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Spin label and ^{2}H-NMR studies on the interaction of melanotropic peptides with lipid bilayers

Marcia H. Biaggi [1], **Teresa J. T. Pinheiro** [2,*], **Anthony Watts** [2], **M. Teresa Lamy-Freund** [1]

[1] Instituto de Física, Universidade de São Paulo, CP 66318, CEP 05389-970, São Paulo, Brasil (Tel.: 0055-11-818-6829, Fax: 0055-11-813-4334, e-mail: MTFREUND @ IF.USP.BR.)
[2] Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

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Abstract. The interaction of the cationic tridecapeptide α-melanocyte stimulating hormone (α-MSH) and the biologically more active analog [Nle^4, DPhe^7]-α-MSH with lipid membranes was investigated by means of ESR of spin probes incorporated in the bilayer, and NMR of deuterated lipids. All spin labels used here, stearic acid and phospholipid derivatives labeled at the 5th and 12th position of the hydrocarbon chain, and the cholestane label, incorporated into anionic vesicles of DMPG (1,2-dimyristoyl-sn-glycero-3-phosphoglycerol) in the liquid-crystalline phase, indicated that both peptides decrease the motional freedom of the acyl chains. No peptide effect was detected with neutral lipid bilayers. Changes in the α-deuteron quadrupolar splittings and spin lattice relaxation time of DMPG deuterated at the glycerol headgroup paralleled the results obtained with ESR, showing that the peptides cause a better packing both at the headgroup and at the acyl chain bilayer regions. The stronger effect caused by the more potent analog in the membrane structure, when compared to the native hormone, is discussed in terms of its larger lipid association constant and/or its deeper penetration into the bilayer.

Key words: Melanotropic peptide – Peptide-lipid interaction – Spin label – ^{2}H-NMR

Introduction

α-Melanocyte Stimulating Hormone (α-MSH; Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) is secreted by the *pars intermedia* of the pituitary gland, and is derived from the precursor protein pro-opiomelanocortin. It has the same first thirteen amino acids in the sequence as the largest pituitary hormone adrenocorticotropin (ACTH), with which it shares a number of common hormonal properties (Sawyer et al. 1980; Castrucci et al. 1990). In most vertebrates, α-MSH is the physiologically relevant hormone regulating skin pigmentation (causing darkening), as well as being involved in many other biological functions, such as fetal growth and behavior (Castrucci et al. 1990).

Several modifications made within the primary structure of α-MSH have yielded peptides with superpotent activity (Sawyer et al. 1980). For example, the native hormone derivative [Nle^4, DPhe^7]-α-MSH (hereafter referred to as MSH-I), which exhibits increased potency and prolonged activity in the frog skin bioassay when compared to the native molecule (Sawyer et al. 1980). It was suggested that the increased potency of MSH-I could be attributed to a reverse turn within the α-MSH$_{4-10}$ sequence which is conformationally-stabilized by the D-Phe^7 substitution. The conformational structure of the analog could facilitate its direct binding to membrane protein receptors. On the other hand, considering the possible catalytic effect of the lipid phase of the cell membrane on peptide-membrane protein interaction (Sargent and Schwyzer 1986; Schwyzer 1986), this present study focuses on the interaction of the two peptides, α-MSH and MSH-I, with pure lipid bilayers. A comparison between the strength and the nature of the interaction of the two peptides with multilamellar liposomes, used here as *in vitro* models of biological membranes, may help in an understanding of the role played by the lipid phase in the peptide action.

It was reported that both the native hormone α-MSH and the analog MSH-I interact with acidic lipid bilayers and not with neutral ones (Ito et al. 1993). This is in accord with them being cationic at neutral pH, with approximately one net positive charge. Variations in the tryptophan fluorescence properties indicated structural changes on incorporation of the two peptides into DMPG (1,2-dimyristoyl-sn-glycero-3-phosphoglycerol), DMPS (1,2-dimyristoyl-sn-glycero-3-phosphoserine) and POPS (1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphoserine) bilayers (Ito et al. 1993). Larger association constants were ob-

* *Present address:* Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Correspondence to: M. Teresa Lamy-Freund

tained for the analog when compared to the native hormone. This result parallels the reported biological activities of the peptides. Additionally, ESR studies using the 3-doxyl cholestane spin label (CSL) incorporated in oriented bilayers indicated that the peptides have opposite effects on the order and rate of movement of the label in DMPG membranes (Biaggi et al. 1993). The native hormone induced greater rate of chain motion while the analog increased the chain packing.

The present work studies the interaction of α-MSH and the analog MSH-I with pure acidic DMPG liposomes, using different spin label molecules incorporated in the bilayer, and deuterated headgroup lipids. Spin label ESR and ^{2}H NMR are particularly suitable methods for the study of membrane structural and/or dynamics changes caused by peptide-lipid interaction (see for example, Sankaran et al. 1989; Watts 1993). The acyl chain spin labels, stearic acid and phospholipid derivatives, report on the gauche-trans isomerization of the acyl chains, whereas the cholestane spin label gives information about the bilayer orientation or the lipid rotational motion as a whole. The spin labels used here give complementary information on the fluidity of the membrane hydrocarbon region. The bilayer surface was monitored via the deuterium quadrupolar interaction of the α, β and γ segments of the glycerol headgroup.

Materials and methods

Materials

The peptides α-MSH and MSH-I, the spin labels 5- and 12-SASL (5- and 12-doxylstearic acid spin label) and the cholestane spin label CSL were purchased from Sigma Chemical Co. (St. Louis, MO). Phospholipids, DMPG (1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol) and DMPC (1,2-Dimyristoyl-sn-glycero-3-phosphocholine), and phospholipid spin labels 5- and 12-PCSL (1-palmitoyl-2-[4- or 12-doxyl stearoyl-sn-glycero-3-phosphocholine) were obtained from Avanti Polar Lipids (Birmingham, AL). The buffer used was 10 mM hepes (4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid) at pH 7.4. All reagents were used without further purification. DMPC-d_4 and DMPG-d_5 were synthesized as described in Sixl and Watts (1982) and Sixl et al. (1984).

ESR sample preparation

Stock solutions of spin labels were prepared in chloroform and stored at –80 °C. The membranes were multilamellar dispersions of lipids (10 mM) containing 1 mol% of spin label. A lipid film was formed from a chloroform solution of lipids and spin labels, dried under a stream of N_2 and left under vacuum for a minimum of 5 h, to remove all traces of the organic solvent. Liposomes were prepared by the addition of the buffer solution, without or with the desired concentration of melanotropic peptides, followed by vortexing.

NMR sample preparation

Pure deuterated lipid samples were prepared as described above, but with a higher concentration of lipids (0.3 M).

ESR spectroscopy

ESR measurements were performed in a Bruker ER 200D-SRC spectrometer interfaced with an IBM-PC like computer for spectrum digitalization. A field modulation amplitude of 0.08 mT and microwave power of 10 mW were used. The temperature was controlled to about 0.5 °C with a Bruker B-ST 100/700 variable temperature device. The temperature was always varied from 40 to 0 °C and monitored with a Fluke 51 K/J thermometer. As the stearic acid spin labels presented a small partition in solution (12-SASL mainly), the ESR spectra shown here are the result of the subtraction of free aqueous label signal from the composite spectrum, using the software *EPRANALA* (J. Rowntree, P. Fajer and B. Bennet, University of Oxford, UK). For the measurement of the spectrum parameters the ORIGIN software (MicroCal Software, Inc., MA, USA) was used.

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

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

were $2A_{//}$ is the maximum hyperfine splitting and

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

where $2A_{min}$ is the measured inner hyperfine splitting (Griffith and Jost 1976; Gaffney 1976). The polarity was normalized from a_o'/a_o where

$a_o' = (1/3)\,(A_{xx} + A_{yy} + A_{zz})$

and

$a_o = (1/3)\,(A_{//} + 2\,A_{\perp})$

A_{xx}, A_{yy} and A_{zz} being the principal values of the hyperfine tensor for doxylpropane (Griffith and Jost 1976). The data shown here are the mean of at least three experiments.

NMR spectroscopy

^{2}H NMR spectra were recorded on a home built 360 MHz (H_o = 8.4 T) spectrometer (Department of Biochemistry, University of Oxford), equipped with a Nicolet pulse programmer and operating at 55.3 MHz for the ^{2}H nucleus. Single pulse modes were used with $\pi/2$ pulse widths varying from 7 to 9 µs. Deuterium spin-lattice relaxation times T_1 were measured by the inversion-recovery technique, a 180°-τ-90° pulse sequence being employed. Temperature was controlled to an accuracy of ±0.5 °C by a nitrogen gas flow temperature unit. The data shown here are the mean of at least three experiments.

Results and discussion

ESR of stearic acid and phospholipid spin labels

Figure 1 shows the ESR spectra of stearic acids labeled at the 5th and 12th position of the hydrocarbon chain incorporated in anionic vesicles of DMPG, in the absence and presence of melanotropic peptides. The spectra were recorded at 40 °C, above the gel-to-liquid crystalline phase transition temperature of DMPG (T_m ~ 21 °C). At the two positions of the spin label group the ESR spectrum is sensitive to the presence of the cationic peptides. Both α-MSH and MSH-I increase the spectral anisotropy, giving rise to spectra that are broader and have larger hyperfine splittings than the spectra for the lipid alone. This corresponds to a restriction of the motion of the spin-labeled chains in the presence of the peptides (Griffith and Jost 1976). No change in the ESR spectra was observed when the two peptides were added to bilayers of the zwitterionic lipid DMPC.

The effect of the peptides on the dynamics and structural properties of DMPG bilayers was quantified by measuring the outer hyperfine splitting A_{max}. This is a useful empirical parameter which contains contributions from both the amplitude and the rate of motion of the spin-labeled chains (Marsh 1981 and references therein). The value of A_{max} decreases as the spin label micro-environment becomes less organized and/or more mobile. The temperature dependence of A_{max} is shown in Fig. 2, in the presence and absence of peptides, for stearic acid and phospholipid labels. All labels monitor a phase transition around 21 °C. This T_m value is in accord with that found before (Heimburg and Biltonen, 1994) for 10 mM DMPG at low ionic strength. Above the transition temperature both peptides increase the A_{max} values of labels placed either near the headgroup (5-SASL and 5-PCSL) or deep in the bilayer (12-SASL and 12-PCSL). The analog MSH-I is more efficient in restricting the lipids mobility than the natural hormone. This result could be related to either the larger value of the peptide-lipid association constant found for the analog relative to the native hormone (Ito et al. 1993), or could be attributed to a deeper penetration of the analog into the bilayer, or to both effects.

The bilayer phase transition monitored by the acyl chain spin-labels clearly reflects a broadening of the DMPG main chain melting phase transition caused by the peptide-lipid interaction, with a more pronounced effect with MSH-I. This effect is similar to that observed with other molecules, such as polar carotenoids and cholesterol, which are thought to penetrate the lipid bilayer (Subczynski et al. 1992). For the nitroxide monitoring the 12th position of the hydrocarbon chain (12-SASL and 12-PCSL) the effect of the peptides on the membrane structure below the phase transition is rather complex and subtle, and will not be discussed here.

The slightly different effects obtained with the stearic acid labels when compared to their equivalent phospholipid labels can probably be attributed to their relatively different positions in the lipid bilayer. Owing to the surface negative charge of the DMPG membrane, the stearic acid labels should be mainly protonated at pH 7.4 (pK_a ≥8.0, Ptak et al. 1980), and could penetrate more deeply in the bilayer. This is in agreement with the lower values of A_{max} obtained for 5-SASL and 12-SASL when compared to 5-PCSL and 12-PCSL, respectively (mainly above T_m).

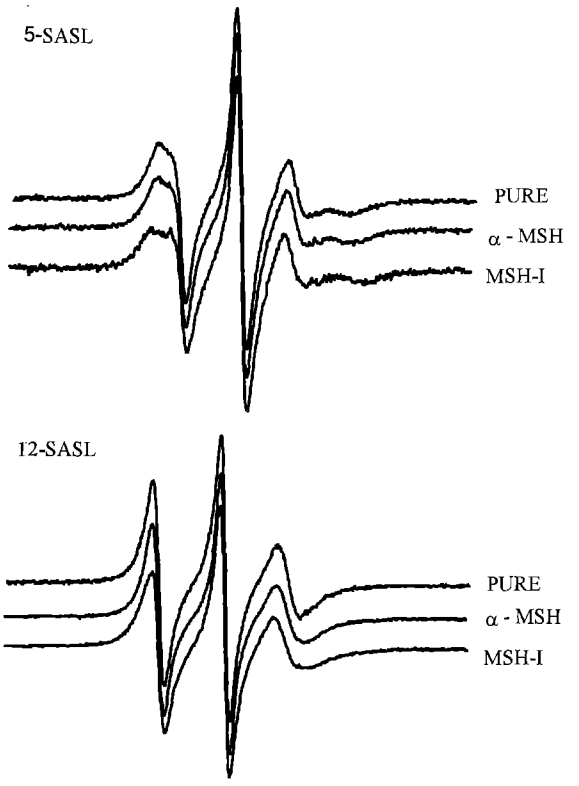

Fig. 1. ESR spectra of 5-SASL and 12-SASL incorporated in DMPG liposomes in the absence and in the presence of 10 mol% peptide. Total spectrum width is 100 G. T = 40 °C

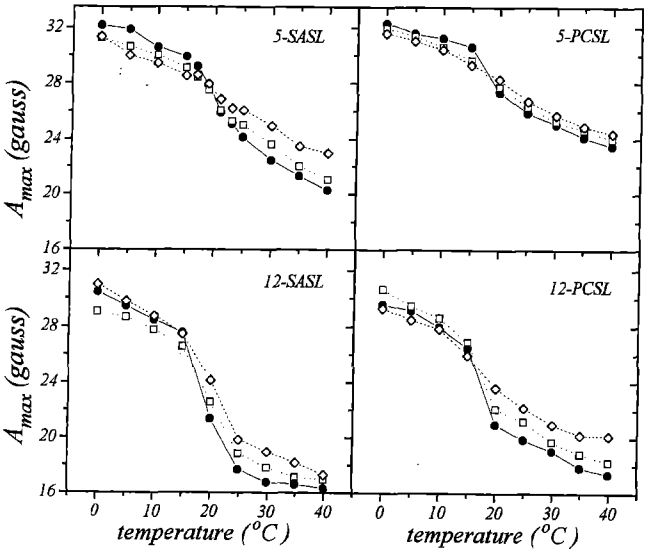

Fig. 2. Temperature profiles of maximum hyperfine splitting (A_{max}) of different acyl chain spin labels incorporated in DMPG liposomes in the presence of 0, (●), and 10 mol% α-MSH (□) and MSH-1 (◇)

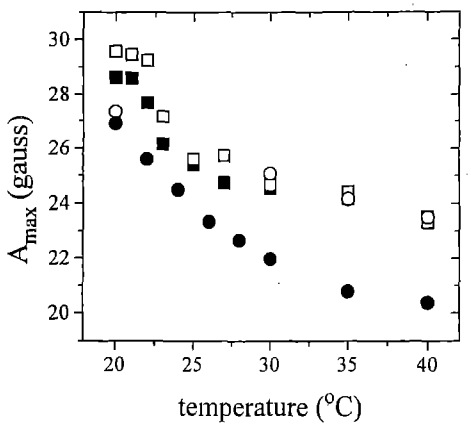

Fig. 3. Temperature dependence of A_{max} values of 5-SASL (filled symbols) and 5-PCSL (open symbols) in DMPC (■ and □) and DMPG (● and ○) membranes

Table 1. Effect of melanotropic peptides (10 mol%) on the isotropic hyperfine constant a_o (Gauss) of two different acyl chain spin labels

	5-PCSL	12-PCSL
DMPG	14.95	14.21
+ α-MSH	15.05	14.00
+ MSH-I	14.97	13.63

Table 2. Effect of melanotropic peptides (10 mol%) on the effective order parameter S_{eff} of two different acyl chain spin labels. T=35 °C

	5-SASL	12-SASL
DMPG	0.324	0.103
+ α-MSH	0.350	0.125
+ MSH-I	0.434	0.164

To further illustrate the dependence of the surface charge on the relative positions of stearic acid and phospholipid labels in bilayers, the same labels were incorported in DMPC vesicles. In this zwitterionic membrane one would expect that the stearic acid would be mainly deprotonated at pH 7.4 (pK_a ~6.5, Ptak et al. 1980). Therefore the charged SASL incorporated in DMPC would be located in a position closer to the bilayer surface than when in DMPG vesicles. On the other hand, the depth position of the PCSL should not be a function of the membrane surface charge. Accordingly, Fig. 3 shows that the A_{max} values of 5-SASL and 5-PCSL in DMPC are very similar and also similar to that of 5-PCSL in DMPG, whereas 5-SASL in DMPG yields smaller A_{max} values. If we assume similar packing for DMPC and DMPG bilayers [1] this result incidates that 5-PCSL in both DMPG and DMPC and 5-SASL in DMPC are located at similar depths, and the neutral 5-SASL in DMPG is clearly located deeper into the bilayer, presenting a smaller A_{max} value. The above discussion can be understood with reference to the spin label average position, as it is known that acyl labels placed deep in the membrane undergo large vertical fluctuations (Godici and Landsberger 1974; Ellena et al. 1988; Yin et al. 1988).

To investigate the possible effect of the peptides on the polarity of the phospholipids spin label environment, effective isotropic hyperfine constants (a_o) were calculated (see Materials and methods) in the absence and in the presence of peptides. The values of a_o are known to be dependent on the net unpaired spin density on the N-atom of the nitroxide, which is a function of the nitroxide micro-environment (Seelig et al. 1972). An increase in polarity is accompanied by an increase in the isotropic hyperfine splitting. Above 25 °C a_o values were approximately independent of the temperature and Table 1 displays the averaged values from 25–40 °C. As expected, the nitroxide closer to the polar-apolar interface (5-PCSL) senses a more polar region than that closer to the terminal methyl ends of the chains (12-PCSL). The polarity of the upper region of the bilayer is not significantly affected by the presence of the peptides, as monitored by the a_o value of 5-PCSL. However, the polarity of the micro-region monitored by the 12-PCSL decreases upon the interaction with the peptides. Again, the analog is more effective in changing the a_o value than α-MSH. The above data are consistent with the binding of the peptides resulting in a decrease in the motional freedom of the acyl chains, therefore causing a decrease on the vertical fluctuations of the labels. The spin label positioned at the 5th carbon would not be much affected, as its vertical fluctuation is not very large (Yin et al. 1988), but the label at the 12th position would be restricted to a region deep in the bilayer, resulting in an average lower polarity for its micro-environment.

Effective order parameters (S_{eff}) were calculated (see Materials and methods) from ESR signals above the gel-fluid phase transition, when the spin-label motion is fast on the ESR time-scale (correlation time $\tau \leq 3\times10^{-9}$ s). It has been shown that spectra similar to those shown in Fig. 1 also contain important contributions from slow molecular motions (Lange et al. 1985). Therefore, S_{eff} is a parameter similar to A_{max} in the sense that it contains contributions from both order and rate of motion, although the principal contribution to S_{eff} is the amplitude of the segmental motion of the alkyl chain (Schindler and Seelig 1973). Unlike A_{max}, S_{eff} is not influenced by the polarity of the spin label environment, as the proper correction was made (see Materials and methods). Calculated S_{eff} values for 5- and 12-SASL at 35 °C (Table 2) show similar dependences on the presence of α-MSH and MSH-I as the A_{max} values measured above the lipid transition temperature (Fig. 2).

The S_{eff} dependence on peptide concentration is shown in Fig. 4. When monitored by the 12-SASL, 5 mol% is a membrane saturating concentration for both peptides. Slightly different results were obtained with other spin labels (not shown). It is seen that the effect of MSH-I on the membrane structure is greater than that of the native hormone, at all concentrations studied here.

[1] It is a reasonable assumption as the two lipids have similar transition temperatures, despite the areas per lipid head group being slightly different, according to Pasher et al. 1992.

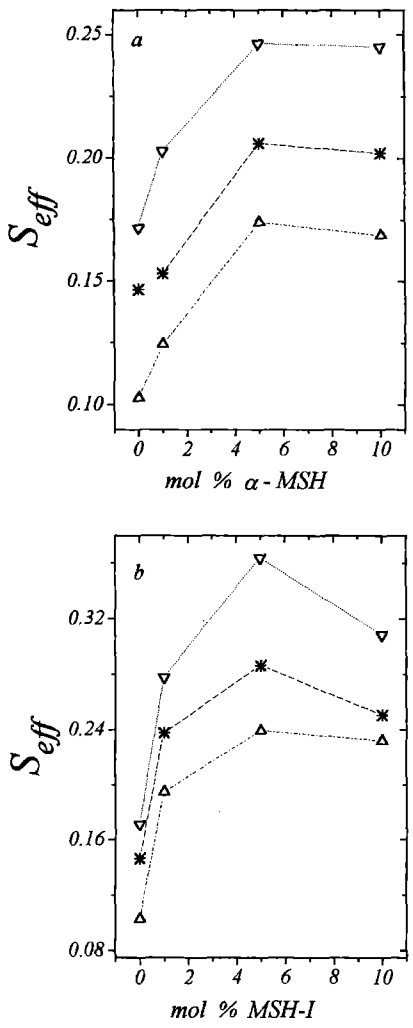

Fig. 4. Dependence of the effective order parameter (S_{eff}) of 12-SASL in DMPG liposomes on the mole fraction of α-MSH **a** and MSH-I **b** at 30 °C (▽), 35 °C (*) and 40 °C (△)

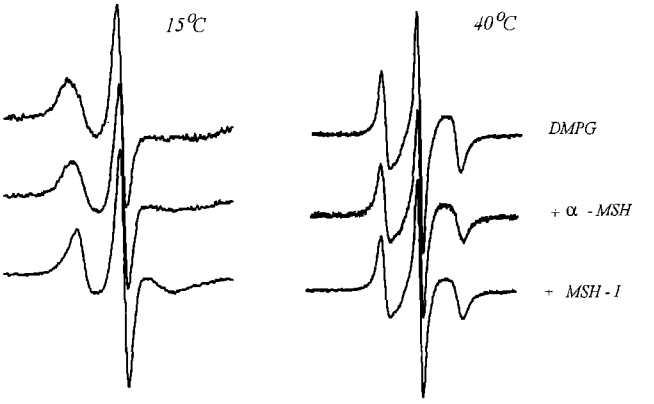

Fig. 5. ESR spectra of CSL incorporated in DMPG liposomes in the absence and in the presence of 10 mol% peptide, below and above the lipid main phase transition. Total spectra width is 100 G

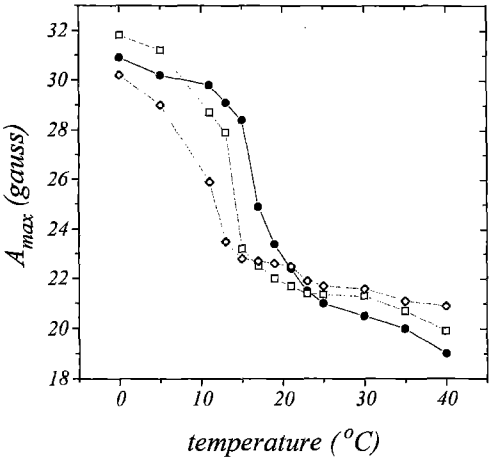

Fig. 6. Temperature profiles of maximum hyperfine splitting (A_{max}) of CSL incorporated in DMPG liposomes in the presence of 0 (●), and 10 ml% α-MSH (□) and MSH-I (◇)

ESR of cholestane spin label

For a further understanding of the effects of melanotropic peptides on lipid bilayers, another type of spin probe was used, the cholesterol analog CSL. This label has a fairly rigid structure and gives information on the rate of lipid rotational long axis motion, whereas the acyl chain spin labels monitor mainly the amplitude and rate of segmental motion (*gauche-trans* isomerism) (Schindler and Seelig 1973, 1974). Figure 5 displays typical spectra of CSL incorported in DMPG bilayers in the absence and presence of peptides, below and above the main phase transition temperature. MSH-I significantly changes the form of the CSL ESR signal at low temperatures, indicating a decrease in the spectral anisotropy caused by the peptide-lipid interaction. In a similar way to the results obtained with the acyl chain labels, the peptides cause no significant alteration in the ESR spectra of CSL incorporated into DMPC liposomes.

The ESR spectra of CSL in DMPG bilayers were analyzed by measuring the outer hyperfine splitting A_{max} (Fig. 6) over a wide range of temperature, though the A_{max} values at low temperatures are rather difficult to measure accurately. In line with the results obtained with the acyl chain labels, both peptides increase the A_{max} values above the main phase transition, having therefore a packing effect on the lipid bilayer. However, in contrast to the other spin labels, CSL monitors a significant shift on the lipid main phase transition temperature caused by the presence of the analog MSH-I. This is consistent with partial penetration of the peptide into the hydrocarbon region of the bilayer preventing crystallization of the chains until a lower temperature is reached.

It is interesting to note that the main DMPG melting temperature, monitored via the cholestane spin label (Fig. 6) is shifted a few degrees to lower values, when compared with that yielded by the acyl chain spin labels (Fig. 2). Different temperature profiles, yielding different T_m values, with sterol and acyl chain labels have been seen before, though not extensively discussed (for instance see Marsh 1980; Subczynski et al. 1992). CSL has also been reported to induce an additional temperature transition at ~20 °C in different phospholipid (Presti and Chan 1982), although DMPG was not studied. In DMPC, which has a

transition temperature similar to that of DMPG, around 23 °C (Watts et al. 1978), CSL was found to yield a T_m value a few units lower, around 17 °C (Fig. 2 in Presti and Chan 1982). This result is in accord with that shown here (Fig. 6).

On the other hand, particularly for DMPG, it has been recently shown that the phospholipid bilayer thermotropic behavior is rather complex (Heimburg and Biltonen 1994), depending on many different factors, such as lipid concentration, ionic strength, and rate of heating or cooling. It was suggested that DMPG exists as several slightly different structures, over a broad temperature range. Therefore, the reason why CSL, incorporated in DMPG, monitors a somewhat different T_m than the acyl chain labels needs to be further investigated. For instance, it could be related to an alteration of the equilibrium between the different DMPG structures caused by the sterol label (even at concentrations as low as 1 mol%), or the cholestane label could be preferentially distributed in a region different to that labeled by the acyl chain probes. The role the melanotropic peptides play in the thermotropic equilibrium of the possible different DMPG structures is also not resolved.

It is important to point out that the data shown here, for the different spin labels incorporated in DMPG liposomes, do not agree with the results obtained with CSL in oriented bilayers (Biaggi et al. 1993). It was reported that while the natural hormone decreased both the order parameter and the rotation correlation time, the analog caused an opposite effect (Biaggi et al. 1993). Although the oriented films contained 10 mol% cholesterol, which was necessary for promoting the orientation of the lipid bilayers, and the spectra were recorded at 40 °C only, the discrepancy between the results obtained with the two lipid systems needs further investigation, including a possible cholesterol dependent effect.

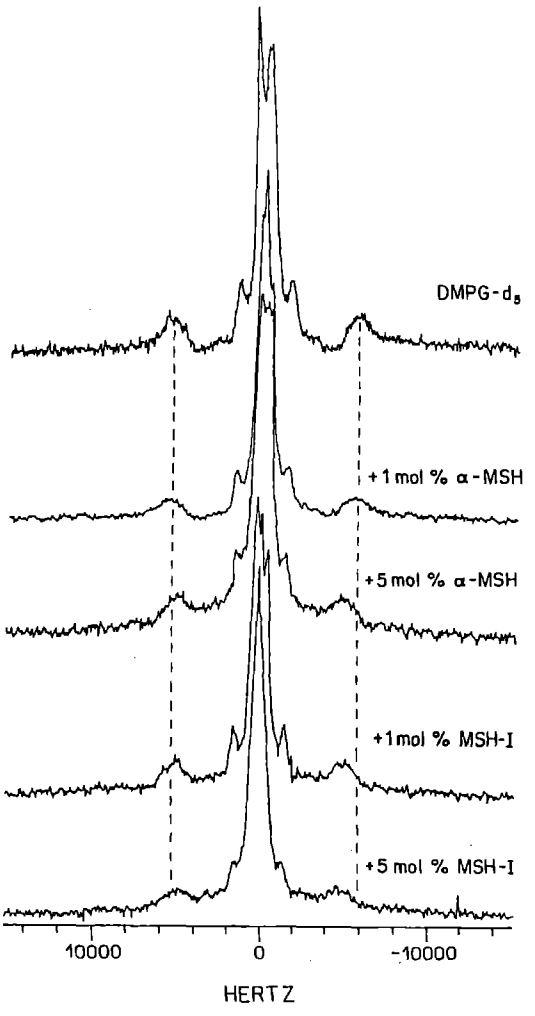

Fig. 7. Deuterium quadrupolar NMR spectra of DMPG-d5 liposomes in the presence of different concentrations of α-MSH and MSH-I

NMR of headgroup deuterated DMPG-d_5 and DMPC-d_4

The ^2H NMR spectra at 35 °C of DMPG deuterated at the α, β and γ segments of the glycerol headgroup are shown in Fig. 7. The spectra are typical of random dispersions of liquid-crystalline lipids in a bilayer arrangement (Seelig 1977). Although they are quite noisy and do not present perfect Pake shapes, the separation between the maxima provides an approximate evaluation of the deuterium quadrupolar splitting ($\Delta\nu_Q$), which are related to changes of the headgroup conformation. The addition of the cationic peptides α-MSH or MSH-I to the negative DMPG membrane decreases the α-quadrupolar splitting without affecting significantly the β- and γ-splitting, within the accuracy of the measurement. Increasing the temperature from 25 to 45 °C causes only a slight variation of the α-quadrupolar splitting (Fig. 8), both in the presence and absence of peptides. For temperatures below 23 °C, a broadening of the ^2H NMR spectra occurs as expected for lipids in the gel phase. Although the interaction of the two peptides with DMPG membrane causes similar effects on the α-quadrupolar splitting, the maximum splitting decrease is achieved with only 1 mol% MSH-I whereas 5 mol% is necessary for the natural hormone (Fig. 7 and Fig. 8). This is certainly a strong indication of a higher partition coefficient for MSH-I than for α-MSH in DMPG vesicles. At the concentrations used here, the peptides do not significantly affect the quadrupolar splitting of headgroup deuterated DMPC at the α and β positions. Under no conditions were separated spectra for free lipid and peptide-bound lipid observed. The deuterium spectra thus indicate a single, time-average headgroup conformation at the NMR time scale in all peptide-lipid systems studied.

The spin lattice relaxation times (T_1) for all the deuterated segments of DMPG are displayed in Table 3. They give information about the dynamic behavior of the deuterated segments. Upon peptide addition, the T_1 values of the α-deuterons of DMPG decrease whereas the T_1 values related to the β- and γ-splitting remain unaffected. From the deuterium spin-lattice relaxation times, in the limit of small order parameters, it is possible to estimate an effective correlation time (τ_c) for the CD fast, local, segmental motion (Brown et al. 1979; Davis 1983), according to the equation

$$\frac{1}{T_1} = \frac{3}{8}\left(\frac{e^2qQ}{\hbar}\right)^2 \tau_c$$

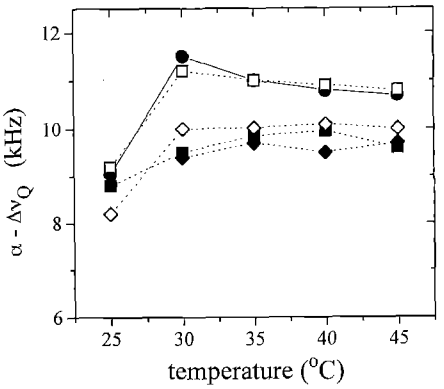

Fig. 8. Temperature dependence of the α-quadrupolar splitting (α-$\Delta\nu_Q$) of headgroup deuterated DMPG in the presence of 0 (●), 1 (□) and 5 mol% α-MSH (■) and 1 (◇) and 5 mol% of MSH-I (◆)

Table 3. Deuterium NMR spin-lattice relaxation times T_1 (ms) for DMPG-d_5 multilamellar liposomes at 35 °C

	α-CD_2	β-CD	γ-CD_2
DMPG	17.0 ± 0.4 (5.4)[a]	14.0 ± 0.9	19.2 ± 0.2
+ 1% α-MSH	15.5 ± 0.8 (5.9)	14.0 ± 0.7	18.7 ± 0.1
+ 5% α-MSH	14.8 ± 0.8 (6.2)	14.9 ± 0.4	18.2 ± 0.1
+ 1% MSH-I	15.2 ± 0.5 (6.1)	14.0 ± 0.8	17.8 ± 0.5
+ 5% MSH-I	14.0 ± 1.0 (6.6)	14.0 ± 0.9	18.6 ± 0.6

[a] Numbers in parentheses refer to correlation times τ_c (see text)

where ($e^2qQ/\hbar$) is the static quadrupolar splitting constant.

Table 3 shows a decrease in the headgroup mobility caused by the peptide-membrane interaction. In agreement with the observed variations in the quadrupolar splitting, the analog MSH-I is more effective in changing the rates of motion than the natural hormone.

The small observed decrease in the DMPG α-CD quadrupolar splitting can be attributed to changes in the headgroup position rather than an increase of the amplitude of angular fluctuations, since the quadrupolar splittings are very sensitive to small variations in torsion angles (Seelig et al. 1987). Considering the change in the deuterium quadrupolar splitting, the effect of the cationic peptides upon DMPG bilayers could be attributed to variations in the surface charge density only (Seelig et al. 1987; Marassi and Macdonald 1991). However, the increase in the α-deuteron spin lattice relaxation time shows that the interaction of the melanotropic peptides with DMPG bilayers not only changes the headgroup dipole moment direction, but also alters the intermolecular interaction between neighboring headgroups. Although the deuterium NMR results do not allow any conclusion about the depth of bilayer penetration by the peptides, they indicate that the melanotropic peptides interact at least at the membrane surface, decreasing the DMPG net charge density and therefore allowing a better packing of the phospholipid headgroups. Upon interaction with PG membranes, other cationic molecules have been found to decrease the lipid α-CD_2 quadrupolar splitting (Beschiaschvili and Seelig 1990; Marassi and Macdonald 1991) and decrease both the splitting values and the spin-lattice relaxation times (Sixl et al. 1984; Sixl and Watts 1985).

The changes that occur in the ^2H NMR quadrupolar spectra of head group deuterated DMPG, due exclusively to surface charge variations, are expected to be much smaller than those for deuterated DMPC since both the orientation and the size of the PC dipole moment facilitates its interaction with the electrical field at the surface (Beschiaschvili and Seelig 1990; Marassi and Macdonald 1991). The size of the PG dipolar moment is around 1.7 times smaller than that of PC. The latter has been extensively used to monitor the interaction of different molecules with PC bilayers (for instance, Brown and Seelig 1977; Roux et al. 1988; Scherer and Seelig 1989; Pinheiro et al. 1994). Like other cationic molecules (Sixl et al. 1984; Sixl and Watts 1985; Marassi and Macdonald 1991), the melanotropic peptides to not alter the DMPC quadrupolar splitting.

General discussion

In line with previous results (Biaggi et al. 1993; Ito et al. 1993) it was found here that an obligatory role is played by electrostatic interactions in the binding of the cationic melanotropic peptides to lipid bilayers. The peptides were not found to interact with the neutral lipid DMPC, in contrast to the acidic lipid DMPG. This result can be attributed to the increase in the concentration of free peptide near the lipid-water interface due to electrostatic attraction between peptides and DMPG headgroups. Using fluorescence spectroscopy it was shown that association constant values for peptide-lipid interaction were approximately three orders of magnitude smaller when calculated accounting for electrostatic effects, through Gouy-Chapman potential functions (Ito et al. 1993). Thus, for monitoring membrane structural alterations caused by melanotropic peptides on the zwitterionic DMPC bilayer a much larger peptide concentration would be necessary.

The peptide-lipid interaction is primarily electrostatic in origin. The increase of the outer hyperfine splitting and the order parameters of the stearic acid and phospholipid spin labels could be attributed to the presence of the cationic peptides on the bilayer surface, which would decrease the membrane surface net charge, leading to a better packing of the hydrocarbon chains. However, several factors must be considered in favor of a partial penetration of the peptides into the hydrocarbon region of the bilayer, as discussed below.

The peptides are relatively small molecules[2], when compared to proteins, with only approximately one net

[2] Molecular dynamics simulations yielded an average area of 260 Å^2 for the peptides, both in aqueous and membrane medium (Pascutti et al., unpublished results). The molecules have not been simulated at the interface yet. Taking the area per lipid molecule of 44.0 Å^2 (Pasher et al. 1992), yields a stoichiometry of ca. 6 lipids per peptide, at the membrane surface.

charge. Considering their low lipid association constants (Ito et al. 1993), the peptide concentration on the membrane surface will be much lower than the total concentation used here, 10 mol% of the total lipids. Thus, the increase of the A_{max} parameter due to the screening of the lipid headgroups by the charged lipids should not be very relevant. We found a very small difference in A_{max} values for 12-PCSL incorporated in DMPG in the absence and in the presence of 0.3 M NaCl (results not shown). Sankaram et al. (1989) found a slight A_{max} increase of 5-PGSL in DMPG for NaCl concentrations above 1.0 M. Therefore, the alterations on the A_{max} values of the different labels used here, caused mainly by the analog MSH-I, could hardly be due to a rigidifying effect as a result of, exclusively, electrostatic surface interaction.

Furthermore, the broadening of the DMPG gel-to-liquid crystalline phase transition monitored by the 5-SASL and 5-PCSL is a strong indication of the penetration of the peptides into the bilayer. A simple screening of the electrostatic charges at the membrane surface would not decrease the A_{max} values below T_m. Those probes indicate that the peptides are bound to the membrane both above and below the transition temperature.

The shift in the DMPG transition temperature, monitored by CSL, due to the presence of the analog MSH-I is rather interesting, and needs further investigation. The effect of a screening of the surface charges would go in the opposite direction, with an increase of T_m, which is observed for uncharged DMPG at low pH values (Watts et al. 1978) and DMPG in high ionic strength medium (Cvec et al. 1980). It has been recently shown by fluorescence spectroscopy, that the analog MSH-I penetrates deeper in DMPG membranes than the native hormone (Macêdo et al. 1996). This is in accord with the larger effect obtained here with MSH-I as compared to α-MSH.

This work shows that both α-MSH and MSH-I interact with DMPG lipid bilayer turning the membrane more rigid at all depth positions monitored here. The analog was found to cause a stronger effect on the membrane than the native hormone, possibly related to both its larger lipid association constant (Ito et al. 1993) and its deeper penetration into the bilayer (Macêdo et al. 1996). The stronger interaction of MSH-I with the lipid phase of biological membranes could partially explain its superpotent activity. As suggested before (Schwyzer 1986), the membrane could play a role as catalyst for the peptide-receptor interaction, either by selecting the proper peptide conformation or just by increasing the chances of peptide-receptor contact, via lateral diffusion. It could also be speculated that the depth of peptide penetration could facilitate its binding to the discriminator receptor site.

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