

Permeability of pure lipid bilayers to melatonin

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Abstract: Melatonin, the chief hormone of the pineal gland, has been reported to interact with a variety of different cells. This ubiquitously acting hormone has been found to interact with protein receptors both at the cell membrane and in the nucleus. Moreover, melatonin was recently shown to be a very potent hydroxyl radical scavenger. The present work focuses on the interaction of melatonin with pure lipid bilayers. It is shown that melatonin can cross multilamellar lipid vesicles, which are used here as model systems for the lipid phase of biological membranes. Thus, the data prove that melatonin can easily pass through the cell membrane and bath every part of the cell, as previously suggested in the literature. Melatonin lipid association constant was calculated based on the change of the hormone fluorescence intensity due to its penetration into the hydrophobic lipid phase. Though melatonin was recently shown to be highly soluble in aqueous media, its lipid association constant is rather high, indicating that the biological action of the hormone is likely to be at the membrane level, either via its interaction with membrane receptors, and/or as a lipoperoxidation radical scavenger.

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Introduction

Diverse physiological roles have been assigned to melatonin (5-methoxy-N-acetyltryptamine), which is the most important hormone synthesized by the pineal gland. Some of its biological functions were shown to be dependent on protein plasma membrane receptors [Morgan and Williams, 1989; Sudgen, 1992; Ebisawa et al., 1994], though it has also been suggested that melatonin can cross the cell membrane barrier and interact in the nucleus [Tan et al., 1993a; Reiter et al., 1993].

It was recently shown that melatonin is highly soluble in aqueous media, being soluble and stable up to the concentration of 5×10^{-3} M [Shida et al., 1994]. Thus, after being secreted by the pineal gland, melatonin could be carried by the blood stream to its target cells, without requiring protein transport. The aqueous solubility of melatonin could explain its presence in the cytosol and hence its interaction with nuclear receptors. However, for a better understanding of the melatonin path across cell membranes, including the possible absence of protein membrane transporters, it remains to be proved that melatonin can cross pure lipid barriers.

The present work focuses on the interaction of melatonin with lipid bilayers. A simple dialysis ex-

periment is used here to show that melatonin can easily penetrate pure lipid bilayers. It is also shown that despite its high solubility in aqueous medium melatonin has a relatively large lipid membrane association constant, in line with its capacity of crossing the bilayers.

Materials and methods

Materials

Melatonin (P6414, lot 98F8374), asolectin (Type II-S, from soybean, L- α -phosphatidylcholine 10-20%), methylene blue (MB), and sodium dodecyl sulfate (SDS, 99% sodium salt) were purchased from Sigma Chemical Co., St Louis, MO, USA and employed without further purification. 2-I-melatonin (M-112) was from Research Biochemicals International (Natick, MA, USA). Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was from SERVA Feinbiochemica (New York, NY, USA).

Sample preparation

A concentrated melatonin solution, 10^{-3} M, was prepared as described elsewhere [Shida et al., 1994]. Diluted samples were prepared from that stock solution.

Multilamellar asolectin vesicles were prepared from direct addition of the desired solution to the lipid powder, followed by vortexing. Considering that asolectin is a mixture of many lipids it will be assumed here an average molecular weight of 750 for the lipids.

Dialysis experiment

Dialysis tubing (D 2272) from Sigma Chemical Co. was used with 0.5 ml of the desired sample inside. Asolectin concentration was 50 mM, calculated as described above. Samples were prepared in water and dialyzed against 2 l of water, at room temperature. Water was changed three times, and each time stirred for 30 min. Optical absorption spectra were registered in a Hewlett Packard 8452A diode array spectrophotometer. SDS was added to the final concentration of 5% (g/100 ml), immediately before the sample absorbance measurement. SDS micelles solubilize the lipids, breaking the vesicles, thus allowing a proper quantification of the chromophore inside and outside them. For comparison among different samples, SDS was used in all samples, though the addition of the detergent is only necessary for the lipid containing ones. Due to the high lipid concentration used here, even after the addition of SDS the lipid containing samples were slightly turbid. Thus, the MB and melatonin measured absorbances were also corrected for light scattering, calculated from pure lipid samples. That correction is particularly important for melatonin, whose absorbance is measured at a low wavelength, 290 nm.

Fluorescence experiment

Melatonin fluorescence emission spectra ($\lambda_{\text{excitation}}=290$ nm) were registered in a Hitachi 3010 spectrofluorimeter, at 25°C and were corrected for the instrumental sensitivity variation with wavelength. Fluorescence intensity measurements were properly corrected for vesicles light scattering. Samples were prepared in 10 mM Hepes buffer pH 7.4. Melatonin was used at the concentration of 10^{-5} M. Large unilamellar asolectin vesicles were prepared by the method of extrusion [Hope et al., 1985] resulting in 2.5 mM stock suspensions. Lipid titration was performed by adding small amounts of the concentrated lipid vesicle suspension to melatonin solution and monitoring the changes in its fluorescence intensity.

Results and discussion

Lipid membrane permeability

To check whether melatonin could cross pure lipid bilayers we used a dialysis experiment based on the

methodology described by Augusto and Carmona-Ribeiro [1989]. Asolectin was chosen as a convenient lipid membrane model system as it is composed of a variety of lipids and form rather stable multilamellar vesicles.

A control experiment was done with MB, which is known to be retained by asolectin vesicles, not being able to cross lipid barriers [Augusto and Carmona-Ribeiro, 1989]. Vesicles were prepared by the addition of a 1.0×10^{-1} mM solution of MB to the lipid powder, resulting in a sample containing the dye both inside and outside the multilamellar vesicles. Two samples were dialyzed simultaneously in the same vessel: one containing pure MB solution and the other containing MB inside and outside the asolectin vesicles, as described above. Before dialysis, MB concentration was 1.0×10^{-1} M for both samples. The optical absorbance of the samples at 660 nm were measured before and after dialysis (Table I). As expected, the absorbance values before dialysis were very similar, and a mean value was used for both samples.

The percentage of MB entrapment can be calculated by

$$\text{ENT (\%)} = 100 \left\{ \frac{A_{\text{MB} + \text{aso}(\text{after})} - A_{\text{MB}(\text{after})}}{A_{\text{MB}(\text{before})}} \right\}$$

where $A_{\text{MB}(\text{before})}$ is the absorbance of both pure MB and MB plus asolectin before dialysis. $A_{\text{MB}(\text{after})}$ and $A_{\text{MB} + \text{aso}(\text{after})}$ are the absorbances of pure MB and MB plus asolectin samples after dialysis, respectively.

The percentage of MB entrapment was calculated to be around 50%. This is in accord with previous results [Augusto and Carmona-Ribeiro, 1989], showing that asolectin vesicles are not permeable to the cationic dye MB.

The same methodology described above was used with melatonin in the place of MB. The initial melatonin concentration was 5×10^{-4} M and the absorbance was measured at 290 nm (Table 1).

Table 1. Optical absorption of methylene blue (MB) and melatonin samples before and after dialysis

Sample	Absorbance before dialysis	Absorbance after dialysis
MB	0.763 ⁽¹⁾	0.022 ⁽¹⁾
MB + asolectin	0.763 ⁽¹⁾	0.428 ⁽¹⁾
Melatonin	1.242 ⁽²⁾	0.009 ⁽²⁾
Melatonin + asolectin	1.242 ⁽²⁾	0.065 ⁽²⁾

Absorbances were measured at 660 nm⁽¹⁾ and 290 nm⁽²⁾. Optical path length 0.1 cm⁽¹⁾ and 0.5 cm⁽²⁾. The data are expressed as mean values of three experiments.

Opposite to the results with MB, the percentage of melatonin entrapment was found to be negligible (ca. 5%), showing that pure lipid bilayers are very permeable to that hormone.

Considering that many biological experiments use the radioactive complex ^{125}I -melatonin, the dialysis experiment was also done with 2-I-melatonin. The results showed that the lipid bilayers are equally permeable to both melatonin and its I complex, within the accuracy of the dialysis experiment.

Lipid bilayer partition coefficient

Melatonin lipid association constant (K_a) was calculated through the increase of the hormone fluorescence intensity due to its penetration into lipid bilayers, which are a more hydrophobic medium (Lakowicz, 1983) (Fig. 1). K_a was calculated as described below.

The amount of bound melatonin [M_b] is given by the equation

$$[M_b] = [M_t](I - I_0)/(I_{max} - I_0)$$

where [M_t] is the total melatonin concentration, I is the fluorescence intensity for different lipid concentrations, I_0 is the initial fluorescence intensity in the absence of lipid and I_{max} is the final fluorescence intensity, in the presence of excess of lipid.

For the calculation of K_a we considered the equilibrium equation

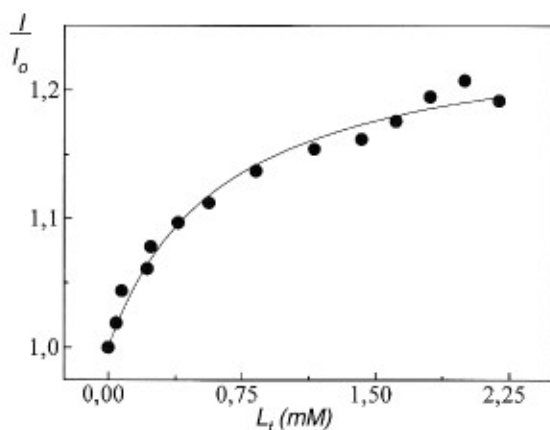
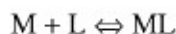


Fig. 1. Relative increase in the fluorescence emission maximum intensity of melatonin (350 nm) on addition of asolectin vesicles, at 30°C. Excitation wavelength 290 nm. L_t is the total concentration of lipid, I and I_0 are the fluorescence intensity at a certain L_t and in the absence of lipid, respectively. [melatonin] = 10^{-5} M. The experimental data (\bullet) are expressed as mean values of three experiments. The best fit (continuous line) was calculated by the chi-square reduced method, using the model described in the text.

where M and L stand for melatonin and lipid, respectively. The association constant can be calculated by $K_a = [ML]/[M_f][L_f]$, where [M_f] and [L_f] are the concentration of free melatonin and lipid, respectively, and [ML] is the concentration of bound molecules. Considering that [M_b] = [ML] and making the simplifying hypothesis that there is an infinity number of binding sites for the hormone, that is [L_f] \approx [L_t], where [L_t] is the total number of lipids. The above equations can be combined to yield

$$\frac{1}{(I - I_0)} = \frac{1}{(I_{max} - I_0)} + \frac{1}{(I_{max} - I_0)} \frac{1}{K_a L_t}$$

from which a melatonin lipid association constant $K_a = 1570 \pm 200 \text{ M}^{-1}$ was calculated from the best fit (continuous line in Fig. 1) to the experimental data. The melatonin lipid partition coefficient K_p can be calculated from the relation $K_p = \rho K_a / MW$, where ρ is the lipid density, which will be considered 1 g/ml, and MW is the lipid molecular weight, which is taken as an average value of 750. Those values yield a lipid partition coefficient K_p around 2000 for melatonin. Therefore, though melatonin is a highly aqueous soluble molecule [Shida et al., 1994] its affinity for the hydrophobic lipid micro environment is much higher.

Final comments

Considering the variety of lipid composition of most biological cells, and the number of different lipids present in asolectin, the system used here can be considered a good model for regions of the lipid phase of many biological membranes. The present work shows that melatonin has a relatively high lipid partition coefficient and can easily cross lipid bilayers. Its ability to cross lipid membranes is in accord with its reported effect at the cell nuclear level [Tan et al., 1993a].

The multiple biological roles played by melatonin should be analyzed in the light of its ability of being almost everywhere in the cell, but preferentially in hydrophobic portion of the membrane. Its reported radical scavenging action [Tan et al., 1993b; Reiter et al., 1993] should certainly be extended to the membrane lipid phase. Accordingly, it has recently been reported [Melchiorri et al., 1995; Reiter et al., 1995], that radicals formed by lipid peroxidation can be efficiently on-site destroyed by melatonin.

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