conclusions concerning the bilayer polarity profile can be surely drawn. Considering that an increase in a_o is related to an increase in the local polarity, it is evident that the cationic peptide, which interacts at the membrane surface, partially penetrating the bilayer [6,8], increases the membrane polarity at the micro-regions monitored by the labels 5- and 16-PCSL, and 5- and 16-SASL. A general effect of electrostatic shielding at the vesicle surface, yielded by the monovalent ion Na^+, does not seem to significantly alter the DMPG polarity (or fluidity) profile, while cholesterol causes a considerable decrease on the membrane polarity at the center of the bilayer (at the 16-PCSL and 16-SASL positions) and also at an intermediate bilayer position, where 5-SASL is localized. It does not change much the hydrophobic degree of the shallower site monitored by 5-PCSL.

It is interesting to note that, although both α-MSH and cholesterol increase the membrane packing, their known different ways of interacting with the DMPG bilayer are reflected on the distinct alterations the two molecules cause on the bilayer polarity. The different behavior of the DMPG bilayer in the presence of α-MSH and cholesterol is better evinced by the parameters (Table 1) obtained for the best theoretical simulations (see Section 2) of the ESR spectra of 5-PCSL and 12-PCSL incorporated in the bilayers, in the absence and presence of the two molecules (Fig. 4). (The simulation of the highly isotropic 16-PCSL was not very informative, as different sets of parameters were found to equally adjust the experimental signal.) Though there were some ambiguities in the theoretical simulations of the signals in Fig. 4, a few trends were evident (Table 1). (i) In the 5-PCSL simulations, the A_{zz} values for the DMPG and DMPG + cholesterol samples were found to be rather similar, and lower than that of DMPG + α-MSH. This is in line with the higher a_o value experimentally obtained for the latter sample (Fig. 2a). (The theoretical and experimental a_o values are shown in Table 1.) (ii) In the 5th carbon atom position cholesterol clearly increases both the bilayer order (S_{20}) and the probe rotational correlation time ($\tau_\perp$), whereas

Table 1
Parameters obtained from non-linear least-squares fitting[a] of ESR spectra of 5- and 12-PCSL in DMPG dispersions at 45°C, in the absence and presence of α-MSH[b] and cholesterol[c,d]

	$A_\perp$ (G)	A_{zz} (G)	a_o^e (G)	$\tau_\perp$ (ns)	S_{20}	a_o^f (exp.)(G)	$S_{eff}^g 0$ (exp.)
5-PCSL							
DMPG	5.12	34.85	15.03	1.05	0.32	15.00 ± 0.05	0.42
+ α-MSH	5.12	35.40	15.21	1.48	0.35	15.12 ± 0.06	0.46
+ Chol.	5.12	34.85	15.03	1.63	0.46	14.97 ± 0.03	0.57
12-PCSL							
DMPG	5.10	33.36	14.52	1.05	0.06	–	–
+ α-MSH	5.10	33.63	14.61	1.52	0.09	–	–
+ Chol.	5.10	32.15	14.12	2.15	0.14	–	–

[a] For the discussion of the simulating program used, and the spectral parameters, see Section 2.
[b] Sample total concentration 10 mol% relative to DMPG.
[c] Concentration in the bilayer 30 mol% relative to DMPG.
[d] The experimental and theoretical spectra are shown in Fig. 4.
[e] $a_o = (2A_\perp + A_{zz})/3$.
[f] Average values from 40 to 50°C (Fig. 2a).
[g] From Fig. 2c.

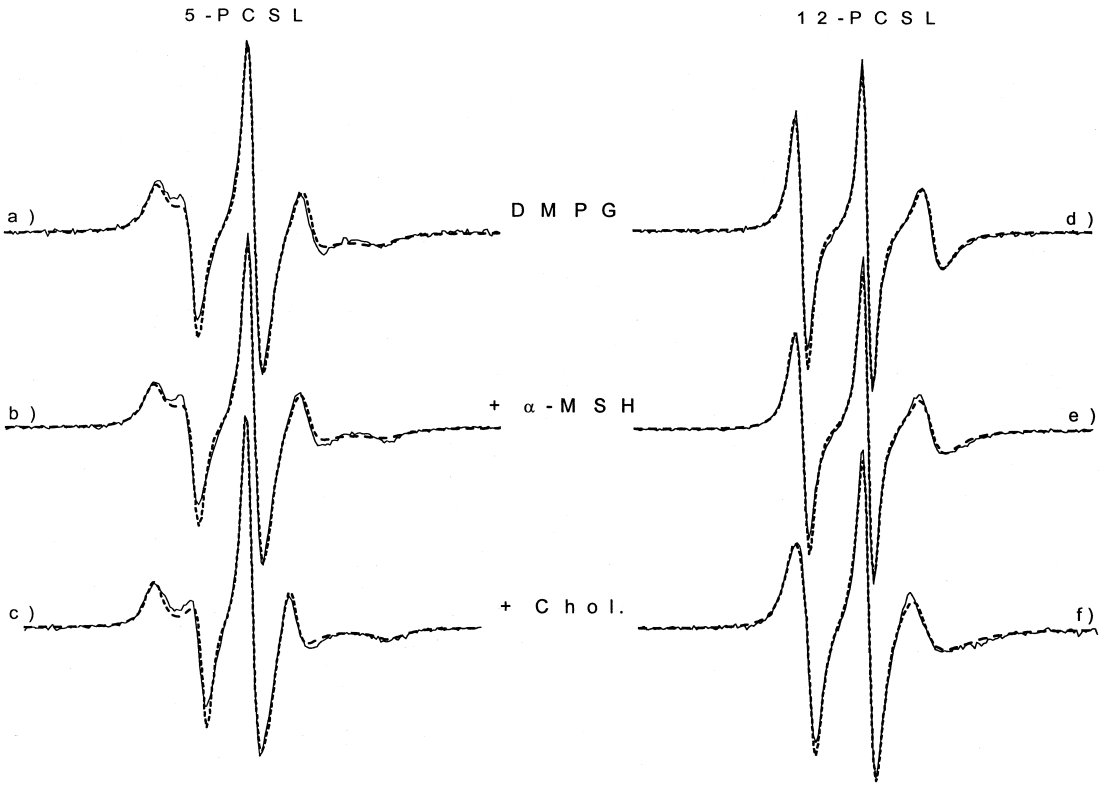

Fig. 4. ESR spectra of 5- PCSL (a, b and c) and 12-PCSL (d, e and f) incorporated in liquid crystalline DMPG vesicles (45°C), in the absence (a and d) and in the presence of 10 mol% α-MSH (b and e) and 30 mol% of cholesterol (c and f). Computer simulations of the spectra (see text) are shown by dashed lines. The spectral parameters derived from the fits are given in Table 1.

the effect of α-MSH is principally in decreasing the probe rate of movement (increase $\tau_{\perp}$). (The experimental order parameters S_{eff}, shown in Fig. 2c, are also presented in Table 1. As expected, they are higher than the theoretical S_{20} values, as S_{eff} incorporates both order and mobility.) (iii) The simulation of the 12-PCSL allows the conclusion that there is an increase (decrease) in the hyperfine splitting A_{zz} in the presence of α-MSH (cholesterol). This result agrees with the increase (decrease) in a_{o} monitored by 16-PCSL and 16-SASL (Fig. 3a,b), indicating that α-MSH (cholesterol) is decreasing (increasing) the hydrophobicity at the lamella center. (iv) Though cholesterol causes a much larger increase in both S_{20} and $\tau_{\perp}$ than α-MSH, at the bilayer region monitored by 12-PCSL, the two molecules increase the bilayer order and decrease the rate of movement.

Considering that the bilayer polarity profile is mainly determined by the extent of water penetration into the membrane [13], the decrease in membrane fluidity caused by the melanotropic peptide is correlated to an increase in water concentration in the acyl chains region, in line with a previous suggestion that peptides induce water penetration into the hydrocarbon chain region [39]. Interestingly, though the peptide probably only penetrates the lipid bilayer down to a certain depth [6], it decreases the bilayer fluidity and stabilizes the presence of water molecules all along the hydrocarbon chain. Therefore, one could speculate that the water molecules are contributing to the increase in order or decrease in mobility of the acyl chains. Otherwise, it is important to have in mind that the peptide–membrane system is heterogeneous, as membrane domains may arise due to the peptide–lipid interaction,

and that the fluidity and polarity information yielded by the spin label ESR spectrum is an average over all the different membrane regions monitored by the probe. Hence, the data shown here could be also rationalized considering that, in a certain instant, the region where the peptide is localized is more packed, but the molecule is deforming the bilayer in such a way as to allow more water penetration through the neighboring lipid regions. The average effects monitored by the spin labels would be the increase in packing and polarity, although the alterations could be happening in different bilayer domains.

The penetration of cholesterol inside the bilayer, a highly hydrophobic molecule, does not significantly change the hydrophobicity at the bilayer region monitored by 5-PCSL, but seems to turn the membrane considerably less polar at the bilayer core. It is important to note that the results obtained with cholesterol could have a somewhat different interpretation, bearing in mind that: (i) there is a descending polarity profile towards the center of the bilayer; (ii) in the liquid crystalline phase the acyl chains have a large amplitude of movement; and (iii) the measured a_o parameters are average values. Therefore, instead of a decrease in water molecules at a certain bilayer depth, the decrease in a_o could be simply a consequence of the increase in the bilayer packing, hence decreasing the vertical fluctuation of the chains.

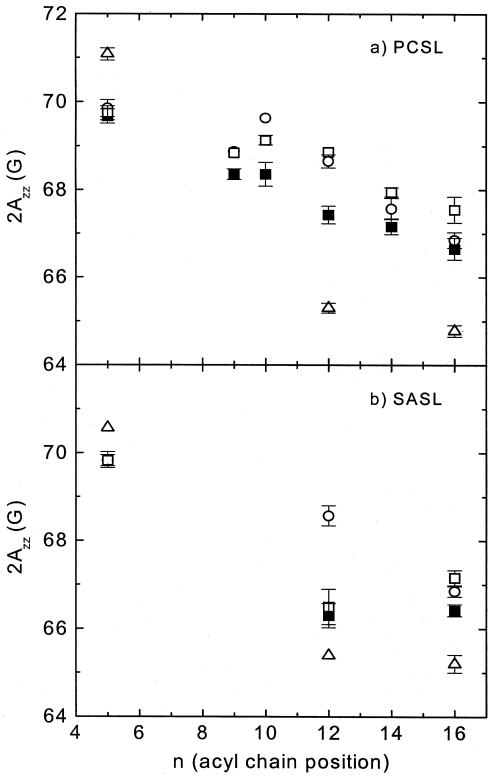

Fig. 5. The z component of the hyperfine splitting (A_{zz}), measured at -150°C, as a function of the nitroxide position in amphiphilics (n-PCSL and n-SASL) in pure 10 mM DMPG dispersion (■), and with the addition of 10 mol% α-MSH (○), 30 mol% of cholesterol (△) and 100 mM NaCl (□).

3.3. Local polarity measured in frozen samples

Lipid bilayer polarity profiles have been extensively discussed based on the hyperfine splitting z component (A_{zz}) obtained from frozen samples [13,16,20,22]. The great advantage of that method is that the measured A_{max} value is the actual A_{zz} parameter, completely independent of label movements. Fig. 5a displays hyperfine splitting parameters directly measured in the ESR spectra of frozen DMPG samples (-150°C), obtained with phospholipids labeled at the six different acyl chain positions, the 5th, 9th, 10th, 12th, 14th and 16th C-atom. Though our discussion will be based on the data obtained with phospholipid spin labels, it is interesting to note that the results yielded by stearic acid derivatives labeled at three acyl chain positions, shown in Fig. 5b, are somewhat different from those obtained with the phospholipid labels.

The A_{zz} values of phospholipid spin labels incorporated in pure DMPG frozen vesicles indicate a mild polarity gradient towards the center of the bilayer: $2A_{zz}$ varies from (69.67 ± 0.16) G at the 5-PCSL position to (66.65 ± 0.25) G at the 16-PCSL environment (Fig. 5a) ($2A_{zz}$ value in the aqueous phase was found to be approx. 72.5 G; [16]). These values are similar to those obtained with stearic acid labels in DMPG (Fig. 5b) and in DMPC membranes [16]. It is noteworthy that the variations on the DMPG polarity profile of frozen samples due to the presence of the different interacting molecules (Fig. 5a), are not the same

as those obtained with a_o (Fig. 2a; Fig. 3a), the latter measured with the lipid in the liquid crystal phase. For instance, at the regions monitored by 5- and 16-PCSL, where a_o values could be measured in the fluid DMPG membrane, and indicated that the peptide increases the average micro-region water concentration (Fig. 2a; Fig. 3a), no significant alteration is monitored by the A_{zz} parameter in frozen samples (Fig. 5a). However, α-MSH is found to increase the bilayer polarity of frozen samples at the central regions monitored by the 9-, 10-, 12- and 14-PCSL (Fig. 5a), with expressive alterations observed at the sites reported by the 10th and 12th labeled lipids.

The results obtained with NaCl are rather surprising: the salt has almost no effect at room temperature (Fig. 2a; Fig. 3a), but significantly increases the polarity of the DMPG bilayer core in frozen samples, monitored by phospholipids labeled from the 9th to the 16th C-atom. Hence, though the effects yielded by α-MSH and Na^+ are not identical at the different acyl chain positions, both cations decrease the bilayer hydrophobic barrier in frozen membranes. This is indicating that the presence of ions at the vesicle surface stabilizes the water molecules inside the bilayer core. It is important to have in mind that this is specific for frozen samples, as in fluid DMPG bilayers the NaCl effect on the membrane polarity does not seem to be relevant (Fig. 2a; Fig. 3a).

The increase in the A_{zz} values of spin labels incorporated in frozen membrane samples has also been observed in the presence of gramicidin A (GA) [22]. Considering that GA is a hydrophobic pentadecapeptide, supposed to complete penetrate lipid bilayers, it was suggested that, in general, membrane proteins and peptides increase the water penetration into lipid bilayers [22]. However, to illustrate that the problem is more complex, it is interesting to note that a_o measurements obtained above 20°C have indicated that the Mojave toxin increases both a_o and S_{eff} of spin labels incorporated in the upper regions of synaptosomal membranes, and decreases the two parameters measured in the ESR spectra of stearic acids labeled towards the center of the bilayer [15].

The effect of cholesterol at the bilayer center of frozen samples is similar to that observed at room temperature, with the steroid causing a great increase in the membrane core hydrophobicity (decrease in A_{zz}). However, the cholesterol increase in the bilayer polarity at sites closer to its surface (monitored by 5-PCSL) is only evident in frozen samples. A similar cholesterol effect in frozen samples can be detected with stearic acid spin labels (Fig. 5b), and has been well documented by Subczynski et al. [16], with labels in different saturated lipid systems.

The variation of the A_{zz} values due to the presence of α-MSH, Na^+ and cholesterol, monitored by the stearic derivative spin labels (Fig. 5b), will not be discussed in detail here. Though the effects observed on the DMPG bilayer polarity profile are not very different from those obtained with the phospholipid labels (Fig. 5a), they are somewhat distinct in magnitude. It is important to note that even in pure DMPG bilayers the behavior of the two types of labels, SASL and PCSL, is not identical: at the 5th and 16th C-atom position their A_{zz} values are rather similar, but the A_{zz} value obtained for the 12-PCSL (Fig. 5a) is significantly higher than that obtained for 12-SASL (Fig. 5b). Opposite to the phospholipid labels, which are well anchored at the bilayer surface, stearic acid spin labels are likely to present vertical shifts upon temperature phase transition, frozen process or the presence of interacting molecules [40]. Moreover, the presence of charged molecules at the vesicle surface could increase the local pH value inducing a deprotonation of the stearic acid, therefore affecting its vertical position in the bilayer [41]. Though not shown, the A_{zz} values obtained with the methyl ester derivative of the stearic acid, spin labeled at different positions along the acyl chain, were also found to be somewhat different from the values yielded by the phospholipid labels.

3.4. Correlation between alterations on the polarity of frozen bilayers and changes on the lipid gel phase fluidity

It is interesting to compare the alterations α-MSH causes on the DMPG polarity gradient

measured via the A_{zz} values, in frozen samples, with the changes due to the peptide–lipid interaction on the fluidity of DMPG vesicles in the more packed gel state. The rationale for doing it is that the membrane frozen state is certainly closer to its gel state, obtained at low temperatures, than to the high temperature more fluid liquid crystal structure.[5] However, it is important to have in mind that the freezing process can possibly lead to other peptide–lipid structural arrangements.

Fig. 6 shows the A_{max} values measured in the 5- and 12-PCSL spectra and the 16-PCSL central field linewidths, ΔH_0 (the measurement of A_{max} is rather inaccurate for the 16-PCSL), for temperatures below the gel–liquid crystal transition. The two spectral parameters, A_{max} and ΔH_0, show a strong temperature dependence, indicating that they are mainly monitoring the lipid bilayer packing. They get smaller as the temperature and the spin label mobility increases, as an indication of a less viscous micro-environment. (It has been shown by ESR saturation transfer that the A_{max} parameter down to approx. 0°C, is more sensitive to the label movements than to polarity changes [42].) Though not shown, the results obtained with 9- and 10-PCSL are rather similar to those obtained with 12-PCSL, showing that α-MSH causes a significant increase in the bilayer packing of DMPG gel phase lipids at regions monitored by those three labels (increase in A_{max} shown in Fig. 6b, or in the linewidths, ΔH_0 and ΔH_{+1}, relative to the central and low field lines, not shown here). Opposite to that, it is very interesting to note that the peptide–lipid interaction brings a small decrease upon the bilayer packing at the 5th C-atom position (decreases A_{max}, Fig. 6a) and at the regions monitored by the 16- and 14-PCSL (data not shown) (decreases ΔH_0 shown in Fig. 6c, or increases h_{+1}/h_0, not shown). Therefore, the peptide effect in the

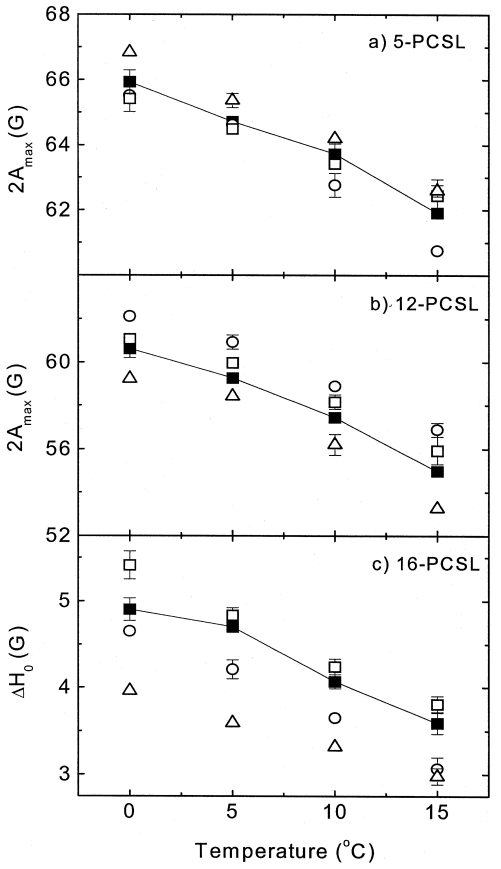

Fig. 6. Temperature dependence of the outer hyperfine splitting (A_{max}) (a and b) and the linewidth of the central field line (ΔH_0) (c) measured on ESR spectra of *n*-PCSL in pure 10 mM DMPG dispersion (■, solid line), and with the addition of 10 mol% α-MSH (○), 30 mol% of cholesterol (△) and 100 mM NaCl (□).

DMPG gel phase fluidity is quite different from that in the bilayer liquid crystalline state. Though the correlation between the alterations in the gel packing and the polarity of frozen samples are not straightforward, it is interesting to note that the peptide rigidifying effect in a certain micro-environment in the gel phase (9th to 12th C-atom region) is related to a greater increase in water concentration at that region in frozen samples.

Different from the results obtained with fluid DMPG (Fig. 2c; Fig. 3c), in the lipid gel phase, sodium ions cause some effect on the membrane structure, though not very large. Apart from

[5] Frozen samples (−150°C) yielded the same ESR signal when prepared by two different procedures: (i) a sample capillary at room temperature quickly dipped in the microwave cavity at −150°C; (ii) sample equilibrated at 5°C (lipid gel phase) before being taken to −150°C. This result is consistent with the frozen bilayer structure being closer to the gel phase packing than to the liquid crystal.

5-PCSL,[6] all other labels (9-, 10-, 12-, 14- and 16-PCSL) indicate that the presence of Na^+ slightly increases the lipid packing, monitored by either A_{max} or the linewidths, directly measured in the ESR spectra (Fig. 6 shows the results obtained with 5-, 12- and 16-PCSL). That result could be interpreted just as a consequence of the Na^+ electrostatic shielding at the bilayer surface, bringing the DMPG head groups closer together, therefore approximating the hydrocarbon chains. However, once more, the correlation of the results obtained in the lipid gel phase with those with frozen samples would indicate that there is an association between the local packing effect and the increase in the presence of water molecules inside the membrane.

In liquid crystal bilayers, the interaction of the peptide–bilayer was rationalized considering that α-MSH partially penetrates the lipid bilayer, increasing the membrane packing, and stabilizing the presence of a greater number of water molecules along the hydrocarbon chain. There, it was impossible to distinguish whether the increase in packing and polarity were happening at the same membrane region or in the structurally different domains created by the peptide–lipid interaction. Opposite to that, if a correlation is drawn between the lipid gel packing state and the frozen sample polarity, the results observed with Na^+ ions, which are unlikely to yield different patches at the membrane surface, indicate that the increase in both bilayer packing and polarity is a general effect over the whole bilayer.

The interaction of the peptide with the lipid gel phase seems to be rather complex. The increase in the bilayer packing at the regions monitored by 9-–12-PCSL could be indicative of the presence of the cationic peptide at the anionic surface of DMPG bilayers, decreasing the electrostatic repulsion between the head groups, therefore causing a further increase on the membrane packing. This is in line with the decrease in fluidity caused by the presence of sodium ions. However, the presence of Na^+, different from α-MSH, decreases the bilayer fluidity at all depths, whereas the peptide increases the chain mobility/disorganization at the membrane core, monitored by 14- and 16-PCSL. Considering the possible correlation between the gel phase and frozen samples results, it could be speculated that the presence of the peptide at the membrane surface stabilizes water molecules only down to a certain depth of the bilayer, turning that region more packed and polar, but somehow spacing the hydrocarbon chains, when compared to pure lipid bilayers. Therefore, close to the bilayer core, where the number of water molecules was not altered by the presence of the peptide, the chain segments would have more mobility and/or less order.

The known effect of cholesterol in increasing the bilayer fluidity in the lipid gel phase, due to the perturbation the molecule causes in the lipid packing [35], well monitored by the 12- and 16-PCSL (Fig. 6b,c), may be related to a decrease in the polarity at the center of the frozen bilayer (see A_{zz} values for 12- and 16-PCSL in Fig. 5a, and also for 12- and 16- SASL in Fig. 5b). Therefore, the presence of the highly hydrophobic cholesterol molecule inside the lipid membrane, either rigidifying the fluid state, or decreasing the organization of the packed gel phase, brings a considerable increase in the bilayer core hydrophobicity, if an association is made between the results observed in the gel and frozen states. As cholesterol increases the membrane fluidity of the gel phase, the discussion made before, for the lipids crystal state, does not apply here: the observed decrease in A_{zz}, in the presence of cholesterol, cannot be attributed to a decrease in the spin label amplitude of movement.

The increase in polarity observed in DMPG frozen samples in the presence of cholesterol close to the bilayer surface (increase in 5-PCSL and 5-SASL A_{zz} parameter, see Fig. 5a,b, and Subczynski et al. for other lipid systems [16]) could possibly be correlated to the increase in packing observed with 5-PCSL in the lipid in the gel phase (Fig. 6a). It seems possible that, in the gel phase, the membrane incorporation of cholesterol drives the head groups apart, allowing the penetration of more water molecules (monitored

[6] The rather small alterations observed with 5-PCSL could be due to the low sensitivity of the conventional ESR spectroscopy for the slow movements of this probe.

in frozen samples), therefore causing an increase in the local polarity and micro-viscosity at the 5th C-atom region.

4. Conclusions

The polarity profile variation due to peptide–lipid interaction is a complementary information to the effect α-MSH causes on the membrane fluidity. The peptide modulating effect on both the packing and the degree of hydration of the lipid phase, could lead to an ideal environment which would favor the hormone–receptor interaction. Alternatively, the peptide effect in decreasing the membrane hydrophobic barrier could be biologically relevant by itself. It is important to have in mind that DMPG is being used here as a model system for the lipid phase of biological membrane cells.

In liquid crystal DMPG membranes, the melanotropic peptide was found to increase both the packing and the polarity at regions monitored by 5- and 16-PCSL, and 5- and 16-SASL (and 12-PCSL, as indicated by the simulated spectra). This is in contrast to the effect caused by the complete penetration of hydrophobic molecules, like cholesterol and carotenoids, into the fluid lipid membrane, which increase the packing but decrease the bilayer polarity [16,20]. It would be interesting to find out whether this is a specific effect of α-MSH, or some peptides, or if it is a general consequence of the interaction of anionic membranes with cationic peptides which partially penetrate the bilayer. For instance, the presence of a charged amino acid, like Arg, next to the hydrophobic residue Trp, as it happens to occur in α-MSH, could induce (or stabilize) the presence of water molecules inside the bilayer. Alternatively, the peptide–lipid interaction may deform the bilayer structure allowing more water to penetrate the membrane. It is important to note that, in fluid bilayers, this is not an effect due to the increase of cations at the DMPG membrane surface, as no alteration was observed in the presence of 100 mM NaCl.

It was shown here that the changes on the polarity profile measured in frozen samples may be different from those obtained with the lipids in the liquid crystal phase. The discrepancy seems to be particularly relevant in the presence of polar molecules that cause distinct alterations on the gel and fluid membrane phases. To both α-MSH and NaCl a certain correlation can be drawn between the packing effect in the gel phase, and the increase in polarity measured in frozen samples (increase in A_{zz}). Hence, it seems that the increase in water molecules along the hydrocarbon chain could be a general effect due to the presence of cations at the negative vesicle surface of bilayers in the gel state.

Acknowledgements

This work was supported by USP, FAPESP and CNPq. Fellowships for R.M.F. (FAPESP) and M.T.L.F. (CNPq, research) are acknowledged. We thank Vera B. Henriques and Carla Goldman for important contributions to the paper discussion.

References

[1] M.E. Hadley, S.D. Sharma, V.J. Hruby, N. Levine, R.T. Dorr, Melanotropic peptides for therapeutic and cosmetic tanning of the skin, Ann. N.Y. Acad. Sci. 680 (1993) 424–439.

[2] H. Vaudry, A.N. Eberle (Eds.), The melanotropic peptides. Ann. N.Y. Acad. Sci., vol. 680 (The New York Academy of Sciences, New York, 1993).

[3] A.M.L. Castrucci, M.E. Hadley, T.K. Sawyer et al., Alpha-melanotropin: the minimal actice sequence in the lizard skin bioassay, Gen. Comp. Endocrinol. 73 (1989) 157–163.

[4] V.J. Hruby, B.C. Wilkes, M.E. Hadley et al., Alpha-melanotropin — the minimal active sequence in the frog skin bioassay, J. Med. Chem. 30 (1987) 2126–2130.

[5] A.S. Ito, A.M.L. Castrucci, V.J. Hruby, M.E. Hadley, D.T. Krajcarsk, A.G. Szabo, Structure–activity correlations of melanotropin peptides in model lipids by tryptophan fluorescence studies, Biochemistry 32 (1993) 12264–12272.

[6] Z.S. Macêdo, T.A. Furquim, A.S. Ito, Estimation of average depth of penetration of melanotropins in dimyristoylphosphatidylglycerol vesicles, Biophys. Chem. 59 (1996) 193–202.

[7] M.H. Biaggi, T.J.T. Pinheiro, A. Watts, M.T. Lamy-Freund, Spin label and ^{2}H-NMR studies on the interaction of melanotropic peptides with lipid bilayers, Eur. Biophys. J. 24 (1996) 251–259.

[8] M.H. Biaggi, K.A. Riske, M.T. Lamy-Freund, Melanotropic peptides–lipid bilayer interaction. Comparison of the hormone α-MSH to a biologically more potent analog, Biophys. Chem. 67 (1997) 139–149.

[9] R. Lawaczeck, On the permeability of water molecules across vesicular lipid bilayers, J. Membrane Biol. 51 (1979) 229–261.

[10] M.C. Winner, S.H. White, Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of X-ray and neutron diffraction data, Biophys. J. 61 (1992) 434–447.

[11] F. Volke, A. Pampel, Membrane hydration and structure on a subnanometer scale as seen by high resolution solid state nuclear magnetic resonance: PCPC and POPC/$C_{12}EO_4$ model membranes, Biophys. J. 68 (1995) 1960–1965.

[12] Z. Zhou, B.G. Sayer, D.W. Hughes, R.E. Stark, R.M. Epand, Studies of phospholipid hydration by high-resolution magic-angle spinning nuclear magnetic resonance, Biophys. J. 76 (1999) 387–399.

[13] O.H. Griffith, P.J. Dehlinger, S.P. Van, Shape of the hydrophobic barrier of phospholipid bilayers (evidence for water penetration in biological membranes), J. Membrane Biol. 15 (1974) 159–192.

[14] P. Fretten, S.J. Morris, A. Watts, D. Marsh, Lipid–lipid and lipid–protein interactions in chromaffin granule membranes, Biochim. Biophys. Acta 598 (1980) 247–259.

[15] J. Harris, T.J. Power, A.L. Bieber, A. Watts, An electron-spin-resonance spin-label study of the interaction of purified mojave toxin with synaptosomal membranes from rat brain, Eur. J. Biochem. 131 (1983) 559–565.

[16] W.K. Subczynski, A. Wisniewska, J.J. Yin, J.S. Hyde, A. Kusumi, Hydrophobic barriers of lipid bilayer membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol, Biochemistry 33 (1994) 7670–7681.

[17] C. Ho, S.J. Slater, C.D. Stubbs, Hydration and order in lipid bilayers, Biochemistry 34 (1995) 6188–6195.

[18] M. Ge, J.H. Freed, Polarity profiles in oriented and dispersed phosphatidylcholine bilayers are different: an electron spin resonance study, Biophys. J. 74 (1998) 910–917.

[19] R.N. Schwartz, M. Peric, S.A. Smith, B.L. Bales, Simple test of the effect of an electric field on the ^{14}N-hyperfine coupling constant in nitroxide spin probes, J. Phys. Chem. 101 (1997) 8735–8739.

[20] A. Wisniewska, W.K. Subczynski, Effects of polar carotenoids on the shape of the hydrophobic barrier of phospholipid bilayers, Biochim. Biophys. Acta 1368 (1998) 235–246.

[21] K.A. Riske, M.J. Politi, W.F. Reed, M.T. Lamy-Freund, Temperature and ionic strength dependent light scattering of DMPG dispersions, Chem. Phys. Lipids 89 (1997) 31–44.

[22] M. Ge, J.H. Freed, An electron spin resonance study of interactions between gramicidin A′ and phosphatidylcholine bilayers, Biophys. J. 65 (1993) 2106–2123.

[23] B.L. Bales, Inhomogeneously broadened spin-label spectra, in: L.J. Berliner, J. Reuben (Eds.), Spin Labelling. Theory and Applications, 8, Plenum Press, New York, 1989, p. 77.

[24] H.J. Halpern, M. Peric, C. Yu, B.L. Bales, Rapid quantitation of parameters from inhomogeneously broadened EPR-spectra, J. Magn. Reson. 103 (1993) 13–22.

[25] O.H. Griffith, P.C. Jost, Lipid spin label in biological membranes, in: L.J. Berliner (Ed.), Spin Labelling. Theory and Applications, Academic Press, New York, 1976, p. 453.

[26] B.J. Gaffney, Practical considerations for the calculation of order parameters for fatty acid or phospholipid spin labels in membranes, in: L.J. Berliner (Ed.), Spin Labeling. Theory and Applications, Academic Press, New York, 1976, p. 567.

[27] W.L. Hubbell, H.M. McConnell, Molecular motion in spin-labeled phospholipids and membranes, J. Am. Chem. Soc. 93 (1971) 314–326.

[28] D. Marsh, K. Schorn, Corrections for anisotropically averaged hyperfine splittings and order parameters from pseudopowder electron paramagnetic resonance (EPR) line shapes, in: L.J. Berliner (Ed.), Spin Labelling. The Next Millennium, 14, Plenum Press, New York, 1998, p. 405.

[29] D.J. Schneider, J.H. Freed, Calculating slow motional magnetic resonance spectra: a user's guide, in: L.J. Berliner, J. Reuben (Eds.), Spin Labelling. Theory and Applications, 8, Plenum Press, New York, 1989, p. 1.

[30] D.E. Budil, S. Lee, S. Saxena, J.H. Freed, Nonlinear-least-squares analysis of slow-motion EPR spectra in one and two dimensions using a modified Levenberg–Marquardt algorithm, J. Mag. Res. 120 (1996) 155–189.

[31] M. Ge, J.H. Freed, Electron-spin resonance study of aggregation of gramicidin in dipalmitoylphosphatidylcholine bilayers and hydrophobic mismatch, Biophys. J. 76 (1999) 264–280.

[32] K.A. Earle, J.K. Moscicki, M. Ge, D.E. Budil, J.H. Freed, 250-GHz electron spin resonance studies of polarity gradients along the aliphatic chains in phospholipid membranes, Biophys. J. 66 (1994) 1213–1221.

[33] A. Hudson, G.R. Luckhurst, The electron resonance line shapes of radicals in solution, Chem. Rev. 69 (1969) 191–225.

[34] H. Schindler, J. Seelig, EPR spectra of spin labels in lipid bilayers, J. Chem. Phys. 59 (1973) 1841–1850.

[35] S. Schreier-Muccilo, D. Marsh, H. Dugas, H. Schneider, I.C.P. Smith, A spin probe study of the influence of cholesterol on motion and orientation of phospholipids in oriented multibilayers and vesicles, Chem. Phys. Lipids 10 (1973) 11–27.

[36] J.B. Feix, C.A. Popp, S.D. Venkataramu, A.H. Beth, J.H. Park, J.S. Hyde, An electron-electron double-resonance study of interactions between [^{14}N]- and [^{15}N] stearic

acid spin-label pairs: lateral diffusion and vertical fluctuations in dimyristoylphosphatidylcholine, Biochemistry 23 (1984) 2293–2299.

[37] J.F. Ellena, S.J. Archer, R.N. Dominey, B.D. Hill, D.S. Cafiso, Localizing the nitroxide group of fatty acid and voltage-sensitive spin-labels in phospholipid bilayers, Biochim. Biophys. Acta 940 (1988) 63–70.

[38] D. Marsh, Experimental methods in spin-label spectral analysis, in: L.J. Berliner, J. Reuben (Eds.), Spin Labelling. Theory and Applications, 8, Plenum Press, New York, 1989, p. 255.

[39] R.E. Jacobs, S.H. White, The nature of the hydrophobic binding of small peptides at the bilayer interface: implications for the insertion of transbilayer helices, Biochemistry 28 (1989) 3421–3437.

[40] R.F. Turchiello, L. Juliano, A.S. Ito, M.T. Lamy-Freund, How bradikinin alters the lipid membrane structure. A spin label comparative study with bradykinin fragments and other cations. Biopolymers (2000) in press.

[41] M. Ptak, M. Egret-Charlier, A. Sanson, O. Boloussa, A NMR study of the ionization of fatty acids, fatty amines and *n*-acylamino acids incorporated in phosphatidylcholine vesicles, Biochim. Biophys. Acta 600 (1980) 387–397.

[42] M.E. Johnson, Apparent hydrogen bonding by strongly immobilized spin-labels, Biochemistry 20 (1981) 3319–3328.