Exploring the Organization and Dynamics of Hippocampal Membranes Utilizing Pyrene Fluorescence

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The organization and dynamics of cellular membranes in the nervous system are crucial for the function of neuronal membrane receptors and signal transduction. Previous work from our laboratory has established hippocampal membranes as a convenient natural source for studying neuronal receptors. In this paper, we have monitored the organization and dynamics of hippocampal membranes and their modulation by cholesterol using pyrene fluorescence. The apparent dielectric constant experienced by pyrene in hippocampal membranes turns out to be $\sim 20 \pm 3$, depending on the experimental condition. Our results show that the polarity of the hippocampal membrane is increased upon cholesterol depletion, as monitored by changes in the ratio of pyrene vibronic peak intensities (I_1/I_3). This is accompanied by an increase in lateral diffusion, measured as an increase in the pyrene excimer/monomer ratio. These results are relevant in understanding the complex organization and dynamics of hippocampal membranes and could have implications in neuronal diseases characterized by defective cholesterol metabolism.

Introduction

Biological membranes are complex noncovalent assemblies of a diverse variety of lipids and proteins that allow cellular compartmentalization, thereby imparting an identity to the cell. Since a significant portion of integral membrane proteins remains in contact with the membrane lipids,[1] the structure and function of membrane proteins often depend on their interactions with the surrounding lipids.[2] The lipid composition of cells that make up the nervous system is unique and has been correlated with increased complexity in the organization of the nervous system during evolution.[3] The organization and dynamics of cellular membranes in the nervous system play a crucial role in the function of neuronal membrane receptors. Cholesterol is an important lipid in this context since it is known to regulate the function of neuronal receptors.[4,5] Interestingly, a number of neurological diseases share a common etiology of defective cholesterol metabolism in the brain,[6] yet the organization and dynamics of neuronal membranes as a consequence of alterations in membrane cholesterol is poorly understood.[7] In addition, in view of the importance of cholesterol in relation to membrane domains,[8] monitoring the effect of alteration in the cholesterol content of neuronal membranes on their organization and dynamics assumes significance.

Earlier work from our laboratory has established native membranes, prepared from the bovine hippocampus, as a convenient natural source for studying the serotonin$_{1A}$ receptor, which is an important member of the seven-transmembrane-domain G-protein-coupled receptor family.[9] The serotonin$_{1A}$ receptor is involved in the generation and modulation of several cognitive, behavioral, and developmental functions. Interestingly, we have recently shown the requirement of membrane cholesterol in modulating ligand binding activity of the serotonin$_{1A}$ receptor.[4,10] To correlate these cholesterol-dependent functional changes with alterations in membrane organization and dynamics, we have previously utilized Laurdan generalized polarization.[11] In this work, we have monitored the organization and dynamics of hippocampal membranes and their modulation with cholesterol and protein content, utilizing characteristic fluorescence properties of the hydrophobic aromatic membrane probe pyrene.

Experimental Section

Materials. Cholesterol, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), methyl-β-cyclodextrin (MβCD), and pyrene were purchased from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) reagent for protein estimation was from Pierce (Rockford, IL). The Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). The concentration of the stock solution of pyrene in methanol was estimated from its molar absorption coefficient (ε) of 54 000 M^{-1} cm^{-1} at 335 nm.[12] The solvents (methanol, ethanol, 1-propanol, 1-butanol, 1-hexanol, and 1-octanol) used were of spectroscopic grade. All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Preparation of Native Hippocampal Membranes. Native hippocampal membranes were prepared as described previously,[11] flash frozen in liquid nitrogen, and stored at −70 °C. The protein concentration was assayed using BCA reagent with bovine serum albumin as a standard.[13]

Cholesterol Depletion of Native Hippocampal Membranes. Native hippocampal membranes were depleted of cholesterol using MβCD as described previously.[11] The cholesterol content was estimated using the Amplex Red assay kit.[14]

Lipid Extraction from Native and Cholesterol-Depleted Membranes. Lipid extraction was carried out according to the method of Bligh and Dyer.[15] The lipid extract was finally dissolved in a mixture of chloroform-methanol (1:1, v/v).

Estimation of Inorganic Phosphate. The concentration of lipid phosphate was determined subsequent to total digestion

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by perchloric acid using Na_2HPO_4 as a standard.[16] DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

Sample Preparation. Membranes (native and cholesterol-depleted) containing 50 nmol of total phospholipid were suspended in 1.5 mL of 10 mM MOPS buffer (pH 7.4). Pyrene was added from a methanolic stock solution such that the final pyrene concentration was 1 mol % with respect to the total phospholipid content. The resultant probe concentration was 0.33 μM in all cases, and the methanol content was always low (<0.07%, v/v). This ensures optimal fluorescence intensity with negligible membrane perturbation. Pyrene was added to the membranes while they were being vortexed for 1 min at room temperature (~23 °C), and then the samples were kept in the dark for 1 h before the measurements. Background samples were prepared the same way except that pyrene was omitted.

Lipid extracts containing 50 nmol of total phospholipid in chloroform-methanol (1:1, v/v) were mixed well with 0.5 nmol of pyrene in methanol. The samples were mixed well and dried under a stream of nitrogen while being warmed gently (~45 °C). After the samples were further dried under a high vacuum for at least 6 h, 1.5 mL of 10 mM MOPS, pH 7.4 buffer was added, and lipid samples were hydrated (swelled) at ~70 °C while being intermittently vortexed for 3 min to disperse the lipid and form homogeneous multilamellar vesicles (MLVs). The MLVs were kept at ~70 °C for an additional hour to ensure proper swelling as the vesicles were formed. Such high temperatures were necessary for hydrating the samples due to the presence of lipids with high melting temperature in neuronal tissues.[17] The samples were kept in the dark at room temperature (~23 °C) overnight before fluorescence measurements.

Fluorescence Spectroscopic Measurements. Steady-state fluorescence measurements of samples containing pyrene were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. For measuring pyrene fluorescence, the samples were excited at 335 nm. Excitation and emission slits with a nominal bandpass of 5 nm were used for all measurements. The background intensities of samples in which pyrene was omitted were negligible in most cases and were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The optical density of the samples at 335 nm was ~0.05, which rules out any possibility of scattering artifacts in the fluorescence measurements. The excimer/monomer fluorescence intensity ratio was determined by measuring the fluorescence intensity at the monomer (393 nm) and excimer (480 nm) peaks. The ratio of the first (373 nm) and third (384 nm) vibronic peak intensities (I_1/I_3) was calculated from pyrene emission spectra.

Results and Discussion

The fluorescence emission spectrum of pyrene is sensitive to environmental polarity.[18] Pyrene also forms excimers with very different fluorescence characteristics, and the ratio of excimer to monomer is known to be dependent on the membrane dynamics.[19,20] It has been recently shown that pyrene is localized predominantly in the interfacial region of the membrane.[21] Figure 1 shows the fluorescence emission spectra of pyrene in native hippocampal membranes and in liposomes of lipid extract from native membranes under control and cholesterol-depleted conditions. A characteristic feature of the structured emission spectra is the maxima at 373, 384, and 393 nm. This type of structured vibronic band intensities, displayed by fluorophores such as pyrene and dehydroergosterol,[22] is known to be

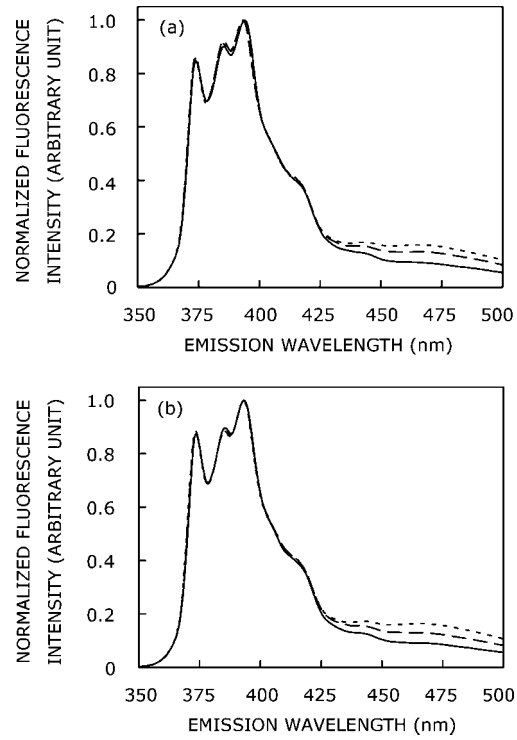

Figure 1. (a) Representative fluorescence emission spectra of pyrene in native hippocampal membranes (–) and membranes treated with 20 (— —) and 40 (-----) mM MβCD. (b) Fluorescence emission spectra of pyrene in liposomes of lipid extract from native membranes (–) and membranes treated with 20 (— —) and 40 (-----) mM MβCD. The spectra are intensity-normalized at the respective emission maxima. Measurements were carried out at room temperature (~23 °C). The excitation wavelength used was 335 nm. The ratio of pyrene to total phospholipid was 1:100 (mol/mol), and the total phospholipid concentration was 33 μM in all cases. See the Experimental Section for other details.

environmentally sensitive. This property has previously been effectively used for elucidating microenvironments of pyrene.[20,23] The ratio of the first (373 nm) and third (384 nm) vibronic peak intensities (I_1/I_3) in the pyrene emission spectrum provides a measure of the apparent polarity of the environment. An increase in the I_1/I_3 ratio is indicative of increased polarity.

MβCD is a water-soluble compound and has previously been shown to selectively and efficiently extract cholesterol from hippocampal membranes by including it in a central nonpolar cavity (see the inset of Figure 2).[10] Figure 2 shows that, upon treatment with increasing concentrations of MβCD, the cholesterol content of hippocampal membranes shows progressive reduction. Thus, when native membranes are treated with 10 mM MβCD, the cholesterol content is reduced to ~87% of the initial value. This effect levels off with increasing concentrations of MβCD, with the cholesterol content of hippocampal membranes being reduced to ~13% of the original value when the membranes are treated with 40 mM MβCD (see Figure 2). Importantly, the change in phospholipid content under these conditions is negligible (data not shown). This ensures that depletion of cholesterol by MβCD is predominantly specific. The change in the ratio of vibronic peak intensities (I_1/I_3) in pyrene emission spectra in hippocampal membranes treated with increasing concentrations of MβCD is shown in Figure 3a. The figure shows that increasing cholesterol depletion resulted in an increase in the vibronic peak intensity ratio. This could imply an increase in apparent polarity experienced by pyrene in cholesterol-depleted membranes, possibly due to an increase in

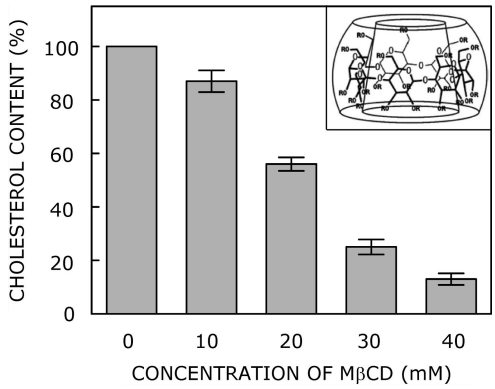

Figure 2. Effect of increasing concentrations of MβCD on the cholesterol content of hippocampal membranes. The cholesterol content was estimated using the Amplex Red assay kit. Values are expressed as percentages of cholesterol content in native hippocampal membranes without MβCD treatment. Data for cholesterol content represent means $\pm$ SE of at least four independent measurements. See the Experimental Section for other details. The inset shows the chemical structure of MβCD (R denotes a methyl group). MβCD can entrap cholesterol in its inner nonpolar cavity and render it soluble in an aqueous medium.

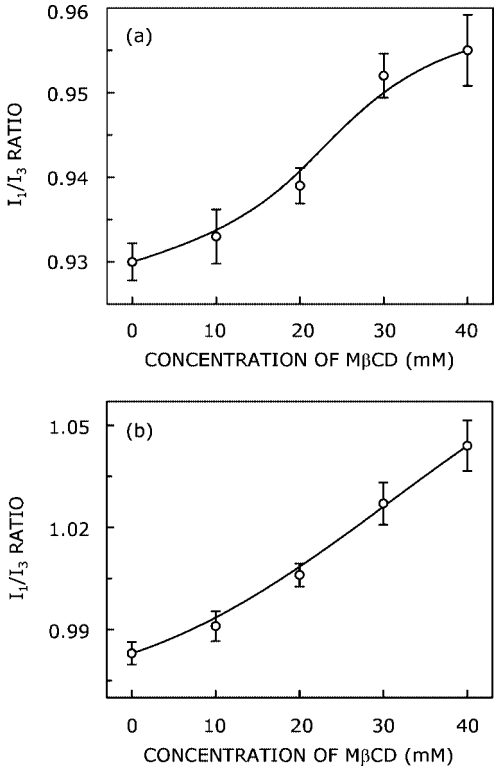

Figure 3. Change in fluorescence intensity ratio of the first (373 nm) and third (384 nm) vibronic peaks of pyrene (I_1/I_3) in (a) hippocampal membranes and (b) liposomes of lipid extract from native membranes with increasing concentration of MβCD. Data points shown are means $\pm$ SE of at least four independent measurements. The lines joining the data points are provided merely as viewing guides. All other conditions are as in Figure 1. See the Experimental Section for other details.

water penetration in the bilayer upon cholesterol depletion. This is in agreement with our earlier observation that the fluorescence lifetime of the hydrophobic probe Nile Red in hippocampal membranes decreases with increasing cholesterol depletion due to an increase in the microenvironmental polarity.[24]

The increase in water penetration is due to the loss of membrane order induced by depletion of cholesterol. These results are supported by our earlier observations that cholesterol

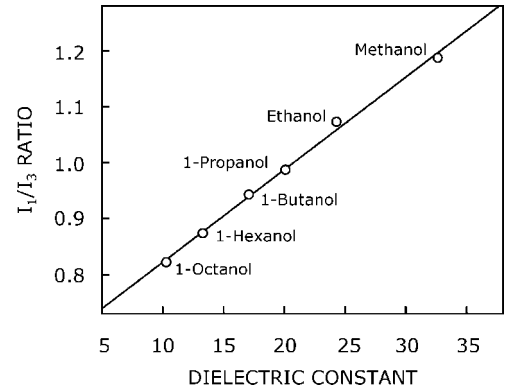

Figure 4. Calibration plot between the fluorescence intensity ratio of the first (373 nm) and third (384 nm) vibronic peaks of pyrene (I_1/I_3) and static dielectric constant (ε) of various alcohols at 25 °C, ranging between 10.3 (1-octanol) and 32.6 (methanol) (taken from ref 38). The calibration plot indicates a very good linear correlation ($y = 0.0165x + 0.6565$; $r = 0.9981$) between the ratio I_1/I_3 and the static dielectric constant of various alcohols. See the Experimental Section for other details.

depletion of native hippocampal membranes leads to a decrease in membrane order as monitored by fluorescence polarization measurements[10] and from previously reported shifts in the Laurdan emission spectrum.[11] To evaluate the effect of proteins in modulating membrane order, we monitored the change in the ratio of vibronic peak intensities in pyrene emission spectra in liposomes prepared from a lipid extract of native hippocampal membranes treated with increasing concentrations of MβCD (see Figure 3b). The figure shows that the change in the ratio of vibronic peak intensities with increasing cholesterol depletion exhibits a similar dependence, i.e., cholesterol depletion resulted in an increase in the peak intensity ratio, implying an increase in apparent polarity in this case also.

To obtain an estimate of the increase in apparent polarity experienced by pyrene, we utilized the linear relationship between the ratio of the vibronic peak intensities (I_1/I_3) and dielectric constant (ε).[23,25,26] We generated a calibration plot between I_1/I_3 and the static dielectric constant of various alcohols ranging from 10.3 to 32.6 (see Figure 4). Since the only polar group in these alcohols is the hydroxyl group, the I_1/I_3 ratio corresponding to each one of them can be attributed to their relative polarity. A higher value of I_1/I_3 corresponds to a relatively polar solvent (such as methanol), while a lower value of I_1/I_3 corresponds to a relatively nonpolar solvent (such as 1-octanol). We utilized this calibration plot to gain information on the apparent polarity experienced by pyrene in hippocampal membranes with progressive cholesterol depletion. The apparent dielectric constants experienced by pyrene in hippocampal membranes and in liposomes from a lipid extract derived from the calibration plot (shown in Figure 4) are summarized in Table 1. The apparent dielectric constant experienced by pyrene in hippocampal membranes turns out to be $\sim$20 $\pm$ 3, depending on the experimental conditions, in agreement with membrane interfacial dielectric characteristics reported previously.[27,28] As is apparent from the table, the apparent dielectric constant experienced by pyrene exhibits an increase upon cholesterol depletion in all cases, irrespective of the presence of proteins in the membrane. This reinforces our earlier conclusion from Figure 3.

Another commonly used parameter related to pyrene fluorescence is the excimer/monomer fluorescence intensity ratio.[19,20] This parameter is indicative of the extent of pyrene excimerization, which is believed to depend on the monomer lateral

TABLE 1: Apparent Dielectric Constants (ε) Determined from Pyrene Vibronic Peak Intensity Ratio (I_1/I_3) Measurements[a]

membrane type	ε
(a) Hippocampal Membranes	
native	16.57
native membranes treated with	
(i) 10 mM MβCD	16.90
(ii) 20 mM MβCD	17.10
(iii) 30 mM MβCD	17.88
(iv) 40 mM MβCD	18.07
(b) Lipid Extract of Hippocampal Membranes	
native	19.68
native membranes treated with	
(i) 10 mM MβCD	20.23
(ii) 20 mM MβCD	21.14
(iii) 30 mM MβCD	22.39
(iv) 40 mM MβCD	23.40

[a] The dielectric constant was obtained from the calibration plot shown in Figure 4 using I_1/I_3 values from pyrene vibronic peak intensities in the emission spectra. The pyrene first (373 nm) and third (384 nm) vibronic peak intensity ratio (I_1/I_3) is from Figure 3. The concentration of total phospholipid was 33 μM, and the ratio of pyrene to total phospholipid was 1:100 (mol/mol). See the Experimental Section for other details.

distribution and dynamics (diffusion) in the membrane, although the exact mechanism of excimerization is not clear.[29] Lateral diffusion in membranes is often described in terms of the free volume model.[30,31] The free volume framework is a semiempirical approach based on statistical mechanical considerations of density fluctuations in the lipid bilayer. According to this model, transient voids that are created in the lipid bilayer by such density fluctuations are filled by the movement of neighboring lipid molecules into the void. It is generally agreed that the greater the free volume available for pyrene, the higher the level of excimer formation.[20]

Figure 5a shows the excimer/monomer ratio in hippocampal membranes treated with increasing concentrations of MβCD. The extent of excimer formation appears to increase almost linearly with an increase in the extent of cholesterol depletion. Assuming the excimer/monomer ratio to be indicative of the free volume in the membrane bilayer,[20] cholesterol depletion appears to increase the free volume with increasing concentrations of MβCD. This results in increased diffusion (lateral mobility) in the hippocampal membrane upon cholesterol depletion. The increase in lateral diffusion upon cholesterol depletion appears to be valid in the case of liposomes prepared from a lipid extract of native hippocampal membranes treated with increasing concentrations of MβCD (see Figure 5b). These results are in agreement with our previous observation, using the fluorescence recovery after photobleaching (FRAP) approach, that lateral diffusion of lipid probes in hippocampal membranes increases upon cholesterol depletion.[32]

In this paper, we have monitored the organization and dynamics of hippocampal membranes and their modulation by cholesterol using pyrene fluorescence. Knowledge of dynamics would help in the analysis of functional data generated by modulation of the membrane lipid composition.[10] Our results show that the polarity of the hippocampal membrane is increased upon cholesterol depletion, as monitored by the change in the ratio of the pyrene vibronic band intensities. This is accompanied by an increase in lateral diffusion, measured as an increase in the pyrene excimer/monomer ratio. Since pyrene is localized predominantly in the interfacial region of the membrane,[21] the information obtained about the polarity change would originate

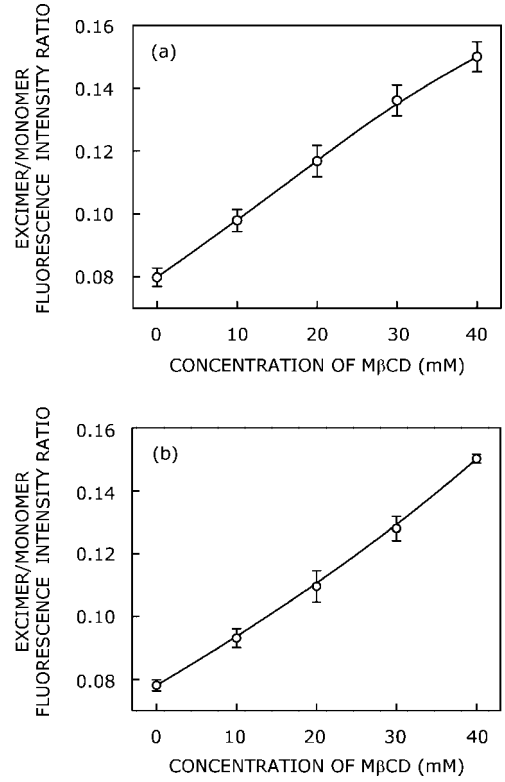

Figure 5. Pyrene excimer (480 nm)/monomer (393 nm) fluorescence intensity ratio in (a) hippocampal membranes and (b) liposomes of a lipid extract from native membranes with increasing concentration of MβCD. Data points shown are means $\pm$ SE of at least four independent measurements. The lines joining the data points are provided merely as viewing guides. All other conditions are as in Figure 1. See the Experimental Section for other details.

from this region of the membrane. This is a functionally important region of the membrane characterized by unique motional and dielectric properties.[33]

Taken together, our results constitute one of the first reports on the organization and dynamics of hippocampal membranes and their modulation by cholesterol using pyrene fluorescence. The membrane organization and dynamics represent important determinants in protein-protein interactions in cell membranes and have a significant impact on the overall efficiency of the signal transduction process.[34−36] In particular, these results could be relevant in understanding the complex spatiotemporal organization of neuronal membranes and may have functional implications in neuronal diseases such as the Smith-Lemli-Opitz syndrome,[6,37] which is characterized by defective cholesterol biosynthesis leading to metabolic cholesterol depletion.

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