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Use of a fluorescent cholesterol derivative to measure lateral mobility of cholesterol in membranes

(4-nitrobenzo-2-oxa-1,3-diazole-labeled cholesterol/fluorescence recovery after photobleaching)

M. R. ALECIO*, D. E. GOLAN*†‡, W. R. VEATCH*§, AND R. R. RANDO*¶

*Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115; and †Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520

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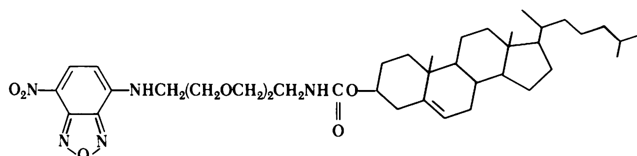
ABSTRACT N^1-Cholesterylcarbamoyl-N^8-(4-nitrobenzo-2-oxa-1,3-diazole)-3,6-dioxaoctyl-1,8-diamine (NBD-Chol), a new fluorescent derivative of cholesterol, was incorporated into L-α-dimyristoylphosphatidylcholine ($Myr_2PtdCho$)-based liposomes. The lateral mobility of this derivative, as well as that of N-(4-nitrobenzo-2-oxa-1,3-diazole)phosphatidylethanolamine (NBD-PtdEtn), was measured by fluorescence recovery after photobleaching techniques. In $Myr_2PtdCho$ liposomes, the diffusion coefficients (D) of the two probes are the same within experimental error below (D, $\approx 2 \times 10^{-10}$ $cm^2 \cdot sec^{-1}$) and above (D, $\approx 2 \times 10^{-8}$ $cm^2 \cdot sec^{-1}$) the main phase transition temperature of the bulk lipid (T_m). There is, however, a distinct difference between the mobilities of the derivatives at concentrations of added cholesterol between 5 and 20 mol % at temperatures below the main phase transition. Under these conditions, the diffusion coefficient of NBD-Chol is approximately twice that of NBD-PtdCho, a result consistent with the idea that cholesterol undergoes a lateral phase separation in these membranes at concentrations less than 20 mol %. At cholesterol concentrations greater than 20 mol % or temperatures above the T_m, the D values of the two probes are identical. The lateral mobility of a cholesterol derivative has thus been monitored directly in cholesterol-containing membranes.

Cholesterol is an important constituent of most eukaryotic cell membranes and, as such, a great deal is known about certain aspects of its function. In model systems, cholesterol distributes evenly on both sides of the bilayer with its polar hydroxyl group held in the vicinity of the phosphate groups of the phospholipids (1, 2). The molecule undergoes rapid uncatalyzed transmembrane and intermembraneous transfer (3, 4). In addition, the bulk effects of cholesterol on membrane phase transitions and, as a consequence, on membrane fluidity and permeability have been well documented (5, 6). On the other hand, very little is known about the lateral mobility and distribution of cholesterol in either model or natural membranes. To study the lateral mobility of cholesterol in membranes, we have prepared the fluorescent derivative N^1-cholesterylcarbamoyl-N^8-(4-nitrobenzo-2-oxa-1,3-diazole)-3,6-dioxaoctyl-1,8-diamine (NBD-Chol; I).



I

The lateral mobility of this probe in L-α-dimyristoylphosphatidylcholine ($Myr_2PtdCho$)-based liposomes was examined by fluorescence recovery after photobleaching techniques (7). The lateral mobility of NBD-Chol in these liposomes was measured as a function of temperature and as a function of cholesterol concentration and compared with that of N-(4-nitrobenzo-2-oxa-1,3-diazole)-3,6-dioxaoctyl-1,8-diamine (NBD-PtdEtn), a fluorescent phospholipid derivative.

MATERIALS AND METHODS

$Myr_2PtdCho$ and NBD-PtdEtn, prepared from "transphosphatidylated" egg phosphatidylcholine, were purchased from Avanti Biochemicals. Cholesterol (cholest-5-ene-3β-ol) was obtained from Sigma. NBD-Chol was prepared as described (8). The buffer used was 150 mM sodium chloride/10 mM monosodium phosphate adjusted to pH 7.2 (sodium hydroxide), through which a fast stream of nitrogen had been passed for at least 20 min to remove dissolved oxygen.

Preparation of Liposomes. A published method was modified as follows (9). Solutions of $Myr_2PtdCho$ and cholesterol (if required) in chloroform were mixed in the desired proportion in a screw-cap vial (15 × 120 mm) to give a total of 1.5 μmol of material. NBD-PtdEtn or NBD-Chol (2.2 nmol, 0.15 mol %) in chloroform was added and, after mixing, the solvent was removed at 20°C by using a rotory evaporator. The mixed lipids were left in the form of a thin film, which was handled under nitrogen to exclude oxygen as thoroughly as possible. Chloroform (200 μl) was added to dissolve the lipids and solvent was removed as before. Traces of solvent were removed by maintaining the sample for at least 8 hr at a pressure of ca. 0.5 torr (1 torr = 133 Pa) at 20°C.

The lipid was allowed to hydrate for 15 min at 40°C under nitrogen in buffer (1 ml) and then agitated vigorously on a Vortex for 15 sec. This procedure produced a suspension of uniformly fluorescent large multilamellar liposomes (diameter, >30–40 μm), which was deoxygenated with a stream of nitrogen for 1 hr at room temperature in a glove bag. Two microliters of this solution was applied to a microscope slide and sealed with epoxy resin inside the glove bag.

Fluorescence Recovery After Photobleaching. The apparatus used was modified from that previously described (10). An interferometer (Ealing) was equipped with electromechanical shutters (Ilex), which were microprocessor controlled (MOS. KIM-1) to enable the measurement of very rapid diffusion, as described by Koppel (11). A ×100 or a ×40 microscope objec-

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Abbreviations: NBD, 4-nitrobenzo-2-oxa-1,3-diazole; NBD-PtdEtn, N-(4-nitrobenzo-2-oxa-1,3-diazole)phosphatidylethanolamine; NBD-Chol, N^1-cholesterylcarbamoyl-N^8-(4-nitrobenzo-2-oxa-1,3-diazole)-3,6-dioxaoctyl-1,8-diamine; $Myr_2PtdCho$, L-α-dimyristoylphosphatidylcholine; D, diffusion coefficient; $f(\infty)$, fractional recovery of fluorescence; T_m, main phase-transition temperature.

‡ Present address: Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115.
§ Deceased.
¶ To whom reprint requests should be addressed.

Table 1. Lateral mobility of NBD-PtdEtn and NBD-Chol in pure $Myr_2PtdCho$ liposomes: Direct comparisons

	Temp, °C	*n*	*D*, $cm^2 \cdot sec^{-1}$	$f(\infty)$
NBD-PtdEtn	10.3	6	$1.1 \pm 0.1 \times 10^{-10}$	0.89 ± 0.05
NBD-Chol	10.5	6	$1.0 \pm 0.4 \times 10^{-10}$	0.89 ± 0.04
NBD-PtdEtn	19	4	$2.1 \pm 0.2 \times 10^{-10}$	0.79 ± 0.04
NBD-Chol	19	5	$1.7 \pm 0.6 \times 10^{-10}$	0.79 ± 0.04
NBD-PtdEtn	26	4	$1.8 \pm 0.2 \times 10^{-8}$	0.91 ± 0.06
NBD-Chol	26	4	$1.6 \pm 0.3 \times 10^{-8}$	0.91 ± 0.04

Measurements at a given temperature were made on identical liposome preparations on the same day. Results are mean ± SD.

tive was used to produce a final gaussian beam diameter of 1.8 or 4.4 μm, respectively. Photobleaching times were 10–150 msec, with photobleaching power at the sample approximately 2 mW. Sample temperatures were controlled to ±0.5°C by using a water-jacketed (Lauda) aluminum microscope slide holder. Complete experimental details will be published elsewhere. Diffusion coefficients (*D*) and fractional recoveries of fluorescence [$f(\infty)$] were calculated by the three-point fitting procedure of Axelrod *et al.* (7).

RESULTS AND DISCUSSION

In our initial studies, the rate of lateral diffusion of NBD-Chol was compared with that of NBD-PtdEtn, a probe often used to monitor lipid mobility (9, 12–14). Multilamellar liposomes containing $Myr_2PtdCho$ and either NBD-Chol or NBD-PtdEtn were prepared. The lateral mobilities of these probes were measured at temperatures above and below the main phase-transition temperature (T_m) of $Myr_2PtdCho$ (24°C) (Table 1 and Fig. 1).

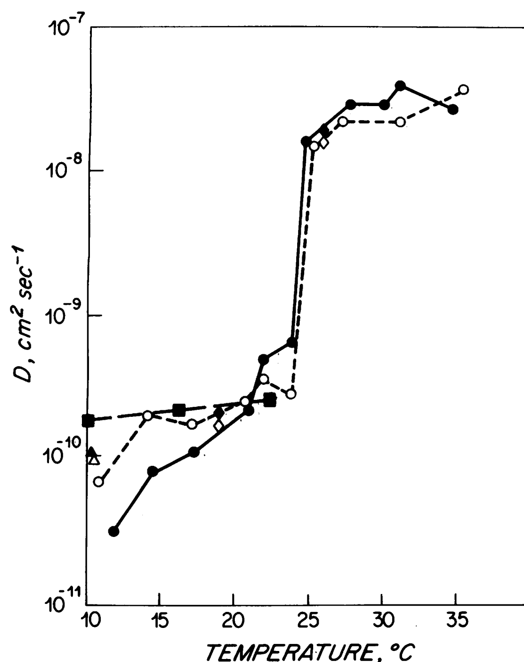


FIG. 1. Lateral *D* values of NBD-PtdEtn (●, ▲, ■, and ◆) and NBD-Chol (○, △, and ◇) in $Myr_2PtdCho$ multilamellar liposomes as a function of temperature. Lipid probes were added to a final mole fraction of 0.15%. Points connected by curves represent measurements made with a single liposome preparation by scanning from the lowest to the highest temperature. Similar symbols (triangles, diamonds) represent direct comparisons between the two fluorescent lipid probes in identical liposome preparations measured on the same day. Each point represents the average of two (● and ○), four (◆ and ◇), or six (▲, △, and ■) independent measurements. There is no significant difference between the *D* values of the two probes at any temperature examined. $f(\infty)$ values averaged 0.85 for both probes at all temperatures.

The *D* values of the two probes are increased by approximately two orders of magnitude as the temperature is raised through the T_m of the lipid, reflecting the increased fluidity of the membrane at the higher temperatures (Fig. 1). In addition, the *D* values of the probes are approximately equal in the temperature range studied. Although there is a rather large scatter in the measured diffusion coefficients below the phase transition temperature of the bulk phospholipid, direct comparisons between the diffusion coefficients of NBD-Chol and NBD-PtdEtn carried out on the same day on identical liposome preparations revealed no differences in the lateral mobilities of the two probes. The measured *D* and $f(\infty)$ values in these experiments are summarized in Table 1. The *D* values for NBD-PtdEtn in $Myr_2PtdCho$ above and below the main phase transition are very close to those previously reported (9, 12–14).

The experiments reported in Fig. 1 show that the *D* values of NBD-PtdEtn and NBD-Chol are equal both above and below the T_m of $Myr_2PtdCho$. Recent studies have suggested that, at membrane cholesterol concentrations less than 20 mol %, lateral separation occurs, so that cholesterol-rich areas containing 20 mol % cholesterol exist side by side with cholesterol-depleted areas (12–15). Since, below the T_m, the cholesterol-rich regions should be more fluid than the cholesterol depleted regions, the lateral mobility of NBD-Chol should be greater than that of NBD-PtdEtn at these temperatures. To test this possibility, two series of multilamellar $Myr_2PtdCho$ liposomes were

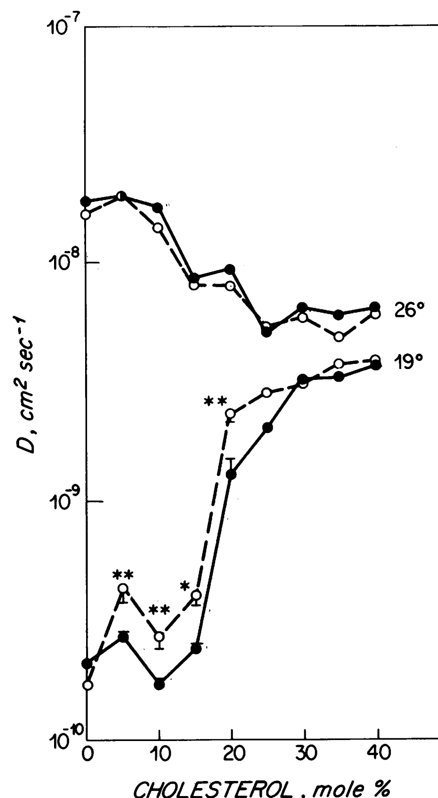


FIG. 2. Lateral *D* values of NBD-PtdEtn (●) and NBD-Chol (○) in binary $Myr_2PtdCho$/cholesterol mixtures as a function of cholesterol mole fraction at temperatures above and below the T_m for the pure phospholipid (24°C). Measurements at any given lipid composition were carried out on identical liposome preparations on the same day. Individual points represent the average of three–six independent measurements. Error bars (mean ± SEM) are shown for comparisons in which there are significant differences (*, $P < 0.03$; **, $P < 0.02$) between diffusion coefficients of the phospholipid and cholesterol probes. The sample temperature was measured to an accuracy of ±0.5°C with a thermistor. There are significant differences between diffusion coefficients of the two probes only for cholesterol mole fractions between 5% and 20% at the temperature below the T_m for the pure phospholipid.

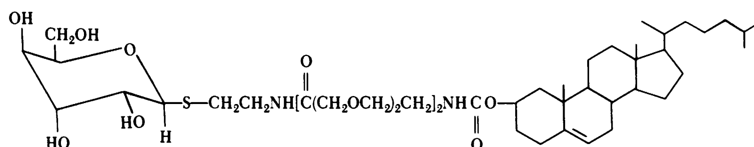
Table 2. Lateral mobility of NBD-PtdEtn and NBD-Chol in $Myr_2PtdCho$/cholesterol multilamellar liposomes

Cholesterol,	NBD-PtdEtn			NBD-Chol			
mol %	$D \times 10^{10}$, $cm^2 \cdot sec^{-1}$	$f(\infty)$	n	$D \times 10^{10}$, $cm^2 \cdot sec^{-1}$	$f(\infty)$	n	Significance*
			Measurement at 19°C ($T<T_m$)				
0.00	2.1 ± 0.2	0.79 ± 0.04	4	1.7 ± 0.6	0.77 ± 0.04	5	NS
0.05	2.7 ± 0.3	0.75 ± 0.05	5	4.3 ± 1.0	0.78 ± 0.05	6	$P < 0.02$
0.10	1.7 ± 0.2	0.85 ± 0.01	4	2.7 ± 0.7	0.84 ± 0.02	6	$P < 0.02$
0.15	2.4 ± 0.1	0.86 ± 0.03	3	4.0 ± 0.7	0.91 ± 0.02	4	$P < 0.03$
0.20	13 ± 6	0.91 ± 0.06	6	23 ± 3	0.93 ± 0.04	4	$P < 0.02$
0.25	20 ± 3	0.93 ± 0.10	3	28 ± 5	0.86 ± 0.04	3	NS
0.30	32 ± 4	0.86 ± 0.05	4	31 ± 3	0.93 ± 0.02	4	NS
0.35	33 ± 2	0.91 ± 0.05	6	37 ± 6	0.92 ± 0.06	6	NS
0.40	37 ± 6	0.95 ± 0.04	4	38 ± 1	0.95 ± 0.03	4	NS
			Measurement at 26°C ($T>T_m$)				
0.00	18 ± 2	0.91 ± 0.06	4	16 ± 3	0.91 ± 0.04	4	NS
0.05	19 ± 4	0.84 ± 0.08	6	19 ± 3	0.88 ± 0.04	6	NS
0.10	17 ± 1	0.88 ± 0.04	4	14 ± 1	0.95 ± 0.01	4	NS
0.15	8.6 ± 0.4	0.92 ± 0.03	4	8.0 ± 0.5	0.92 ± 0.03	4	NS
0.20	9.4 ± 1.2	0.86 ± 0.04	4	7.9 ± 1.8	0.92 ± 0.05	4	NS
0.25	5.1 ± 0.9	0.87 ± 0.02	3	5.3 ± 0.3	0.88 ± 0.03	3	NS
0.30	6.4 ± 0.6	0.92 ± 0.04	4	5.8 ± 0.9	0.94 ± 0.01	4	NS
0.35	5.9 ± 0.7	0.92 ± 0.02	4	4.8 ± 0.8	0.95 ± 0.03	4	NS
0.40	6.4 ± 0.5	0.94 ± 0.02	4	6.0 ± 0.7	0.97 ± 0.92	4	NS

Measurements at a given cholesterol concentration were carried out on identical liposome preparations on the same day.
* Test of significance between D values of NBD-PtdEtn and NBD-Chol (Student's two-tailed t test). NS, not significant ($P > 0.03$).

prepared containing one or the other of the probes plus 0–40 mol % cholesterol. The D values of the probes were then measured at 19°C and 26°C. The results of this experiment are presented in Fig. 2 and Table 2. Above the main phase transition of $Myr_2PtdCho$, the diffusion coefficients of the two probes are identical. Interestingly, however, at a temperature below the T_m, with concentrations of cholesterol between 5 and 20 mol %, a significant difference in D values between the two probes becomes evident. Under these conditions, the lateral mobility of the cholesterol probe was faster than that of the phospholipid probe by a small but clearly significant factor (approximately 2). Since the orientations of the cholesterol-rich and cholesterol-depleted areas in a multilamellar system are averaged in space, the apparent D for NBD-Chol is not expected to be orders of magnitude larger than that for NBD-PtdEtn. This result is expected based on model calculations for randomly oriented fluid and solid domains in multilamellar systems (15).

In the experiments reported here, we have used a fluorescent probe to measure the lateral mobility of cholesterol in membranes. The design of the probe was based on work previously done on synthetic glycolipids containing the structural elements shown below (**II**; ref. 16).



II

These compounds have been shown to interact with membranes in a manner similar to that of cholesterol. That is, they exhibited a condensing effect on egg lecithin-based membranes, they broadened the main phase transition of dipalmitoylphosphatidylcholine-based membranes, and they decreased the permeability of egg lecithin-based liposomes to sucrose (17). In addition, these compounds distributed evenly across the bilayer and underwent intermembraneous exchange (17). Since the cholesterol hydroxyl group is blocked in these compounds, this moiety may not be required for the bulk effects of cholesterol, a hypothesis not at odds with current ideas on structure–activity relationships in the cholesterol series (18).

The important structural elements of the synthetic glycolipids were carried over into the design of NBD-Chol. The water-soluble ethylene glycol-based spacer group that was used increases the water solubility of the derivative as well as increasing the likelihood that the NBD moiety will be held away from the membrane. Increasing the water solubility of the probe is important if useful rates of intermembranous transfer of the probe are to be achieved. This in turn is critical if the probe is to be a general tool that can be incorporated into diverse cell types at appreciable rates. Indeed, it has been found that NBD-Chol undergoes intermembranous exchange between small unilamellar vesicles (8) and is rapidly transferred from lipid vesicles to HeLa cells, guinea pig peritoneal macrophages, and bovine rod outer segments (unpublished experiments).

The studies reported here showed that, in the absence of cholesterol, the lateral mobility of NBD-Chol is virtually identical to that of NBD-PtdEtn both above and below the T_m of the membrane. Many lipid probes seem to have D values of approximately $10^{-8} cm^2 \cdot sec^{-1}$ in fluid membranes, a property shared by NBD-Chol (9, 12–14). Interestingly, a distinct difference was found between the lateral mobility of NBD-Chol and NBD-PtdEtn at cholesterol concentrations between 5 and 20 mol % at temperatures below the T_m of the lipid, a result of interest in light of current models of cholesterol–phospholipid interactions (9, 12–15, 19, 20). This cholesterol probe could also be used to determine other ways in which the behavior of cholesterol and phospholipid are divergent in model and natural membranes.

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