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Effect of cholesterol on lateral diffusion of fluorescent lipid probes in native hippocampal membranes

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Abstract

Cholesterol is an abundant lipid of mammalian membranes and plays a crucial role in membrane organization, dynamics, function and sorting. The role of cholesterol in membrane organization has been a subject of intense investigation that has largely been carried out in model membrane systems. An extension of these studies in natural membranes, more importantly in neuronal membranes, is important to establish a relationship between disease states and changes in membrane physical properties resulting from an alteration in lipid composition. We have monitored the lateral diffusion of lipid probes, $DiIC_{18}(3)$ and *FAST* DiI which are similar in their intrinsic fluorescence properties but differ in their structure, in native and cholesterol-depleted hippocampal membranes using the fluorescence recovery after photobleaching (FRAP) approach. Our results show that the mobility of these probes is in general higher in hippocampal membranes depleted of cholesterol. Interestingly, the increase in mobility of these probes does not linearly correlate with the extent of cholesterol depletion. These results assume significance in the light of recent reports on the requirement of cholesterol to support the function of the G-protein coupled $serotonin_{1A}$ receptor present endogenously in hippocampal membranes.

Keywords: Hippocampal membranes; Cholesterol; Lateral diffusion; FRAP; Indocarbocyanine probes; $DiIC_{18}(3)$; *FAST* DiI

1. Introduction

Cholesterol is an abundant lipid in mammalian membranes and plays a crucial role in membrane organization, dynamics, function and sorting (Schroeder et al., 1995). It is often found distributed non-randomly in domains or pools in biological and model membranes (Schroeder et al., 1995; Simons and Ikonen, 1997; Xu and London, 2000; Rukmini et al., 2001; Mukherjee and Maxfield, 2004). The preferential interaction between certain classes of lipids such as cholesterol, sphingolipids and lipids with saturated fatty acyl chains is thought to play a role in the domain-like organization of cell membranes (Silvius, 2003; Mukherjee and Maxfield, 2004). These domains are considered to be laterally segregated entities with lipid and protein composition and physical properties distinct from that of the bulk membrane. Evidence gathered from model membranes with similar lipid composition as that found in these domains have suggested that they represent a liquid-ordered like phase in an otherwise fluid (liquid

Abbreviations: BCA, bicinchoninic acid; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; $DiIC_{18}(3)$, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DPH, 1,6-diphenyl-1,3,5-hexatriene; *FAST* DiI, 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine 4-chlorobenzenesulfonate; FRAP, fluorescence recovery after photobleaching; MβCD, methyl-β-cyclodextrin; PMSF, phenylmethylsulfonyl fluoride

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crystalline) membrane (Brown, 1998; London and Brown, 2000). The liquid-ordered phase is characterized by the presence of tightly packed lipids, as in an ordered gel phase (Ipsen et al., 1987; Sankaram and Thompson, 1990) but exhibits rapid lateral diffusion (Rubenstein et al., 1979) similar to that observed in a liquid crystalline phase. The idea of such specialized membrane domains assumes importance in cell biology since physiologically important functions such as membrane sorting and trafficking (Simons and van Meer, 1988) and signal transduction processes (Simons and Toomre, 2000) have been attributed to these domains on account of their unique lipid-protein composition and their potential to sequester signaling molecules in the plane of the membrane.

The nervous system characteristically contains very high concentration of lipids (which is only exceeded by the adipose tissue). 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 (Sastry, 1985). The central nervous system accounts for only 2% of the body mass yet contains ∼25% of free cholesterol present in the whole body (Dietschy and Turley, 2001). Brain cholesterol is synthesized in situ and is developmentally regulated. The organization, traffic and dynamics of brain cholesterol are stringently controlled through exclusive in situ synthesis in the brain (Dietschy and Turley, 2001). As a result, a number of neurological diseases, such as the Smith-Lemli-Opitz syndrome (SLOS), share a common etiology of defective cholesterol metabolism in the brain (Waterham and Wanders, 2000). Although several epidemiological studies indicate a possible role of lipids in a variety of neurological disorders (Porter, 2002), the organization and dynamics of neuronal membranes as a consequence of alterations in membrane lipid composition (specifically cholesterol) is still poorly understood (Wood et al., 1999; Pfrieger, 2003). In view of the importance of cholesterol in relation to membrane domains, the effect of alteration in the cholesterol content of neuronal membranes on membrane dynamics and protein/receptor function represents an important determinant in the analysis of several neuropathologies (Wood et al., 1999; Pfrieger, 2003).

Several studies in model membranes have investigated the manner in which cholesterol modulates key aspects of membrane structure and dynamics such as promoting a domain-like organization (Dietrich et al., 2001; McConnell and Radhakrishnan, 2003; Veatch and Keller, 2003; Crane and Tamm, 2004) and modulating lateral diffusion of membrane constituents (Rubenstein et al., 1979; Almeida et al., 1992; Ladha et al., 1996; Korlach et al., 1999; Dietrich et al., 2001; Crane and Tamm, 2004). Results from similar analysis on the role of cholesterol in modulating lateral diffusion of membrane constituents in cell membranes however appears to lack consensus. Thus, cholesterol depletion has been found to either selectively enhance lateral diffusion of putative raft-localized membrane proteins (Niv et al., 2002; Shvartsman et al., 2003), or reduce diffusion of membrane proteins and lipids irrespective of their putative raft localization (Kenworthy et al., 2004; Vrljic et al., 2005; Nishimura et al., 2006). A possible reason for this inconsistency could be the result of non-specific effects of cholesterol depletion from cells in culture. This is apparent from the recent observation that although acute (cyclodextrin-mediated) or chronic (statin-mediated) means of cholesterol depletion from cells effectively leads to loss in membrane cholesterol, these treatments can have a significantly different effect on the lateral diffusion characteristics of membrane lipids and proteins (Goodwin et al., 2005). Further, the simultaneous effects of cholesterol depletion on the lateral segregation and membrane anchoring of the widely used model system of peripherally-anchored membrane proteins (Rotblat et al., 2004) complicates analysis of the role of cholesterol-dependent lateral diffusion. In such a scenario, analyzing the lateral diffusion characteristics of lipid probes, which have earlier been widely used in model membranes, in response to a controlled alteration in the cholesterol content of a natural yet cell-free membrane system assumes significance. Further, such studies in membranes of neuronal origin would be significant in establishing a relationship between changes in membrane physical properties resulting from alteration in membrane composition to neurological disorders.

Work from our laboratory has established native membranes isolated from the bovine hippocampus as a natural source for the seven transmembrane domain, G-protein coupled, serotonin$_{1A}$ receptor (Pucadyil et al., 2005; Pucadyil and Chattopadhyay, in press). Importantly, we have shown the requirement of cholesterol in the ligand binding and G-protein coupling of the endogenously present serotonin$_{1A}$ receptor in hippocampal membranes (Pucadyil and Chattopadhyay, 2004, 2005, in press). A controlled alteration in the cholesterol content of hippocampal membranes in these studies was achieved using methyl-β-cyclodextrin (MβCD) which selectively extracts cholesterol from membranes by including it in a central non-polar cavity (Christian et al., 1997; Härtel et al., 1998). In order to correlate alterations in membrane protein function (in this case the serotonin$_{1A}$ receptor function) in response

to cholesterol depletion (Pucadyil and Chattopadhyay, 2004, 2005) to alterations in membrane dynamics, we report here a detailed analysis of the lateral diffusion characteristics of lipid probes, $DiIC_{18}(3)$ and *FAST* DiI, in native and cholesterol-depleted hippocampal membranes.

2. Experimental procedures

2.1. Materials

Amplex Red cholesterol assay kit, $DiIC_{18}(3)$ and *FAST* DiI were from Molecular Probes (Eugene, OR, USA). BCA, MβCD, DMPC and poly-L-lysine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BCA reagent kit for protein estimation was from Pierce (Rockford, IL, USA). Stock solutions of DiI probes were made in ethanol and stored at −70 °C under argon. Concentrations of stock solutions of $DiIC_{18}(3)$ and *FAST* DiI were estimated from their molar extinction coefficients (ε) of 148,000 $M^{-1}\,cm^{-1}$ at 549 nm (Haugland, 1996). All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout. Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at −70 °C till further use.

2.2. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously (Pucadyil and Chattopadhyay, 2004). Briefly, bovine hippocampal tissue (∼100 g) was homogenized as 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900 × *g* for 10 min at 4 °C. The resultant supernatant was filtered through four layers of cheesecloth and centrifuged at 50,000 × *g* for 20 min at 4 °C. The pellet thus obtained was suspended in 10 vol. of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50,000 × *g* for 20 min at 4 °C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was suspended in a minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at −70 °C. Protein concentration was assayed using the BCA assay kit (Smith et al., 1985) with bovine serum albumin as a standard.

2.3. Cholesterol depletion of native membranes

Native hippocampal membranes were depleted of cholesterol using MβCD as described previously (Pucadyil and Chattopadhyay, 2004). Briefly, membranes at a protein concentration of 2 mg/ml were treated with various concentrations of MβCD in buffer C at room temperature (24 °C) with constant shaking for 1 h. Membranes were then spun down at 50,000 × *g* for 5 min at 4 °C, washed with buffer C and resuspended in the same buffer. Cholesterol was estimated using the Amplex Red cholesterol assay kit (Amundson and Zhou, 1999). In order to preserve the size and morphology of the membrane patches for FRAP experiments (see later), all resuspension steps were carried out in a motorized Bellco homogenizer (Vineland, NJ, USA) fitted with a teflon-capped pestle at low speed.

2.4. Estimation of inorganic phosphate

Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid (McClare, 1971) using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.5. Labeling hippocampal membrane patches with lipid probes

Methanol-washed glass coverslips were coated with poly-L-lysine at a concentration of 0.1 mg/ml and dried. An aliquot (100 μl) of hippocampal membranes suspended at a protein concentration of 0.15 mg/ml in buffer C was poured onto these coverslips and the membrane patches were allowed to adhere for 30 min. Unbound membrane patches were washed off by dipping the coverslips in the same buffer several times. An aliquot of $DiIC_{18}(3)$ (typically 0.34 mM in ethanol) or *FAST* DiI (1.3 mM in ethanol) was dried under a gentle stream of argon. A minimum volume of ethanol was added to redissolve the probe and the solution was suspended in buffer C with vortexing to yield a final concentration of 8 μM of the probe with 1% (v/v) ethanol. An aliquot (50 μl) of this solution was used to label membranes on coverslips for 30 min at room temperature (24 °C) in dark. The coverslips were dipped 3–4 times in millipore water and placed upside down on a clean glass slide with a drop of

water. The edges were sealed with nail enamel and used for further experiments.

2.6. Fluorescence imaging of labeled hippocampal membrane patches and FRAP experiments

Fluorescence images of membrane patches were acquired on a Meridian Ultima 570 confocal laser scanning microscope using a 40×, 0.85NA objective at room temperature (24 °C). Fluorescence was acquired with the 514 nm line of an Ar laser as the excitation source. Emission was collected with a 580 ± 30 nm bandpass filter with an open pinhole. FRAP experiments were carried out in a Gaussian spot-photobleaching and line-scanning mode. Samples were bleached with a 30 ms pulse of the laser at maximum power (10 mW). Fluorescence recovery profiles were analyzed for diffusion coefficient and mobile fraction according to the equation (Yguerabide et al., 1982):

$$\mu(t) = [\mu(0)t_{1/2} + \mu(\infty)t]/[t_{1/2} + t] \quad (1)$$

where if $F(p)$ is the normalized prebleach intensity, $F(0)$ the intensity at bleach, $F(t)$ the intensity at time t and $F(\infty)$ is the intensity after recovery, then $\mu(0) = F(p) - F(0)$, $\mu(t) = F(p) - F(t)$, and $\mu(\infty) = F(p) - F(\infty)$. The calculated half-time for recovery $t_{1/2}$ is used to estimate diffusion coefficient D, according to the equation:

$$D = \beta\omega^2/4t_{1/2} \quad (2)$$

where β is a parameter which depends on the extent of bleach and is described in (Yguerabide et al., 1982) and ω is the e^{-2} beam radius. The mobile fraction R is given by $[\mu(0) - \mu(\infty)]/\mu(0)$. Data analysis and graphical representation were carried out with the Meridian Ultima Software version v4.15. Statistical analysis using one-way ANOVA was carried out with the Microcal Origin software version 5.0 (OriginLab Corporation, Northampton, MA, USA). Frequency distribution plots were generated using the same software.

3. Results

Fluorescence recovery after photobleaching (FRAP) is among the most widely used approaches in the quantitative analysis of diffusion characteristics in membranes (Petersen et al., 1986; Lippincott-Schwartz et al., 2001). This approach involves generating a concentration gradient of fluorescent molecules by irreversibly photobleaching a fraction of fluorophores in the observation region. The dissipation of this gradient with time owing to diffusion of fluorophores into the bleached region from unbleached regions is an indicator of the mobility of fluorophores. We carried out FRAP measurements in order to explore long-range membrane dynamics in hippocampal membranes. The DiI series of lipid analogues represent well characterized fluorescent probes (Klausner and Wolf, 1980; Spink et al., 1990; Mukherjee et al., 1999; Kalipatnapu and Chattopadhyay, 2004). The DiI analogues are composed of an indocarbocyanine headgroup and two hydrophobic alkyl chains (see Fig. 1) which impart an overall amphiphilic character. They have earlier been shown to preferentially partition into gel (ordered) or fluid (disordered) phases depending on the degree of matching between their acyl chain length and those of lipids that comprise the host membrane (Klausner and Wolf, 1980; Spink et al., 1990). $DiIC_{18}(3)$ and *FAST* DiI (Fig. 1A and B) represent two such probes that are similar in their intrinsic fluorescence properties but differ in their phase partitioning preference. Fluorescence quenching studies have earlier indicated that $DiIC_{18}(3)$ prefers to partition into a more ordered phase (Klausner and Wolf, 1980; Spink et al., 1990). Although a similar analysis has not been performed for *FAST* DiI, it is expected to partition more into disordered regions of the membrane due to the unsaturation in its acyl chains (Fig. 1B) which introduces kinks in the acyl chain resulting in packing defects in the membrane. This is well supported by the observed similarities in cellular trafficking properties of *FAST* DiI with short chain DiI analogues (Mukherjee et al., 1999), which are known to prefer a more disordered phase (Spink et al., 1990).

Patches of native hippocampal membranes were labeled with these probes and used for FRAP experiments (see Fig. 1C and D). The concentration of the probes were chosen so as to ensure optimal fluorescence intensities for FRAP experiments. Regions displaying intense fluorescence (generally in the centre of the patches) were avoided for FRAP measurements since very often there was no recovery seen after photobleaching. Fig. 2 shows typical fluorescence recovery plots of DiI probes in native and cholesterol-depleted hippocampal membranes. As seen from the plots, the kinetics of fluorescence recovery can be fitted well to Eq. (1) which assumes random diffusion of molecules in the membrane. Analysis of such FRAP experiments for $DiIC_{18}(3)$ in hippocampal membranes results in a diffusion coefficient of $\sim 1.0 \times 10^{-9}$ cm^2 s^{-1} with a mobile fraction of ∼58% (Table 1). In comparison, the diffusion coefficient of *FAST* DiI was found to be $\sim 0.9 \times 10^{-9}$ cm^2 s^{-1} with a mobile fraction of ∼54%. It is possible that our estimates of the diffusion coefficients of DiI probes could be affected by the non-uniform alignment of membrane

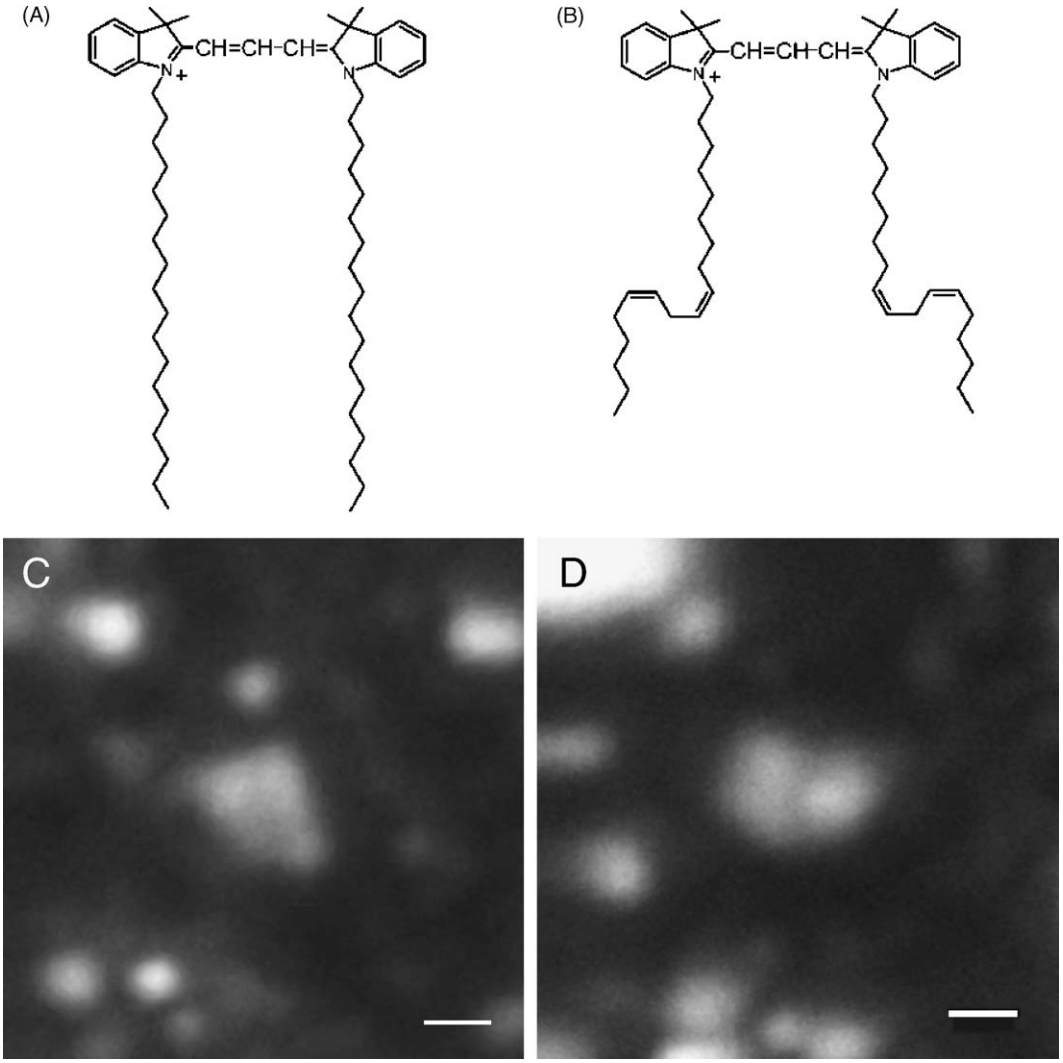

Fig. 1. The chemical structures of fluorescent lipid probes used: (A) $DiIC_{18}(3)$ and (B) *FAST* DiI. Representative fluorescence images of native hippocampal membrane patches labeled with (C) $DiIC_{18}(3)$ and (D) *FAST* DiI, and imaged as described in Section 2 are shown. The fluorescence images of membrane patches were acquired under similar conditions to those used in FRAP measurements. The scale bar represents 5 μm.

Table 1
Lateral diffusion parameters of $DiIC_{18}(3)$ and *FAST* DiI in native and cholesterol-depleted hippocampal membranes[a]

Membranes	$DiIC_{18}(3)$			*FAST* DiI		
	D^b ($\times 10^{-9}$ cm^2 s^{-1})	R^c (%)	N	D^b ($\times 10^{-9}$ cm^2 s^{-1})	R^c (%)	N
Native	1.06 ± 0.13	58 ± 3	32	0.91 ± 0.09	54 ± 3	28
Treated with 20 mM MβCD	2.54 ± 0.40[d]	54 ± 4	23	1.97 ± 0.26[d]	55 ± 3	14
Treated with 40 mM MβCD	1.83 ± 0.26[d,e]	51 ± 4	26	1.04 ± 0.13[e]	53 ± 2	15

[a] FRAP on native and cholesterol-depleted hippocampal membranes was carried out as described in Section 2.
[b] Refers to the diffusion coefficient of probes. The data represent the means ± S.E. of N number of measurements (sum of all measurements across different days).
[c] Refers to the mobile fraction of probes. The data represent the means ± S.E. of N number of measurements (sum of all measurements across different days).
[d] These values are significantly different ($P < 0.05$) from those in native membranes.
[e] These values are significantly different ($P < 0.05$) from each other.

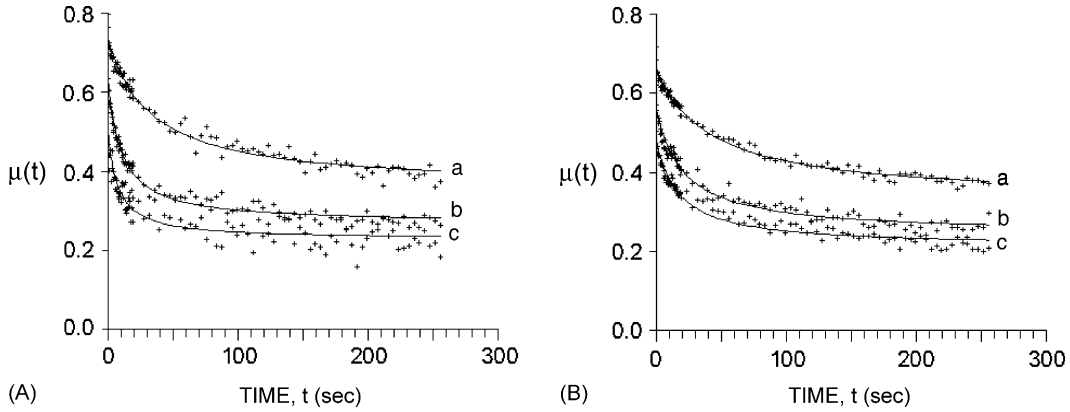

Fig. 2. Representative fluorescence recovery plots of (A) $DiIC_{18}(3)$ and (B) *FAST* DiI in bovine hippocampal membranes (control, shown as (a)) or treated with 20 mM (shown as (b)) and 40 mM MβCD (c). The solid lines are non-linear regression fits of the data to Eq. (1). The parameter μ on the ordinate (y-axis) is the difference between fluorescence intensity before bleach and during recovery. See Section 2 for more details.

patches with respect to the laser beam thereby leading to an underestimation in the area bleached in these membranes. Further, the low mobile fraction of these lipid probes in membrane patches could reflect the limitation in the size of the membrane patch in relation to the area bleached in FRAP experiments. Importantly, while the non-uniformity in alignment of membrane patches and their relatively small size is an unavoidable limitation in these experiments, a comparative analysis of diffusion behavior of $DiIC_{18}(3)$ and *FAST* DiI probes appears valid since these measurements have been performed under identical conditions for both probes. More importantly, the diffusion coefficients of DiI probes in hippocampal membranes (Table 1) agree well with those reported earlier in natural membranes (Thompson and Axelrod, 1980; Jacobson et al., 1981; Edidin and Stroynowski, 1991; Thomas et al., 1994; Mukhopadhyay et al., 2004; Vrljic et al., 2005).

The role of cholesterol in lateral diffusion of $DiIC_{18}(3)$ and *FAST* DiI in hippocampal membranes was monitored by performing FRAP experiments in cholesterol-depleted membranes labeled with these probes. As described earlier (Pucadyil and Chattopadhyay, 2004), cholesterol depletion was carried out using MβCD which selectively extracts cholesterol from membranes by including it in a central non-polar cavity. Thus, treatment of bovine hippocampal membranes with 20 and 40 mM MβCD resulted in depletion of ∼63% and 88% of membrane cholesterol with no appreciable loss of phospholipids (see Table 2). It must be mentioned here that the overall fluorescence distribution of $DiIC_{18}(3)$ and *FAST* DiI in cholesterol-depleted hippocampal membrane patches was similar to that seen in native membranes. Interestingly, treatment of native membranes with 20 mM MβCD significantly increases the diffusion of $DiIC_{18}(3)$ and *FAST* DiI by 2.4- and 2.2-fold respectively (see Table 1). The diffusion data plotted as a frequency distribution (see Fig. 3) indicate that the spread of diffusion coefficients of $DiIC_{18}(3)$ is larger than that observed for *FAST* DiI for hippocampal membranes treated with 20 mM MβCD. Thus, while the mean diffusion coefficients of both the probes in

Table 2
Estimation of cholesterol and phospholipid contents in native and MβCD-treated hippocampal membranes[a]

Membranes	Cholesterol content[b] (nmol/mg protein)	Phospholipid content[c] (nmol/mg protein)	Cholesterol:phospholipid ratio[d]
Native	452 ± 15 (100%)[e]	1192 ± 47 (100%)[e]	0.38
Treated with 20 mM MβCD	167 ± 6 (37%)	1179 ± 85 (99%)	0.14
Treated with 40 mM MβCD	54 ± 3 (12%)	1055 ± 42 (89%)	0.05

[a] Cholesterol depletion using MβCD was carried out as described in Section 2.
[b] Cholesterol content was assayed as described in Section 2 and was normalized with respect to total protein. The data represents the means ± S.E. of duplicate points from three independent experiments.
[c] Phospholipid content of normal and cholesterol-depleted membranes was assayed as described in Section 2. The data represent the means ± S.E. of six independent experiments.
[d] Cholesterol to phospholipid ratio was calculated for native and cholesterol-depleted membranes from data in columns 2 and 3.
[e] Numbers in parentheses indicate percentage of cholesterol and phospholipid contents of membranes normalized with respect to control values.

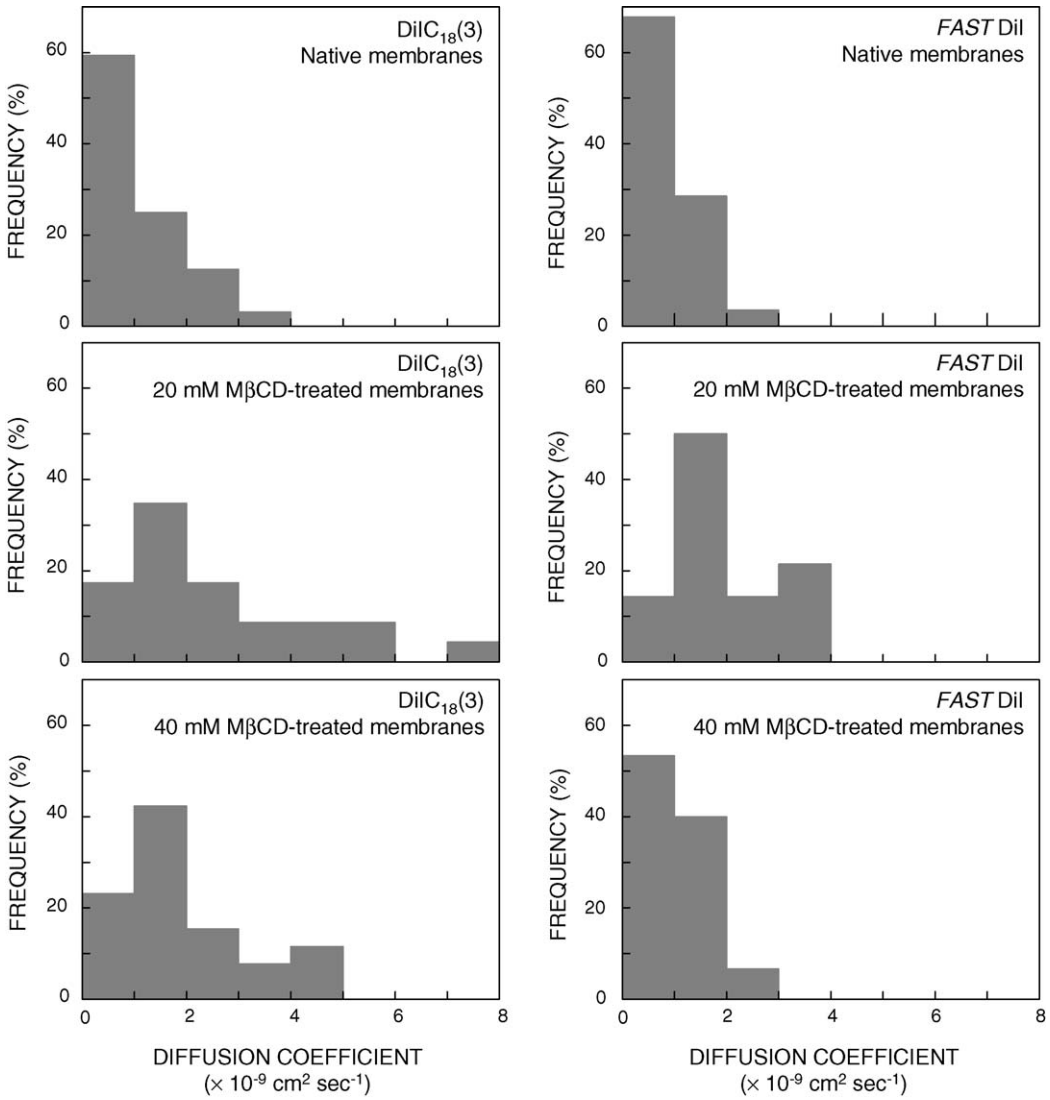

Fig. 3. Frequency distribution plots of diffusion coefficient values of $DiIC_{18}(3)$ and *FAST* DiI in native and cholesterol-depleted hippocampal membranes. See Table 1 and Section 2 for more details.

20 mM MβCD-treated membranes is higher than in native membranes, the differences in the fold increase (2.4 for $DiIC_{18}(3)$ and 2.2 for *FAST* DiI) in the diffusion of these probes does not appear significant due to the heterogeneity in diffusion coefficient values observed in case of $DiIC_{18}(3)$. It is however difficult to correlate the diffusion coefficient of DiI probes to membrane cholesterol content. This is apparent from the fact that a higher degree of cholesterol depletion using 40 mM MβCD results in an increase in the diffusion coefficient of $DiIC_{18}(3)$ of only 1.7-fold over native membranes while that of *FAST* DiI shows a more modest (1.1-fold) increase (see Fig. 3 and Table 1).

Our results point out several interesting features of diffusion behavior of these probes in native hippocampal membranes. There appears to be no pronounced difference between the diffusion parameters of $DiIC_{18}(3)$ and *FAST* DiI in native membranes in spite of their earlier described partitioning preferences in model membranes. Interestingly, differences between the diffusion properties of these probes are enhanced upon cholesterol depletion of native membranes. The lateral diffusion of the ordered phase preferring probe $DiIC_{18}(3)$ is found to be consistently higher in membranes depleted of cholesterol to varying extents compared to that found in native membranes. The corresponding increase in diffusion due

to cholesterol depletion for the disordered phase preferring probe *FAST* DiI appears to be less, particularly at higher extents of cholesterol depletion.

4. Discussion

We have analyzed the diffusion properties of two indocarbocyanine probes, $DiIC_{18}(3)$ and *FAST* DiI, which have earlier been shown to display membrane phase-specific partitioning properties in model membranes. Although fluorescence quenching studies have earlier indicated that $DiIC_{18}(3)$ prefers to partition into a more ordered phase (Klausner and Wolf, 1980; Spink et al., 1990), it has been recently reported that this probe could partition to a significant extent in the gel/fluid boundaries in model membranes having coexisting gel and fluid phases (Loura et al., 2000). Fluorescence images of planar bilayers consisting of gel and fluid phases in the presence of low amounts of cholesterol visualized with $DiIC_{18}(3)$ as a probe indicate a similar phenomenon (Crane and Tamm, 2004).

In spite of the structural differences between $DiIC_{18}(3)$ and *FAST* DiI, the lateral diffusion parameters of these probes in native hippocampal membranes are found to be similar. This may not be surprising considering the heterogeneous nature of membranes isolated from a natural source. In addition, the presence of membrane cholesterol and proteins would tend to reduce any possible phase separation that would have otherwise led to differences in the lateral diffusion of these probes. Interestingly, depletion of membrane cholesterol by ∼63% leads to an increase in the lateral diffusion of both $DiIC_{18}(3)$ and *FAST* DiI. Earlier reports describing the lateral diffusion of $DiIC_{18}(3)$ in natural membranes have indicated a reduction in its mobility in response to cholesterol depletion (Thompson and Axelrod, 1980; Vrljic et al., 2005). Thompson and Axelrod (1980) reported a reduction in the lateral diffusion of $DiIC_{18}(3)$ when erythrocyte membranes were depleted of cholesterol using small unilamellar vesicles of dipalmitoylphosphatidylcholine while Vrljic et al. (2005), reported a reduction in diffusion of $DiIC_{18}(3)$ in Chinese hamster ovary cells upon cholesterol depletion using β-cyclodextrin. It is possible that the disparity between our results and the earlier reports reflects inherent differences in the organization and dynamics of membranes isolated from neuronal and non-neuronal sources. In addition, our observation that the change in the lateral diffusion of *FAST* DiI is not linearly dependent on the cholesterol content of hippocampal membranes argues for a more detailed analysis of the relationship between the cholesterol content and its effects on lateral diffusion of such probes. Our present measurements represent a step in this direction. Significantly, we have used MβCD to modulate membrane cholesterol content in a more controlled manner (Table 2) as against small unilamellar vesicles which alter the native lipid composition of membranes (Thompson and Axelrod, 1980). In addition, our measurements were carried out on membranes depleted of cholesterol to varying extents (using varying concentrations of MβCD) rather than a unique concentration of β-cyclodextrin (Vrljic et al., 2005; Nishimura et al., 2006). Interestingly, we have earlier observed that the lateral diffusion of *FAST* DiI is higher in the plasma membrane of yeast biosynthetic mutants which have reduced amounts of ergosterol, the predominant sterol in yeast, compared to wild type cells (Mukhopadhyay et al., 2004). Our present results with hippocampal membranes depleted of cholesterol indicate a similar effect for both $DiIC_{18}(3)$ and *FAST* DiI for lower extents of cholesterol depletion.

We observe that the lateral diffusion of the ordered phase preferring probe $DiIC_{18}(3)$ is more responsive to modulation in cholesterol content. One reason for this effect might be its preferential partitioning into the putative cholesterol and sphingolipids enriched domains believed to exist in a liquid-ordered phase in natural membranes (London and Brown, 2000). Insolubility in non-ionic detergents such as Triton X-100 serves as a useful biochemical criterion to implicate the presence of membrane constituents in such domains (London and Brown, 2000). It has recently been demonstrated that $DiIC_{16}(3)$ (that is similar to $DiIC_{18}(3)$) displays a significant extent of detergent insolubility compared to *FAST* DiI in cell membranes that possibly reflects partitioning preferences of these probes into the above mentioned domains (Kalipatnapu and Chattopadhyay, 2004; Hao et al., 2001). It is conceivable that the reduction of membrane cholesterol could perturb these domains, as has been indicated in studies carried out in several model and natural membrane systems (Dietrich et al., 2001, 2002; Samsonov et al., 2001) thereby leading to a greater increase in the lateral diffusion of $DiIC_{16}(3)$ compared to *FAST* DiI. Interestingly, confocal microscopic studies of cells labeled with $DiIC_{16}(3)$ and *FAST* DiI probes have previously indicate a large scale segregation of such probes upon cholesterol depletion (Hao et al., 2001). This study showed that while the distribution of $DiIC_{16}(3)$ and *FAST* DiI probes was homogenous in the plasma membrane of normal cells, *FAST* DiI was localized in domains in cholesterol-depleted cells where it showed a reduced rate of exchange with probes in other regions of the membrane. It is possible that a similar alteration in distribution of *FAST* DiI could occur in hip-

pocampal membranes at higher extents of cholesterol depletion thereby leading to no apparent change in its lateral diffusion.

Native membranes isolated from the bovine hippocampus represent a natural source for the seven transmembrane domain, G-protein coupled, serotonin$_{1A}$ receptor (Pucadyil et al., 2005). The major paradigm in GPCR signaling is that their stimulation leads to the recruitment and activation of heterotrimeric GTP-binding proteins (G-proteins) (Hamm, 2001). These initial events, which are fundamental to all types of GPCR signaling, occur in the membrane via protein–protein interactions. An important consequence of this is that dynamics of membrane constituents represents an important determinant in receptor/G-protein interaction, and has significant impact on the overall efficiency of the signal transduction process (Neubig, 1994; Pucadyil et al., 2004). In this regard, the observation that GPCRs are not uniformly present on the plasma membrane but are concentrated in specific membrane domains that are enriched in cholesterol assumes significance (Ostrom and Insel, 2004). We have previously shown the requirement of cholesterol in the ligand binding and G-protein coupling of the endogenously present serotonin$_{1A}$ receptor in hippocampal membranes (Pucadyil and Chattopadhyay, 2004, 2005). Further, we have reported that fluorescence polarization of the membrane probe DPH (which displays no partitioning preference for gel or fluid phases in model membranes (London and Feigenson, 1981)) shows a steady decrease with increasing extents of cholesterol depletion (Pucadyil and Chattopadhyay, 2004). Along with the observed increase in mobility of DiIC$_{18}$(3), our results therefore indicate an overall reduction in membrane order upon cholesterol depletion in terms of both lateral and rotational diffusion of membrane embedded probes. The present results therefore provide an experimental framework in which to correlate alterations in membrane protein function (in this case the serotonin$_{1A}$ receptor) in response to cholesterol depletion (Pucadyil and Chattopadhyay, 2004, 2005) to alterations in membrane dynamics.

While the effect of cholesterol on the lateral diffusion of membrane embedded probes in model membranes has been well established, a similar level of understanding is beginning to emerge for natural membranes (Mukhopadhyay et al., 2004; Goodwin et al., 2005; Vrljic et al., 2005; Nishimura et al., 2006). The compositional heterogeneity associated with natural membranes, in addition to the lack of a precise knowledge of the partitioning preferences of various probes in such membranes, make it difficult to interpret experimental results. Clearly, a more general understanding of the effects of cholesterol on membrane dynamics requires further experimental data from natural membrane systems. Our experiments on analyzing the lateral diffusion properties of DiI probes, which have previously been well characterized in terms of their partitioning preferences in model membranes, in native and cholesterol-depleted hippocampal membranes represent an attempt in generating such a level of understanding.

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