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Short communication

Monitoring cholesterol organization in membranes at low concentrations utilizing the wavelength-selective fluorescence approach

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Abstract

We previously showed using a fluorescent analogue of cholesterol (NBD-cholesterol, or 25-[*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27-norcholesterol), that cholesterol may exhibit local organization at low concentrations in membranes by the formation of transbilayer tail-to-tail dimers of cholesterol (Rukmini, R., Rawat, S.S., Biswas, S.C., Chattopadhyay, A., 2001. Biophys. J. 81, 2122–2134). In this report, we have monitored the microenvironmental features of cholesterol monomers and dimers utilizing wavelength-selective fluorescence spectroscopy. Our results utilizing red edge excitation shift (REES) and wavelength-dependent change in fluorescence anisotropy show that the microenvironment around the NBD moieties in the dimer form is more rigid possibly due to steric constraints imposed by the dimer conformation. These results provide new information and are relevant in understanding the organization of cholesterol in membranes at low concentrations.

Keywords: Cholesterol organization; Cholesterol dimers; NBD-cholesterol; REES; Fluorescence anisotropy

1. Introduction

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting (Yeagle, 1985; Simons and Ikonen, 2000). It is often found distributed nonrandomly in domains or pools in biological and model membranes (Yeagle, 1985; Simons and Ikonen, 1997, 2000). Many of these domains are believed to be important for the maintenance of membrane structure and function. Although a large body of literature exists on the organization of cholesterol in plasma membranes or membranes with high cholesterol content, very little is known about its organization in the membrane when the cholesterol content is very low (<5 mol%) similar to what is found in endoplasmic reticulum (where cholesterol biosynthesis takes place) and inner mitochondrial

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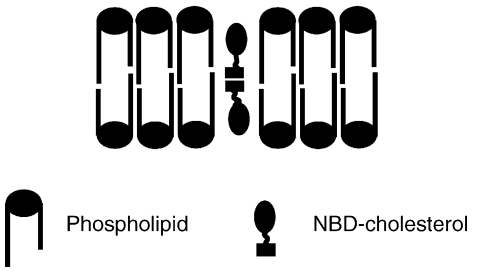

Fig. 1. A schematic diagram of the membrane bilayer showing the transbilayer tail-to-tail dimers of NBD-cholesterol in membranes at low concentrations (adapted and modified from Rukmini et al., 2001).

membranes (Lange et al., 1999). We have previously shown, using a fluorescent analogue of cholesterol (NBD-cholesterol, or 25-[*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27-norcholesterol), in which the flexible acyl chain of cholesterol is labeled with the NBD group, that cholesterol may exhibit local organization even at very low concentrations in membranes. Our results indicated the possible presence of transbilayer tail-to-tail dimers of cholesterol (see Fig. 1) in such membranes (Mukherjee and Chattopadhyay, 1996; Rukmini et al., 2001). These results are supported by similar observations, using cholesterol (Harris et al., 1995) and dehydroergosterol (a naturally occurring fluorescent cholesterol analogue) (Loura and Prieto, 1997; Rukmini et al., 2001), obtained under comparable concentrations. In addition, we further showed that the transbilayer dimer arrangement of cholesterol observed at low concentrations in membranes is sensitive to membrane surface curvature and thickness (Rukmini et al., 2001).

In this paper, we have investigated the microenvironmental features of cholesterol monomers and dimers by utilizing wavelength-selective fluorescence spectroscopy. Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy, which can be used to directly monitor the environment and dynamics around a fluorophore in an organized molecular assembly (Chattopadhyay, 2003). A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. The unique feature of REES is that while other fluorescence techniques yield information about the fluorophore itself, REES provides information about the relative rates of solvent relaxation, which is not possible to obtain by other techniques. We have previously shown that REES and related techniques (wavelength-selective fluorescence approach) serve as powerful tools to monitor the organization and dynamics of probes and peptides bound to membranes and membrane-mimetic media (reviewed in Chattopadhyay, 2003). We show here, using REES data obtained in conditions favoring predominantly either monomers or dimers of cholesterol, that the microenvironment around the dimer of cholesterol offers more restriction to solvent reorientation in the excited state and hence appears to be more rigid. This is further supported by changes in fluorescence anisotropy as a function of excitation wavelength.

2. Experimental procedures

2.1. Materials

DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and NBD-cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The purity of phospholipids and NBD-cholesterol were checked as described previously (Rukmini et al., 2001). Concentration of DPPC was determined by phosphate assay after total digestion by perchloric acid (McClare, 1971). DMPC was used as an internal standard to assess lipid digestion. Concentration of stock solutions of NBD-cholesterol in methanol was estimated using molar extinction coefficient (ε) of 22,000 M^{-1} cm^{-1} at 484 nm (Rukmini et al., 2001). Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. Sample preparation

All experiments were done using large (100 nm diameter) unilamellar vesicles (LUVs) of DPPC in the gel

phase containing 0.1 or 2 mol% NBD-cholesterol. The samples containing 0.1 or 2 mol% NBD-cholesterol represent predominantly either monomer or dimer forms of cholesterol, respectively (Mukherjee and Chattopadhyay, 1996; Rukmini et al., 2001). Samples containing 640 nmol of DPPC, and 0.64 or 12.8 nmol (for 0.1 and 2 mol% samples, respectively) of NBD-cholesterol were taken together in methanol, a few drops of chloroform were added and mixed well, and dried under a stream of nitrogen while being warmed gently (∼35 °C). After further drying under a high vacuum for at least 6 h, 1.5 ml of 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 buffer was added, and lipid samples were hydrated (swelled) well above the phase transition temperature of DPPC while being vortexed for 3 min to disperse the lipid and form homogeneous multilamellar vesicles (MLVs). The lipid suspensions were maintained at ∼60 °C (i.e., above the phase transition temperature) as the vesicles were made. LUVs of 100 nm diameter were prepared by the extrusion technique using an Avanti Mini-Extruder (Alabaster, AL, USA) as previously described (MacDonald et al., 1991). Briefly, the multilamellar vesicles were freeze-thawed five times by cycling in liquid nitrogen and water maintained at 60 °C to ensure solute equilibration between trapped and bulk solutions, and then extruded through polycarbonate filters (pore diameter of 100 nm) mounted in the extruder fitted with Hamilton syringes (Hamilton Company, Reno, NV, USA). The samples were subjected to 11 passes through the polycarbonate filter to give the final LUV suspension. Samples were incubated in dark for 12 h at room temperature (23 °C) for equilibration before measuring fluorescence. Background samples were prepared in the same way except that fluorophore was not added to them. All experiments were performed at room temperature (23 °C) where DPPC membranes are in the gel phase.

2.2.2. *Steady state fluorescence measurements*

Steady state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used for all measurements. Background intensities of samples in which NBD-cholesterol was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ±1 nm of the ones reported. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory as previously described (Raghuraman et al., 2004). All experiments were done with multiple sets of samples and average values of anisotropy are shown in Fig. 4.

3. Results

Fig. 2 shows the emission spectra of NBD-cholesterol in gel phase DPPC vesicles in conditions favoring predominantly either monomers or dimers of cholesterol. We consider the samples containing 0.1 mol% NBD-cholesterol to be representative of predominantly the monomer form of cholesterol in the membrane. As noted earlier (Mukherjee and Chattopadhyay, 1996), the spectrum is smooth and homogeneous at low (0.1 mol%) concentration of NBD-cholesterol with the maximum of fluorescence emission around 522 nm, which corresponds to the spectral feature of the NBD-cholesterol monomers in the membrane. We have previously shown that at a higher (2 mol%) concentration of NBD-cholesterol,

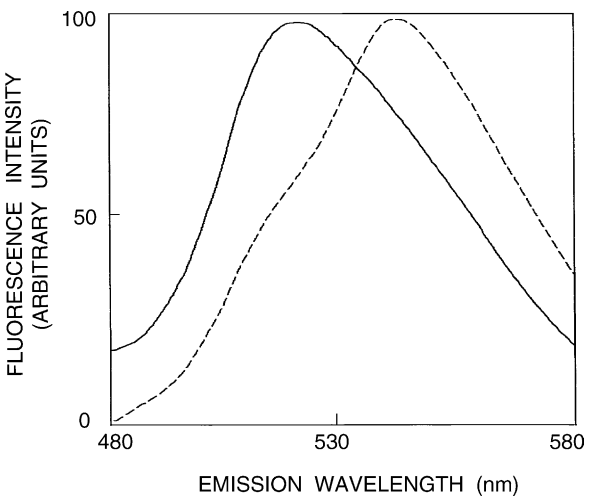

Fig. 2. Fluorescence emission spectra of 0.1 mol% NBD-cholesterol (—) and 2 mol% NBD-cholesterol (- - -) in DPPC large unilamellar vesicles in the gel phase. The concentration of DPPC was 0.43 mM, and excitation wavelength was 460 nm in both cases. The spectra are intensity-normalized at the emission maximum. See Section 2 for other details.

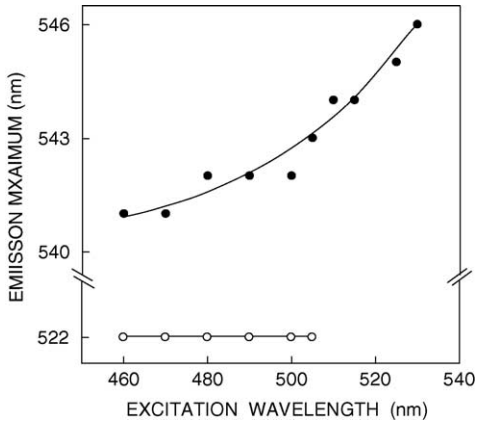

Fig. 3. Effect of changing excitation wavelength on the wavelength of maximum emission for 0.1 mol% NBD-cholesterol (◯) and 2 mol% NBD-cholesterol (●) in DPPC large unilamellar vesicles in the gel phase. All other conditions are as in Fig. 2. See Section 2 for other details.

transbilayer tail-to-tail dimers of NBD-cholesterol are formed in gel phase DPPC membranes and spectral features corresponding to the dimers predominante (Mukherjee and Chattopadhyay, 1996; Rukmini et al., 2001). The fluorescence emission spectrum in this case becomes relatively broad and inhomogeneous and displays an emission maximum at ∼541 nm which corresponds to the transbilayer cholesterol dimer.

The shift in the maxima of fluorescence emission[1] of 2 mol% NBD-cholesterol (representing the predominantly dimer form) in DPPC vesicles in the gel phase as a function of excitation wavelength is shown in Fig. 3. As mentioned above, the fluorescence emission maximum was found to be 541 nm when excited at 460 nm. As the excitation wavelength is changed from 460 to 530 nm, the emission maximum is shifted from 541 to 546 nm, which corresponds to a REES of 5 nm. Such a shift in the wavelength of emission maximum with change in the excitation wavelength is characteristic of REES and indicates that the NBD moieties in the dimer form are localized in a motionally restricted region of the membrane, which offers considerable resistance to solvent reorientation in the excited state (Chattopadhyay, 2003). This microenvironmental restriction could be generated due to the definite spatial orientation of the two NBD groups in the dimer arrangement (Rukmini et al., 2001). In addition, the aromatic-aromatic interaction between the NBD rings, would make the microenvironment more polar. These microenvironmental features would tend to slow down the rate of solvent reorientation giving rise to REES effects. In sharp contrast to this, the emission maximum of 0.1 mol% NBD-cholesterol (representing the predominantly monomer form) in DPPC vesicles in the gel phase remains invariant at 522 nm when the excitation wavelength was changed from 460 to 505 nm. In other words, the monomers of NBD-cholesterol do not display any REES under these conditions. These results show that REES is sensitive to the organization of NBD-cholesterol in the membrane and could potentially be used to distinguish these two forms.

In addition to the dependence of fluorescence emission maxima on the excitation wavelength, fluorescence anisotropy is also known to depend on the excitation wavelength in motionally restricted media (Mukherjee and Chattopadhyay, 1995 and references therein). Due to strong dipolar interactions with the surrounding solvent molecules, there is a decreased rotational rate of the fluorophore in the relaxed state. On red edge excitation, a selective excitation of this subclass of fluorophore occurs. Because of strong interaction with the polar solvent molecules in the excited state, one may expect these “solvent relaxed” fluorophores to rotate more slowly, thereby increasing the anisotropy.

The excitation anisotropy spectra (i.e., a plot of steady state anisotropy *vs*. excitation wavelength) of NBD-cholesterol in both concentrations in DPPC vesicles in the gel phase are shown in Fig. 4. The anisotropy of NBD-cholesterol is in general lower for the dimer form. This could be attributed to depolarization resulting from self-energy transfer in the dimer form. In addition, it was previously shown that fluorescence anisotropy of NBD-cholesterol in DPPC gel phase membranes in general decreases with increasing probe concentration (Mukherjee and Chattopadhyay, 1996). Interestingly, the anisotropy of 2 mol% NBD-cholesterol shows a considerable increase (94%) upon increasing the excitation wavelength from 460 to 525 nm (Fig. 4). Such an increase in anisotropy upon

[1] We have used the term maximum of fluorescence emission in a somewhat wider sense here. In every case, we have monitored the wavelength corresponding to maximum fluorescence intensity, as well as the center of mass of the fluorescence emission. In most cases, both these methods yielded the same wavelength. In cases where minor discrepancies were found, the center of mass of emission has been reported as the fluorescence maximum.

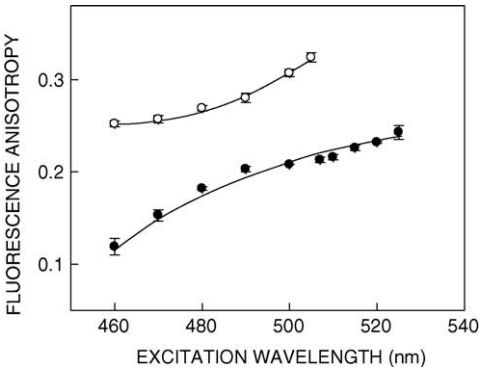

Fig. 4. Fluorescence anisotropy of 0.1 mol% NBD-cholesterol (○) and 2 mol% NBD-cholesterol (●) in DPPC large unilamellar vesicles in the gel phase as a function of excitation wavelength. The emission was monitored at 522 nm for 0.1 mol% NBD-cholesterol and 541 nm for 2 mol% NBD-cholesterol. All other conditions are as in Fig. 2.The data points shown are the means ± S.E. of at least five independent measurements. See Section 2 for other details.

red edge excitation has been previously reported for fluorophores in motionally restricted media (Mukherjee and Chattopadhyay, 1995). On the other hand, the anisotropy of 0.1 mol% NBD-cholesterol shows a much weaker dependence (24% increase) upon increasing excitation wavelength. Thus, the dimer form exhibits a much stronger dependence of its fluorescence anisotropy on excitation wavelength than the monomer form. This is partly due to a reduction in homotransfer since the spectral overlap is less in the dimer form because of red shifted emission. This is true even if one compares the increase in fluorescence anisotropy for identical range of excitation wavelength (i.e., from 460 to 505 nm) in both cases (70 and 24% for the dimer and monomer forms, respectively).

4. Discussion

We have previously shown that cholesterol may exhibit local organization even at very low concentrations in membranes using NBD-cholesterol. Our results indicated the presence of transbilayer tail-to-tail dimers of cholesterol in such membranes (Mukherjee and Chattopadhyay, 1996; Rukmini et al., 2001). In this paper, we have explored the motional characteristics of the microenvironment around the monomer and dimer forms of NBD-cholesterol. Our results utilizing REES and wavelength-dependent change in fluorescence anisotropy show that the microenvironment around the NBD moieties in the dimer form is more rigid possibly due to steric constraints and additional polarity imposed by the dimer conformation.

The transbilayer tail-to-tail cholesterol organization could have a role in pathogenic states. The importance of cholesterol transbilayer domains in atherogenesis was previously demonstrated by Tulenko et al. (1998). In this study, small angle X-ray diffraction was used to examine arterial smooth muscle cell plasma membranes isolated from control and cholesterol-fed (2%) atherosclerotic rabbits. Beyond 9 weeks of cholesterol feeding, X-ray diffraction patterns demonstrated a lateral immiscible cholesterol domain with a bilayer thickness of 34 Å coexisting with the liquid crystalline (fluid) lipid bilayer. The membrane thickness of 34 Å corresponds to a tail-to-tail arrangement of cholesterol dimers as the length of an individual cholesterol molecule is 17 Å. This points out the possible involvement of the transbilayer tail-to-tail organization of cholesterol in atherogenesis. In another study, transbilayer cholesterol domains have been implicated in human ocular lens fiber cell plasma membranes, especially in cataractous condition (Jacob et al., 1999, 2001).

The wavelength-selective fluorescence approach represents a powerful and sensitive tool to study membrane organization and dynamics (Mukherjee and Chattopadhyay, 1995; Chattopadhyay, 2003). We have previously reported changes in membrane dynamics detected using this approach due to change in probe location as a function of membrane penetration depth (Chattopadhyay and Mukherjee, 1999), ionization state (Mukherjee et al., 2004) and charge of the lipid headgroup (Kelkar et al., 2003). The results presented here show that this approach can be used to detect differences in microenvironment around the NBD group of NBD-cholesterol in conditions where the monomer or the dimer form predominates in the membrane. These results are significant in the context of cholesterol organization in membranes at low concentrations.

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