How melatonin interacts with lipid bilayers: a study by fluorescence and ESR spectroscopies

Ernane J.X. Costa[1], Claudio S. Shida, Márcia H. Biaggi, Amando S. Ito, M. Teresa Lamy-Freund*

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

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Abstract ESR spectra of spin labels placed at the membrane surface and at different depths of the bilayer core, and melatonin fluorescence in the presence of lipid vesicles, suggest an average shallow position for the hormone in the membrane. However, according to the melatonin ability to cross lipid bilayers, nitroxides placed deep in the bilayer were able to quench the melatonin fluorescence. Melatonin membrane partition coefficients were calculated for bilayers in different packing states, and similar and rather high values were found. The data presented here may be quite important to the understanding of melatonin physiological actions at the membrane level.

Key words: Melatonin; Lipid bilayer; Fluorescence; Spin label

1. Introduction

Melatonin (5-methoxy-*N*-acetyltryptamine), a pineal hormone derived from tryptophan, has been reported to interact with many different cells, playing a number of distinct physiological roles. The hormone has been shown to participate in many biological process via its interaction with membrane protein receptors (for instance see [1–4]). On the other hand, melatonin's action as a free radical scavenger is well established [5–7], being effective in protecting DNA, membrane lipids and some cytosolic proteins. Supporting the idea that melatonin could cross the cell membrane barrier, it was shown that the hormone could cross pure lipid bilayers [8], therefore being able to act at the cytosol or nuclear levels. It was also shown that although melatonin partitions in lipid bilayers it is highly soluble in aqueous medium (up to 5×10^{-3} M) [9].

Considering the action of melatonin at the membrane level, both its interaction with membrane protein receptors and its role as a membrane antioxidant, it seems important to understand how melatonin interacts with lipid bilayers: how it partitions in the water/membrane system, how the bilayer changes the melatonin structure, how the hormone disrupts the lipid environment, and what the distribution profile of the hormone is inside the bilayer.

The present work uses the ESR signal of spin labels incorporated in lipid bilayers, and the melatonin fluorescence, to analyze the hormone's interaction with lipid vesicles. Stearic acids and phospholipids labeled at different positions along the acyl chain and at the headgroup were used either as spin labels or as quenchers of the melatonin fluorescence. The well studied zwitterionic lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was chosen as the model membrane system, due to its very convenient main transition temperature, $T_m \cong 23°C$, easily allowing studies with the membrane at the gel and liquid crystal phases, below and above T_m, respectively. Those two phases would mimic micro-regions of different packing in a biological membrane.

2. Materials and methods

2.1. Materials

Melatonin, DMPC, 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). The spin labels 5- and 12-PCSL (1-palmitoyl-2-[5- or 12-doxyl stearoyl]-*sn*-glycero-3-phosphocholine) were obtained from Avanti Polar Lipids (Birmingham, AL). SSL (stearamide spin label) was a gift from S. Schreier's laboratory, at the University of São Paulo. The buffer used was 10 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) at pH 7.4. All reagents were used without further purification. Deionized double distilled water was used throughout.

2.2. Melatonin sample preparation

A concentrated melatonin solution, 10^{-3} M, was prepared either as described elsewhere [9], or by directly dissolving the powder in buffer using a bath-type sonicator. The integrity of melatonin prepared by the last method was checked by its light absorption and fluorescent emission spectra, and by biological activity measurements [10]. Diluted samples were prepared from the stock solution. The two preparations avoid the presence of even small amounts of organic solvent in the final melatonin solution.

2.3. 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 melatonin, followed by vortexing.

2.4. Fluorescence sample preparation

DMPC large unilamellar vesicles (LUV) were prepared by the method of extrusion [11], resulting in 2.5 ± 0.1 mM stock suspensions (inorganic phosphate was quantitated based on [12]). Lipid titration was performed by adding small amounts of the concentrated lipid vesicle suspension to 10^{-5} M melatonin aqueous solution. The lipid concentration varied from 0.1 to 2.0 mM. Appropriate corrections for volume changes were made. For the quenching experiments, the samples were prepared as described above for the ESR experiments, but with 10 mol% of spin label, prior to extrusion.

2.5. ESR spectroscopy

ESR measurements were performed in a Bruker ER 200D-SRC spectrometer interfaced with an IBM-PC like computer for spectrum

*Corresponding author. Fax: (55) (11) 8134334.
E-mail: mtfreund@fge.if.usp.br

[1]Present address: Escola Politécnica, Universidade de São Paulo, SP, Brazil.

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digitalization. A field modulation amplitude of 0.08 mT and microwave power of 5 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 monitored with a Fluka 51 K/J thermometer. The LOWFIT program (B. Bales) was used for the SSL spectrum simulations above the lipid transition temperature. For the measurement of several ESR spectrum parameters the ORIGIN software (MicroCal Software, Inc., MA, USA) was used. The data shown here are the mean values of at least three experiments, and the error bars are the calculated standard deviations.

2.6. Fluorescence spectroscopy

Melatonin fluorescence emission spectra ($\lambda_{ex} = 290$ nm) were registered in a Hitachi 3010 spectrofluorimeter, interfaced with an IBM-PC like computer, and were corrected for the instrumental sensitivity variation with wavelength. Slit widths were typically set at 5 nm, for both the excitation and emission. Fluorescence intensity was properly corrected for vesicle light scattering, making use of correction factors calculated from the experiment with pyranin (8-hydroxy-1,3,6-pyrenitrisulfonate) and DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol) vesicles, which are known not to interact with one another [13]. The concentrations of DMPG were such that they yielded the same turbidity as those of DMPC, measured in a Hewlett Packard 8452A diode array spectrophotometer. Alternatively, a home-made front face fluorescence cell was used, with the excitation beam making an angle of 36° with the normal to the cell face, to avoid specularly reflected light from reaching the emission optics [14]. The fluorescence data are also the mean values of at least three experiments, and the error bars are the calculated standard deviations.

3. Results and discussion

3.1. Membrane spin label ESR

To study the effect of the melatonin-lipid interaction in the bilayer structure, spin labels that monitor different depths of the lipid bilayer were used. The alterations caused by melatonin in the ESR signal of SSL, which monitors the lipid headgroup region, can be evaluated by the decrease of the empirical parameter h_{+1}/h_0 (ratio between the amplitude of the lines corresponding to $M_I = +1$ and $M_I = 0$) calculated from the SSL ESR spectra (Fig. 1). The hormone alters the DMPC membrane surface, below and above T_m, indicating that melatonin binds to the bilayer independent of its packing state. For temperatures above 30°C, rotation correlation times were calculated according to [15]. The spectra were simulated using the corrections for inhomogeneous broadening due to unresolved hyperfine structure, as suggested by [16]. Fig. 2 shows that the effect of melatonin on the τ// values (rotation correlation time about the molecular symmetry axis) is analogous to that caused by decreasing the temperature, suggesting that melatonin increases the packing of the PC headgroups. Considering that there is probably a small orienting potential in the membrane interface, the calculated correlation times should be taken as comparative parameters and not as true numerical values. The changes caused by melatonin on both h_{+1}/h_0 and τ// are similar, though less intense, to those yielded by the interaction of melanotropic peptides with DMPG vesicles [17]. Those peptides were shown to partially penetrate anionic bilayers.

Although melatonin was shown to cross lipid bilayers [8], the ESR spectra of spin labels placed at the 5th or 12th carbon of the acyl chain of stearic acid (5- and 12-SASL) or phospholipid (5- or 12-PCSL) derivatives do not change in the presence of up to 50 mol% of melatonin (relative to DMPC). The same result was obtained with the cholesterol analog spin label CSL. Therefore, unlike the melanotropins [17,18] and other small peptides, melatonin does not seem to significantly alter the packing of the bilayer core. The same result was recently found with a fluorescence probe [7]. That could be either due to the small volume of the hormone, as compared to the peptides, and/or to its preferential distribution at the membrane surface. Small aromatic hydrocarbons were also found to penetrate lipid bilayers, without altering the degree of organization of the membrane [19]. It was suggested that the small hydrophobic molecules were dissolved in the central region of the bilayer, close to the acyl terminal methyl group, although the water/lipid interface was not monitored.

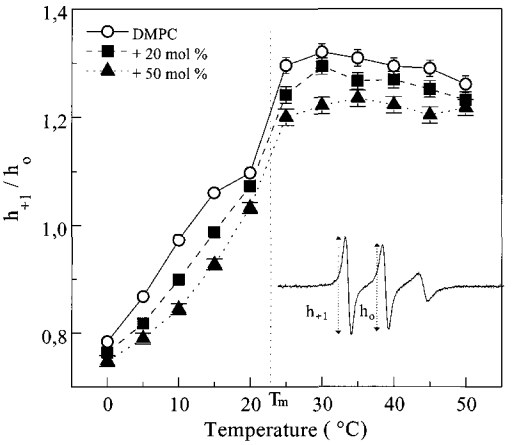

Fig. 1. Effect of melatonin on the ESR spectra of SSL in DMPC, at different temperatures (mol% melatonin relative to lipid).

3.2. Melatonin fluorescence

Like many fluorophores, the melatonin emission spectrum is highly dependent on the medium dielectric constant. Melatonin fluorescence spectra in solutions containing different proportions in volume of water/ethanol were obtained. The corresponding spectral variations were evaluated in terms of the changes in the area under the curves and position of the maximum of emission, and plotted versus the dielectric constant of the medium (ε) (Fig. 3). The values of ε for the different mixtures were calculated from the given values for different mixtures in weight of water/ethanol [20], assuming 1.00 and 0.80 g/cm^3 for the water and ethanol densities, respectively. Fig. 3 shows that the fluorescence emission of melatonin is highly sensitive to its environment. Keeping that in mind, it is interesting to analyze the spectra of melatonin in the presence of excess DMPC (2.5 mM, where the titration experiment discussed below showed that melatonin was completely bound to the vesicles). The hormone fluorescence emission does not change much, yielding $I_{max}/I_{min} \cong 1.25$ and $\Delta\lambda \cong 3$ nm, where I_{min} and I_{max} are the areas under the melatonin emission spectra in the absence and the presence of DMPC vesicles, respectively. Therefore, based on the data in Fig. 3, melatonin in DMPC bilayers seems to be mainly localized in a region of dielectric constant around 75, hence close to the bilayer interface. The small variations on the melatonin fluorescence anisotropy at 30°C – from 0.002 in buffer to 0.033 in the presence of excess DMPC – also suggest the bilayer/water interface as the hormone localization. It is important to note that the fluorescence spectra of melatonin in DMPC vesicles indicated similar hormone-membrane interactions for the two different states of lipid packing.

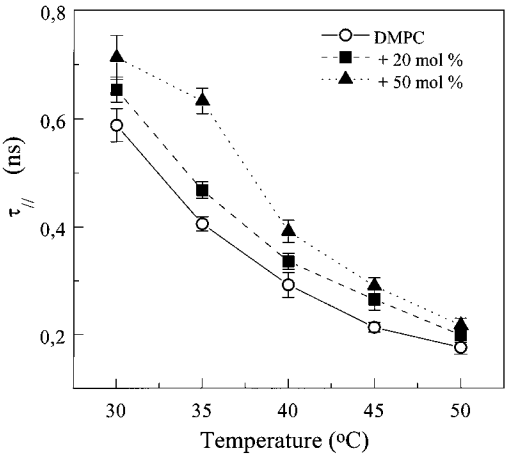

Fig. 2. The melatonin increase of SSL rotation correlation time, in the liquid crystal phase of DMPC.

3.3. Melatonin-lipid membrane partition coefficient

To further understand the binding of melatonin to DMPC vesicles, membrane partition coefficients, K_p, were calculated.

K_p is defined as

$$K_p = \frac{n_M/V_M}{n_{H_2O}/V_{H_2O}}$$

where n_M and n_{H_2O} are the number of moles of melatonin bound to the membrane and in solution, and V_M and V_{H_2O} are the volumes of membrane and solution, respectively. It will be assumed that the total volume $V_t = V_{H_2O}$.

The above equation can be rewritten as a function of the bound and the free molar melatonin concentrations, $[M_b]$ and $[M_f]$, respectively.

$$K_p = \frac{[M_b]}{[M_f]}\frac{V_t}{V_M}$$

Hence, the bound peptide concentration is

$$[M_b] = \frac{K_p[M_t]V_M}{(K_pV_M - V_t)}$$

where $[M_t]$ is the total concentration of melatonin.

The above equation, together with the assumption that melatonin is partitioned between two media, lipid membrane (M_b) and buffer (M_f), where its fluorescence intensity is I_{max} and I_{min}, respectively

$$I = \frac{[M_b]}{[M_t]}I_{max} + \frac{[M_f]}{[M_t]}I_{min}$$

can be put together to yield an expression for the variation of the melatonin fluorescence I, as a function of the total concentration of lipids, $[L_t]$.

$$\frac{I}{I_{min}} = \frac{K_p[L_t]M_w/\rho}{(K_p[L_t]M_w/\rho)+1}\left(\frac{I_{max}}{I_{min}} - 1\right) + 1$$

where M_w and ρ are the lipid molecular weight and density (ρ was assumed to be 1 kg/l). That equation will fit well any set of data where the concentration of bound molecule is much lower than the total concentration of lipid, yielding a constant value for K_p for the range of $[L_t]$ used. That is, when the number of binding sites is not restricted.

The titration data for the melatonin fluorescence in the presence of different DMPC concentrations, above and below T_m, obtained as described in Section 2, were well fitted with the model discussed above, yielding K_p values of 3100 ± 500 and 4300 ± 800, for the partition of melatonin in the gel and liquid crystal DMPC phases, respectively. Within the experimental error, the membrane melatonin partition is not a function of the packing lipids. It is interesting to point out that K_p values around 3000 are rather high, as compared to the values obtained, for instance, for the binding of melanotropic peptides to lipid bilayers [21], after the correction for the electrostatic effect ($K_p \cong 3$, assuming $K_p = K_a/0.7$ and $K_a = 1/nK_d$ where K_a, K_d are the association and dissociation constants, respectively, and n is the number of bound lipid molecules per peptide, as defined in [21]). They could also be compared with the partition of melatonin in a chloroform/water system, which yields a K_p around 20 (unpublished results). Values for solute/membrane partition coefficients around 10^3 were found, for example, for the neutral form of the local anesthetic dibucaine, but rather smaller values were obtained for other local anesthetics [22]. Therefore, although it is highly soluble in aqueous medium [9], melatonin strongly associates with lipid vesicles, both in the gel and in the liquid crystal states of the bilayer.

3.4. Membrane fluorescent quenchers

The interaction of melatonin with DMPC bilayers was also monitored by the quenching of its fluorescence emission by spin labels placed at the 5th and 12th carbon of the acyl chain (5- and 12-PCSL). This approach has been extensively used to localize fluorophores in lipid bilayers. It has been found that fluorophores which are supposed to bind near the membrane surface have their fluorescence emission better suppressed by nitroxides localized close to the lipid headgroups (for instance see [21,23]). Surprisingly, Fig. 4 shows that the melatonin fluorescence is better quenched by spin labels deeper in the bilayer (12-PCSL) than by those closer to the interface (5-PCSL). These results could be rationalized in the light of the known vertical fluctuation of acyl chain spin labels [24–26], and the ability of melatonin to cross lipid bilayers [8]. Hence, even considering that melatonin is preferentially localized at the membrane surface, as it is crossing the bilayer, the label at the 12th position, which is on average deeper in the bilayer [17] but can span most of the membrane, could be

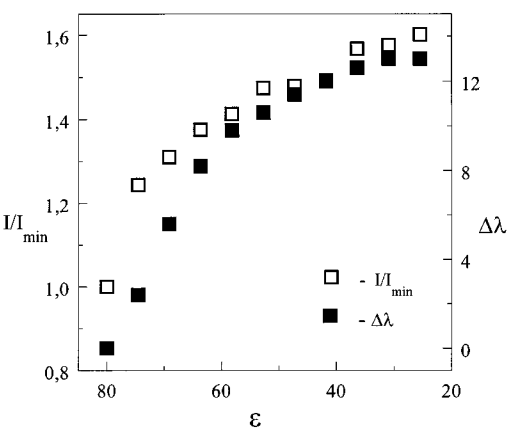

Fig. 3. The dependence of the melatonin fluorescence (area under the emission spectra and peak position) with the medium dielectric constant. I_{min} is the intensity in pure water.

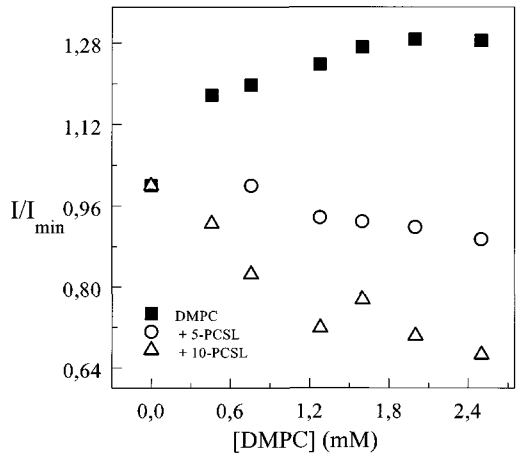

Fig. 4. Quenching of melatonin fluorescence as a function of the DMPC/PCSL (molar ratio 90/10) concentration.

more effective in quenching the hormone fluorescence than the less mobile quencher 5-PCSL.

For the understanding of the average distribution of melatonin near the surface of the bilayer one could make a very simple parallel with the distribution of a particle under a classical harmonic field, centered at the middle of the bilayer ($x = 0$). If the particle oscillates between the bilayer surfaces, its probability distribution would be such that the particle is predominantly found at the maximum value of $|x|$, the bilayer surfaces. Actually, melatonin inside the membrane could be subjected to any potential with a minimum at the middle of the bilayer, which would yield a higher molecular density close to the membrane interfaces. The origin of that potential could possibly be attributed to 'hydrophobic' and 'viscous interactions.

The results presented here, concerning the melatonin/lipid interaction, will certainly be relevant for the discussion of the several melatonin physiological actions at the membrane level.

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