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INFLUENCE OF CHOLESTEROL ON BILAYERS OF ESTER- AND ETHER-LINKED PHOSPHOLIPIDS

PERMEABILITY AND ^{13}C-NUCLEAR MAGNETIC RESONANCE MEASUREMENTS

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^{13}C-NMR and permeability studies are described for sonicated vesicles of phosphatidylcholines bearing two 16-carbon saturated hydrocarbon chains with (a) one ether linkage at carbon 1 (3) or 2 of glycerol and one ester linkage at carbon 2 or 1 (3) of glycerol; (b) two ether linkages and (c) two ester linkages at carbons 1 (3) and 2 of glycerol. The results of ^{13}C-NMR relaxation enhancement measurements using cholesterol enriched with ^{13}C at the 4 position indicate that no significant relocation of the cholesterol molecules takes place in the bilayer when a methylene group is substituted for a carbonyl group in phosphatidylcholine. The 4-^{13}C atom of cholesterol undergoes similar fast anisotropic motions in diester- and diether-phosphatidylcholine bilayers, as judged by spin-lattice relaxation time measurements in the liquid-crystalline phase; although the fast motions are unaltered, linewidth and spin-spin relaxation time measurements suggested some restriction of the slow motions of cholesterol molecules in bilayers from phosphatidylcholines containing an *O*-alkyl linkage at the *sn*-2 position instead of an acyl linkage. At temperatures above the gel to liquid-crystal phase transition, the kinetics of ionophore A23187-mediated $^{45}Ca^{2+}$ efflux from vesicles prepared from each type of phosphatidylcholine molecule were the same; the kinetics of spontaneous carboxyfluorescein diffusion from diester- and diether-phosphatidylcholine vesicles were the same, whereas mixed ether/ester phosphatidylcholine molecules gave bilayers which are less permeable. The rate constants were reduced on cholesterol incorporation into the bilayers of each type of phosphatidylcholine molecule. The reductions were not statistically significant for $^{45}Ca^{2+}$ release. The rate constants for carboxyfluorescein release were also reduced by cholesterol to the same extent in vesicles from diester-, diether-, and 1-ether-2-ester-phosphatidylcholines; however, a smaller reduction was noted in bilayers from the 1-ester-2-ether analog. These results provide further evidence that there are no highly specific requirements for ester or ether linkages in phosphatidylcholine for cholesterol to reduce bilayer permeability. This is a reflection of the fact that in both diester- and diether-phosphatidylcholine bilayers, the 4-^{13}C atom of cholesterol is located in the region of the acyl carboxyl group or the glyceryl ether oxygen atom.

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Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; HPPC, 1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; PHPC, *rac*-1-palmitoyl-2-*O*-hexadecylglycero-3-phosphocholine.

Introduction

Various physical measurements suggest that the average molecular packing density in hydrated monolayers and bilayers of diester and diether

phosphatidylcholines (PC) are similar ([1–3] and references contained therein). Furthermore, both systems respond similarly to the addition of cholesterol molecules, and the degree of condensation of PC molecules above their chain-melting temperature is the same for diether and diester PC [4]. This implies that direct interaction between the cholesterol hydroxyl group and one of the carbonyl oxygens of PC is not essential for the condensation effect (cf. Ref. 5). Spectroscopic studies have provided conflicting information about whether or not an ester-PC oxygen is hydrogen bonded to the hydroxyl group of cholesterol. NMR [6–8] and Raman studies [9] supported the specific interaction between the carbonyl group of PC and hydroxyl group of cholesterol, but the Raman and infrared measurements of the carbonyl stretching of PC indicated no hydrogen bonding to this sterol [10].

Immediate correlations of the above structural findings with the changes in permeability of PC bilayers induced by cholesterol molecules are not obvious. Thus, conflicting information has been reported concerning the influence of cholesterol on ether-linked glycerophospholipids, which are natural components of membranes. For example, it was reported that cholesterol gave a smaller reduction in the rates of passive diffusion of polar solutes and ions across diether-PC bilayers than diester-PC bilayers [11,12]. These results were considered to support a model in which the phospholipid ester carbonyl group and the sterol 3β-hydroxyl group are hydrogen bonded in diester-PC membranes [13,14]. However, similar permeability studies [2,15,16] showed that diester-PC and diether-PC bilayer membranes were affected by cholesterol to similar extents, leading to the conclusion that specific hydrogen bonding in the bilayer-water interface region is not an important stabilizing force. Furthermore, the fluidity of diether- and diester-PC bilayers, as probed with a spin-labeled stearic acid, was found to be reduced equally by cholesterol [12]; also, the total enthalpy changes of the phase transitions were reduced to the same extent by incorporation of up to 25 mol% sterol [2]. Nevertheless, in a model proposed recently, a 1 : 1 phospholipid-cholesterol complex was considered to be stabilized by hydrogen bonding between the phospholipid glyceryl ester oxygen and the cholesterol 3β-hydroxyl group; a second phospholipid molecule is associated loosely with this complex, with the acyl chains in maximal van der Waals interaction with the steroid nucleus [17].

The present paper examines the importance of the postulated hydrogen bond as a stabilizing force in PC-cholesterol interactions. We have compared the molecular motions of cholesterol in sonicated vesicles from phosphatidylcholines bearing two ether, two ester, or one ether and one ester linkages, as judged from ^{13}C-NMR measurements using cholesterol enriched with ^{13}C at the 4 position; similarly, the effects of cholesterol on membrane permeability properties are compared, as judged from the reduction in the rates of spontaneous diffusion of the water-soluble fluorescent probe, carboxyfluorescein, and of ionophore A23187-mediated $^{45}Ca^{2+}$ efflux from small unilamellar vesicles. The presence or absence of carbonyl groups in the PC molecules does not significantly alter the packing of cholesterol molecules in these systems. This is consistent with our observation that the changes in permeability induced by cholesterol are generally independent of the presence of PC carbonyl groups.

Experimental procedures

Materials

The 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (DHPC), and cholesterol were purchased and purified as described previously [15]. The syntheses of 1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (HPPC) and *rac*-1-palmitoyl-2-*O*-hexadecylglycero-3-phosphocholine (PHPC) will be described elsewhere. The sources of the ionophore A23187, $^{45}Ca^{2+}$ and carboxyfluorescein were cited elsewhere [2]. Carboxyfluorescein was found to be chromatographically pure using cellulose MN plates eluted with chloroform/methanol/water (65 : 25 : 4, v/v). Crystalline [4-^{13}C]cholesterol with 90 atom% ^{13}C enrichment was obtained from Merck Co. (Montreal, Canada); the sterol was more than 99% pure as judged by thin-layer chromatography. $^{2}H_2O$ was purchased from Stohler Isotopes (Waltham, MA) or Merck (Rahway, NJ), and was routinely deoxygenated and stored under nitrogen. Manganese

chloride (A.C.S. Grade) and 1,4-dioxane were obtained from Fisher Scientific Co. (Fairlawn, NJ).

Methods

Preparation of vesicles containing trapped Ca^{2+} and carboxyfluorescein. Aqueous dispersions (2 mM total lipid) were prepared at 45–50°C by agitation of a thin lipid film in the presence of glass beads. The aqueous medium used for loading with $^{45}Ca^{2+}$ contained 10 mM imidazole, pH 7.0, with 135 mM NaCl and 150 mM $CaCl_2$ (with $^{45}Ca^{2+}$). For loading with carboxyfluorescein, the aqueous medium contained 50 mM Tris, pH 7.4, with 170 mM NaCl and 200 mM carboxyfluorescein. Unilamellar vesicles were prepared by sonication for about 45 min under nitrogen at 4°C using a Heat Systems Model W375A sonicator with a tapered microtip at power level 6 and 50% duty cycle. Undispersed lipid, multilamellar liposomes, and metal released from the probe were removed by centrifugation for 20 min at 12000 rpm in a SS34 rotor. The vesicles were stored at 4°C overnight prior to gel filtration. The suspensions were passed through Sephadex G-50 columns (1.5 × 30 cm) at room temperature to remove untrapped Ca^{2+} or carboxyfluorescein. For the Ca^{2+} and carboxyfluorescein efflux studies, the vesicles were prepared with PC : cholesterol molar ratios of 1 : 1 and 3 : 1, respectively. The vesicles were eluted from the columns, and phospholipid and cholesterol concentrations were determined as cited previously [2].

Assay of $^{45}Ca^{2+}$ and carboxyfluorescein efflux. Ionophore-mediated $^{45}Ca^{2+}$ efflux and spontaneous diffusion of the water-soluble fluorophore carboxyfluorescein were measured using procedures described elsewhere [2]. The final A23187 concentration was 50 nM, and efflux rates were corrected for the small spontaneous leakage (< 1.2%) of Ca^{2+} in the presence of ethanol.

Preparation of vesicles for NMR studies. Cholesterol (10 mol%) was mixed with 50–70 mg of DPPC, DHPC, or PHPC in chloroform and dried under N_2 to a thin film on the walls of a test tube. Traces of remaining solvent were removed by drying for 2 h at 50°C under vacuum. The dry lipid mixture was taken up in 1.2 ml of 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.6, and immersed in a 50°C water bath. The dispersions were sonicated under N_2 for 60 min (50% cycle) on setting 4 using a Branson Model 350 sonicator equipped with a tapered microtip. After sonication the dispersions were centrifuged for 5 min at low speed to pellet any titanium particles present. The supernatant was removed, 20 μl of 1,4-dioxane was added, and then ^2H_2O as the NMR lock compound was added to increase the volume by 20%. The dispersions were kept at 50°C and used within a few hours of preparation.

NMR measurements. ^{13}C-NMR spectra were recorded at 90.55 MHz by using a Bruker WH 360 spectrometer operating in the Fourier transform mode at 50°C. Chemical shifts were measured to ±0.05 ppm from internal 1,4-dioxane (66.55 ppm). A $(180°\text{-}\tau\text{-}90°\text{-PD})_n$ pulse sequence was employed for spin-lattice relaxation time T_1 measurements [18], where τ is the variable time delay between the 180° and 90° pulses, PD is the pulse delay ($\geq 4\ T_1$), and n is the number of times the pulse sequence was repeated. At least nine different values of τ were utilized and a total of 500 scans was accumulated for each measurement of τ. T_1 values were derived from linear plots of ln (signal intensity) vs. τ by using a linear regression analysis. The T_1 values were usually accurate to ±10%.

Relaxation enhancement experiments were performed by adding Mn^{2+} and repeating the T_1 measurement. The dispersion was titrated with 0.1 M $MnCl_2$ dissolved in ^2H_2O until a Mn^{2+}/PC molar ratio of 0.06 was attained. The dispersion was then sonicated at 50°C for a few minutes to ensure that Mn^{2+} ions entered into the aqueous compartment in the interior of the unilamellar vesicles. The sample was quickly replaced into the NMR tube and returned to the NMR probe, which was maintained at 50 ± 1.5°C.

Results

NMR

The ^{13}C-NMR spectra of small unilamellar vesicles of liquid-crystalline DPPC or DHPC + 10 mol% [4-^{13}C]cholesterol at 50°C are shown in Fig. 1. The 4-^{13}C resonance from cholesterol is well resolved at δ 41.7 ppm in DPPC (cf. Ref. 19) and 41.8 ppm in DHPC. It should be noted that the nuclear Overhauser effect was not suppressed, so that the relative intensities in the spectra of Fig. 1

TABLE I

COMPARISON OF NMR PARAMETERS FROM 10 MOL% [4-^{13}C]CHOLESTEROL/1,2-DIPALMITOYL- AND 1,2-DI-*O*-HEXADECYL-PC VESICLES

δ in ppm. $\Delta\nu_{1/2}$ in Hz (linewidth of 1,4-dioxane internal standard was the same in all spectra). Spin-lattice relaxation time T_1 in ms. n is used to normalize the data for the number of directly bonded hydrogen atoms.

Resonances	DPPC				DHPC			
	δ	$\Delta\nu_{1/2}$	T_1	nT_1	δ	$\Delta\nu_{1/2}$	T_1	nT_1
[4-^{13}C]Cholesterol	41.75	35	210	420	41.80	60	240	480
$N(\underline{C}H_3)_3$	54.15	18	580	1740	54.20	19	630	1890
$\underline{C}H_2OP$	63.65	22	350	700	64.85	24	310	620
sn-$\underline{C}$-2	70.10	51	420	420	70.20	44	430	430
$\underline{C}H_2CH_2C$-O	24.90	33	500	1000	–	–	–	–
$\underline{C}H_2$-CH_2-O	–	–	–	–	26.30	27	530	1060
$(\underline{C}H_2)_n$	29.95	34	560	1120	30.20	29	570	1140

are not a true reflection of the number of carbon atoms contributing to each signal. The volumes of the aqueous compartment in the interior of small unilamellar vesicles formed from both types of PC are very similar (see captions to Figs. 2 and 4), and the thickness of vesicle membranes from diether-PC is the same as that from diester-PC [3]. Consequently, the linewidths ($\Delta\nu_{1/2}$) observed should be the same if the segmental motions of molecules in both diester- and diether-PC bilayers are similar. The $\Delta\nu_{1/2}$ values observed for selected resonances are summarized in Table I. The PC carbon atoms in DPPC and DHPC vesicles give similar values, whereas the [4-^{13}C]cholesterol resonance is broader by a factor of 1.7 in the DHPC vesicles. PHPC was difficult to handle because the vesicles flocculated badly when dispersed at 50 mg/ml at 50°C, giving rise to a spectrum of low intensity. The reasons for this enhanced aggregation compared to the DPPC and DHPC bilayers are not obvious. Although T_1 measurements could not be performed, we were able to estimate that the $\Delta\nu_{1/2}$ values for $N(CH_3)_3$ and [4-^{13}C]cholesterol in PHPC vesicles are 19 Hz and approx. 70 Hz, respectively; within the experimental error, these are the same as for the DHPC system.

The T_1 values for selected ^{13}C resonances in the PC molecules and for [4-^{13}C]cholesterol are listed in Table I. It is clear that T_1 values are similar in DPPC and DHPC vesicles. The relaxation enhancements induced by the addition of Mn^{2+} at Mn^{2+}/PC = 0.06 mol/mol are also similar for the [4-^{13}C]cholesterol and $N(CH_3)_3$ resonances obtained with vesicles formed from both types of PC (Table II). The polar group T_1 values are significantly reduced while the hydrocarbon chain $(CH_2)_n$ resonances are not affected by the presence of Mn^{2+} ions in the aqueous phase.

The relaxation enhancement induced by paramagnetic ions such as Mn^{2+} is a function of the Mn^{2+}-^{13}C distance. This technique has been used to obtain distance information in PC bilayers [20]

TABLE II

EFFECT OF Mn^{2+} ON T_1 OF SELECTED RESONANCES FROM [4-^{13}C]CHOLESTEROL/PC VESICLES

Resonances	Spin-lattice relaxation time (ms)			
	10 mol% [4-^{13}C]cholesterol/DPPC		10 mol% [4-^{13}C]cholesterol/DHPC	
	$-Mn^{2+}$	$+Mn^{2+}$[a]	$-Mn^{2+}$	$+Mn^{2+}$[a]
[4-^{13}C]cholesterol	210	110	240	120
$N(\underline{C}H_3)_3$	580	120	630	150
$(\underline{C}H_2)_n$	560	540	570	530

[a] Mn^{2+}/PC (mol/mol) = 0.06.

and active sites of enzymes (e.g. Ref. 21) The observed spin-lattice relaxation rate enhancement attributed to the influence of the bound Mn^{2+} $(1/\Delta T_1)$ is given by

$$\frac{1}{\Delta T_1} = \frac{1}{T_1(Mn^{2+})} - \frac{1}{T_1(Mg^{2+})} \quad (1)$$

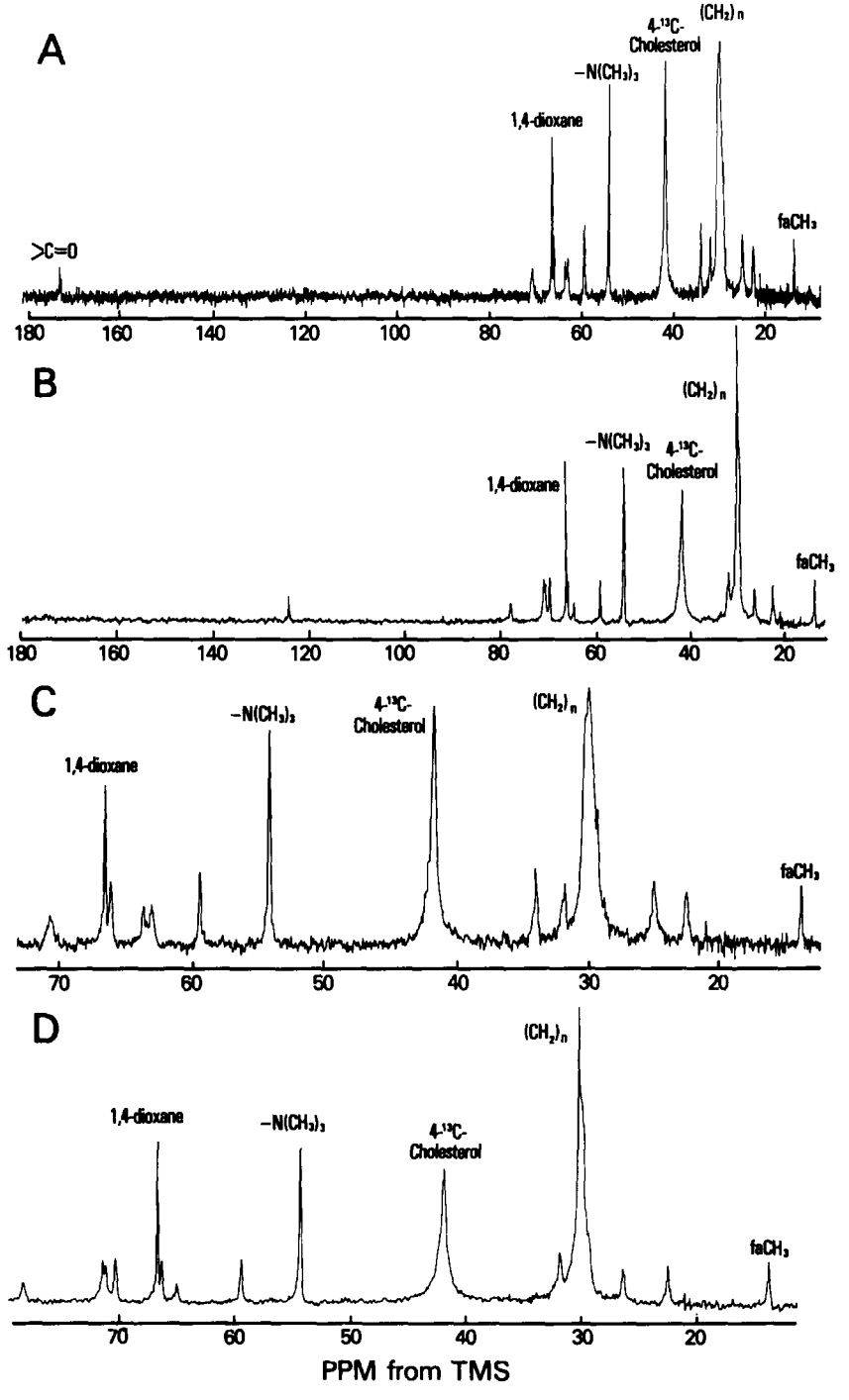

Fig. 1. Proton-decoupled ^{13}C-NMR spectra (90.55 MHz) at 50°C of small unilamellar vesicles of 10 mol% [4-^{13}C]cholesterol mixed with either DPPC or DHPC. A, DPPC, 740 accumulations; B, DHPC, 2500 accumulations; C, D, Expansions of spectra A and B, respectively; the horizontal axes are expanded by a factor of about 2.8. Spectra were obtained using broad-band decoupling and recycle time of 1.2 s. All spectra were processed with 2 Hz exponential filtering.

where $T_1(Mn^{2+})$ and $T_1(Mg^{2+})$ are values measured in the presence of the same concentrations of these two cations. Assuming fast exchange and low occupancy of the phosphate binding sites of the PC bilayers, $1/\Delta T_1$ is related to r, the Mn^{2+} (unpaired electron)-^{13}C nucleus distance, by the simplified Solomon-Bloembergen equation (Eqn. 2):

$$r = C\left[\Delta T_1 \cdot \frac{3\tau_c}{1+\omega^2\tau_c^2}\right]^{1/6} \quad (2)$$

In Eqn. 2, C is a collection of constants whose value depends on the spin of the paramagnetic ion and the gyromagnetic ratio of the nucleus, ω is the Larmor precession frequency, and τ_c is the correlation time of the Mn^{2+}-^{13}C nuclear interaction. Using Eqns. 1 and 2, we can use the relative relaxation enhancements of the [4-^{13}C]cholesterol and $N(CH_3)_3$ nuclei to obtain relative distances of the Mn^{2+} from these nuclei because

$$\frac{r_{Mn-chol}}{r_{Mn-N(CH_3)_3}} = \left(\Delta T_{1,chol}/\Delta T_{1,N(CH_3)_3}\right)^{1/6} \quad (3)$$

Knowing that Mn^{2+} and other paramagnetic cations bind to the phosphate group in PC bilayers [22] and having distance ratios from the cation to various glyceryl-phosphorylcholine protons [23], we can deduce the location of the [4-^{13}C]cholesterol nucleus with respect to the PC molecules in the bilayer.

The distance $r_{Mn\text{-}N(CH_3)_3}$ is the same in DPPC and DHPC bilayers because replacement of an ester linkage by an ether linkage does not affect the average conformation or segmental motion of the PC polar group [24,25]. Consequently, Eqn. 3 gives a measure of the immersion of the cholesterol molecules in diester- and diether-PC bilayers. Substitution of the T_1 values for [4-^{13}C]cholesterol and $N(CH_3)_3$ in DPPC from Table II into Eqns. 1 and 3 gives $r_{Mn\text{-}chol}/r_{Mn\text{-}N(CH_3)_3} = 1.1$, while the equivalent figure for DHPC = 1.0. Given the errors in measuring T_1, this difference is probably not significant. Comparison with the distance ratios of protons in the PC polar group from Gd^{3+} bound to the phosphate group (Table 3 of Ref. 23) indicates that protons in the $N(CH_3)_3$ group and the region of the first carbon atom of the acyl chains are approximately equidistant from the paramagnetic ion. It follows that in both diester- and diether-PC bilayers the 4-^{13}C atom of cholesterol is located in the region of the acyl carboxyl group or the glyceryl ether oxygen atom. This is in agreement with earlier neutron diffraction and ^{13}C-NMR studies of diacyl-PC/cholesterol bilayers, which showed that the cholesterol C-3 atom is at the position of the glycerol-fatty acid ester bonds [26], and the cholesterol C-4 atom is localized in the ester bond region of the bilayer [27].

Efflux of $^{45}Ca^{2+}$

Ionophore A23187-mediated release of $^{45}Ca^{2+}$ from vesicles prepared from DPPC, DHPC, HPPC, and PHPC follow first-order kinetics for at least the first 40 min of the efflux process (Figs. 2, 3). The first-order rate constants listed in Table III for vesicles prepared from the four PC molecules above the gel to liquid-crystalline transition temperature are not significantly different ($P > 0.05$) by the criterion of single-factor analysis of variance. The kinetics of efflux of $^{45}Ca^{2+}$ catalyzed by

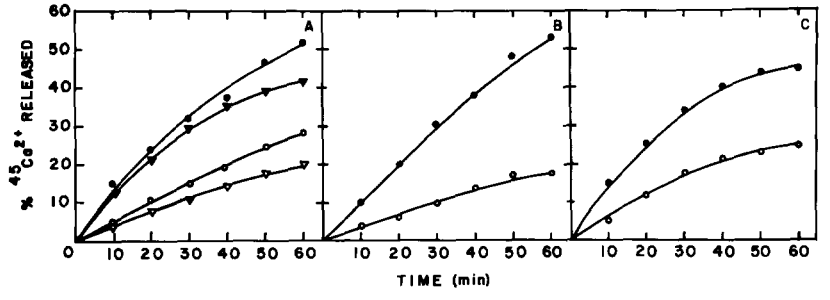

Fig. 2. Time course of A23187-mediated $^{45}Ca^{2+}$ efflux from vesicles at 46°C. Vesicles were composed of the following phosphatidylcholines without cholesterol (closed symbols) or with an equimolar concentration of cholesterol (open symbols): (A) (○) DPPC and (▽) DHPC, (B) (○) HPPC, and (C) (○) PHPC. The vesicle size did not vary with PC structure or with cholesterol incorporation (0.30 ± 0.03 mmol Ca^{2+} trapped/mol of lipid).

TABLE III

FIRST-ORDER RATE CONSTANTS OF A23187-MEDIATED $^{45}Ca^{2+}$ EFFLUX AND CARBOXYFLUORESCEIN RELEASE FROM VESICLES WITH AND WITHOUT CHOLESTEROL

The vesicles contained 50 mol% cholesterol in the A23187-mediated $^{45}Ca^{2+}$ efflux studies and 25 mol% cholesterol in the carboxyfluorescein efflux studies. The results are reported as the mean ± standard deviation of the mean; the errors of the % decreases were calculated according to Hendee [38]. The numbers in parentheses indicate the number of kinetic determinations. The $^{45}Ca^{2+}$ efflux measurements were conducted at 46°C, and the carboxyfluorescein release was monitored at 48°C.

	$^{45}Ca^{2+}$		Carboxyfluorescein	
	$10^2 \cdot k$ (min^{-1})	% decrease with cholesterol	$10^2 \cdot k$ (min^{-1})	% decrease with cholesterol
DPPC	1.17 ± 0.08 ($n = 3$)		1.57 ± 0.05 ($n = 4$)	
DPPC/cholesterol	0.60 ± 0.02	49 ± 8	0.74 ± 0.08	53 ± 6
DHPC	1.08 ± 0.08 ($n = 4$)		1.61 ± 0.06 ($n = 4$)	
DHPC/cholesterol	0.37 ± 0.02	66 ± 9	0.88 ± 0.06	45 ± 5
HPPC	1.21 ± 0.05 ($n = 5$)		1.32 ± 0.03 ($n = 5$)	
HPPC/cholesterol	0.31 ± 0.04	74 ± 6	0.54 ± 0.05	59 ± 5
PHPC	1.31 ± 0.09 ($n = 5$)		1.42 ± 0.04 ($n = 5$)	
PHPC/cholesterol	0.63 ± 0.04	52 ± 8	0.91 ± 0.03	36 ± 4

50 nM A23187 were reduced by the presence of 50 mol% cholesterol in vesicles prepared from DPPC, DHPC, HPPC, and PHPC (Figs. 2,3 and Table III). The % decreases in first-order rate constants (Table III) are not significantly different ($P > 0.05$) indicating that reduction by cholesterol of $^{45}Ca^{2+}$ permeation across the bilayers is not dependent on the presence of acyl linkages in either the *sn*-1 or *sn*-2 position.

Efflux of carboxyfluorescein

Since trapped carboxyfluorescein (200 mM) is self-quenched, the rates of carboxyfluorescein efflux are monitored easily from the increase of fluorescence intensity (de-quenching). The kinetics of efflux from DPPC, DHPC, HPPC, and PHPC vesicles is first order with respect to carboxyfluorescein concentration remaining in the vesicles over a period of 1 h (Figs. 4 and 5). The rate constants for carboxyfluorescein efflux from DPPC and DHPC vesicles are the same, whereas the mixed ester/ether-PC molecules give bilayers that are less permeable to carboxyfluorescein. The difference in rate constants for passive permeation of carboxyfluorescein from DPPC and DHPC vesicles compared to HPPC and PHPC vesicles is just statistically significant ($P < 0.05$) by the criterion of single-factor analysis of variance.

Cholesterol (at 25 mol%) decreases the efflux of carboxyfluorescein from vesicles prepared from phosphatidylcholines with and without acyl linkages (Figs. 4, 5). The first-order rate constants are decreased by incorporation of cholesterol into vesicles from each PC (Table III). The percentage decreases in rate constants are significantly different ($P < 0.05$) when all four PC systems are com-

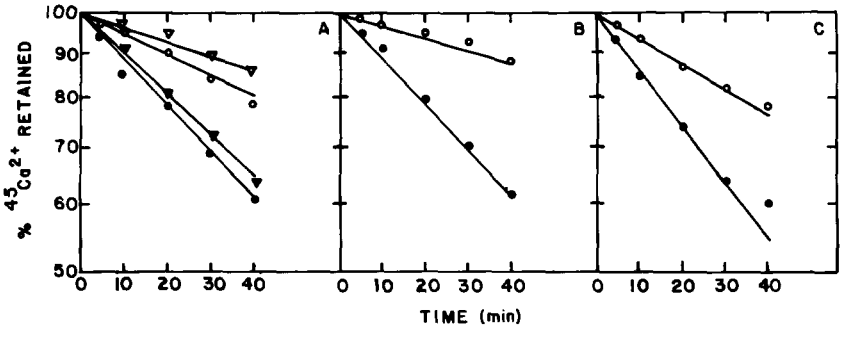

Fig. 3. First-order plots of A23187-mediated $^{45}Ca^{2+}$ release from vesicles at 46°C. The % $^{45}Ca^{2+}$ remaining in vesicles without cholesterol (closed symbols) or with an equimolar concentration of cholesterol (open symbols) is plotted vs. time. The data were taken from Fig. 2, and the symbols are the same as those described in the caption to Fig. 2.

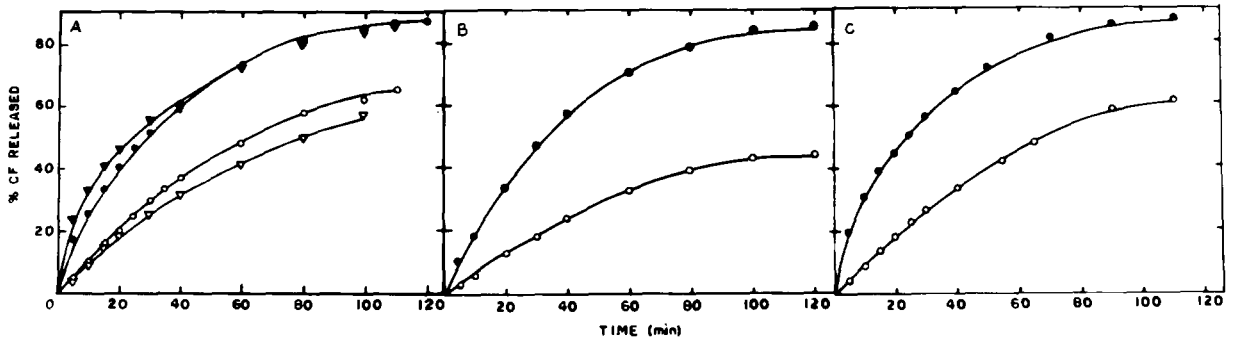

Fig. 4. Time course of carboxyfluorescein (CF) release from vesicles at 48°C. Vesicles were prepared in the absence (closed symbols) and presence (open symbols) of 25 mol% cholesterol from the following phosphatidylcholines: (A) (○) DPPC and (▽) DHPC, (B) (○) HPPC, and (C) (○) PHPC. The vesicle sizes (volume of carboxyfluorescein trapped per total lipid concentration) were as follows: DPPC, 0.50 l of carboxyfluorescein/mol of lipid; DPPC/cholesterol, 0.54; DHPC, 0.47; DHPC/cholesterol, 0.44; PHPC, 0.40; PHPC/cholesterol, 0.43; HPPC, 0.40; and HPPC/cholesterol, 0.44.

pared, but this difference becomes insignificant ($P > 0.05$) when the $36 \pm 4\%$ decrease observed with PHPC/cholesterol bilayers is omitted. Consequently, there is a significant difference between the effect of cholesterol on carboxyfluorescein permeation through PHPC bilayers relative to bilayers from its positional isomer, and from diether- and diester-PC of the same chain lengths. If the data for carboxyfluorescein efflux from PHPC/cholesterol vesicles are omitted, the remaining reductions in carboxyfluorescein and $^{45}Ca^{2+}$ efflux induced by cholesterol are not significantly different ($P > 0.05$).

Discussion

Packing of cholesterol molecules in diacyl- and diether-PC bilayers

Overall, the segmental motions of PC molecules in liquid-crystalline DPPC and DHPC bilayers containing 10 mol% cholesterol are similar, as indicated by the $\Delta\nu_{1/2}$ and T_1 data. This is reasonable given the previous evidence for similar molecular packing in diacyl- and diether-PC bilayers and similar condensations when cholesterol is mixed with either type of PC [1,4]. Furthermore, ^{13}C-NMR studies have shown that short-chain di-*O*-alkylphosphatidylcholines have similar conformations and motions as the diacyl analogs [36]. ^{13}C-NMR studies of PC vesicles [28,29] have demonstrated a gradient of motion along the PC molecules: the $N(CH_3)_3$ and chain terminal CH_3 groups are relatively mobile, whereas the region of most restricted motion and tightest packing is the glycerol backbone. Since the principal mode of ^{13}C spin-lattice relaxation is by dipolar coupling through directly bonded hydrogen atoms, it is informative to normalize T_1 data using the product nT_1, where n is the number of hydrogen atoms

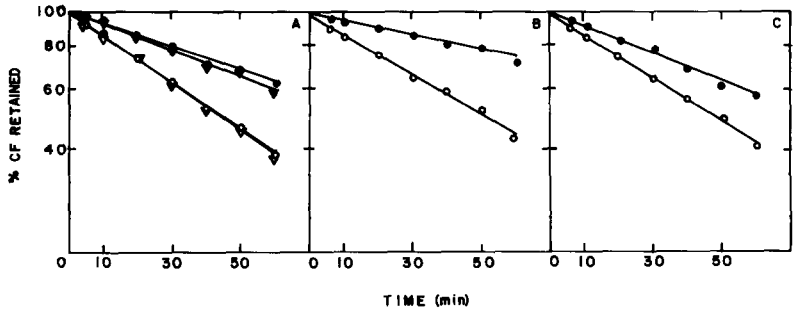

Fig. 5. First-order plots of carboxyfluorescein (CF) release from vesicles. Vesicles were prepared in the absence or presence of 25 mol% cholesterol from (A) (○) DPPC, (●) DPPC/cholesterol, (▽) DHPC, (▼) DHPC/cholesterol; (B) (○) HPPC, (●) HPPC/cholesterol; and (C) (○) PHPC, (●) PHPC/cholesterol. The total fluorescence intensity was measured after the addition of Triton X-100 (0.2% final concentration), which caused release of all of the trapped carboxyfluorescein.

[30,31]. As shown in Table I, the gradient of motion along diester- and diether-PC molecules is the same, with the $N(CH_3)_3$ and chain $(CH_2)_n$ atoms having the highest nT_1 values indicative of faster motions. The bilayer is most tightly packed in the region of the glycerol backbone, which probably constitutes the main permeability barrier in bilayers of both ester- and ether-linked PC molecules. More detailed interpretation is complicated because PC molecules in mixed PC/cholesterol bilayers can exist either free or associated with cholesterol molecules (e.g. Refs. 32, 33). The contributions from PC molecules in either state are not derived easily since at present there is a lack of information concerning the exchange frequency.

The T_1 value of about 200 ms at 50°C for the 4-^{13}C atom of cholesterol is consistent with the cholesterol molecules undergoing similar anisotropic motions in both DPPC and DHPC bilayers. These findings are in agreement with earlier studies showing that the correlation time for fast axial rotation of cholesterol molecules in liquid-crystalline bilayers is about 10^{-10} s [30,34,35]. In contrast to T_1 which is sensitive to fast motions, the apparent T_2 $[=(\pi \cdot \Delta\nu_{1/2})^{-1}]$ is affected principally by slower motions involving reorientation of local segments [30,31,34]. Since the apparent T_2 values for the [4-^{13}C] atom of the cholesterol molecule in 10 mol% cholesterol/DHPC and PHPC bilayers are shorter than those observed with the analogous DPPC system, it can be inferred that the motion of cholesterol seems to be relatively restricted when the PC molecules contain an *O*-alkyl rather than an acyl linkage at the *sn*-2 position. The arrangement of the *sn*-1 and *sn*-2 chains in diacyl-PC bilayers is not the same because the ester linkage of the *sn*-2 chain is extended along the lipid-water interface, whereas the ester group of the *sn*-1 chain is immersed further into the bilayer (for reviews, see Refs. 22, 37). Since $r_{\text{Mn-chol}}/r_{\text{Mn-N(CH}_3)_3}$ is essentially the same for the [4-^{13}C]cholesterol in DPPC and DHPC, replacement of the carbonyl group by a methylene group apparently does not lead to any significant relocalization of the cholesterol molecules in the PC bilayers. Consequently, the restriction in slow cholesterol oscillations presumably reflects the change in bilayer structure in this region when the 2-ester linkage is replaced by an ether bond. The molecular arrangement in this region of tightest packing in the cholesterol/PC bilayers could play a critical role in the partitioning of molecules into and out of the bilayers.

Permeabilities of ether-linked PC bilayers in the absence and presence of cholesterol

The similarities in rates of ionophore A23187-mediated $^{45}Ca^{2+}$ release from vesicles of pure DPPC, DHPC, HPPC, and PHPC (in the absence of sterol) indicate that this transport process is not sensitive to replacement of ester linkages by ether linkages in either the *sn*-1 or *sn*-2 positions, or both (cf. Refs. 2, 15, 16). The passive diffusion of carboxyfluorescein across bilayers of DPPC and DHPC occurs at the same rate, which is consistent with a general conclusion that the permeabilities of bilayers of analogous ester and ether PC molecules are the same. The somewhat lower rates of carboxyfluorescein permeation across HPPC and PHPC bilayers imply that in some cases the packing of mixed ester/ether PC molecules in the bilayers can increase the permeability barrier. However, this is not a generalized effect because the A23187-mediated $^{45}Ca^{2+}$ release from HPPC and PHPC vesicles is not significantly slower than from DPPC or DHPC vesicles.

Diacyl- and di-*O*-alkyl-PC interact similarly with cholesterol because the fast molecular motions and location of cholesterol molecules are essentially the same in bilayers of both lipids, using the criteria of the T_1 and relaxation enhancement ^{13}C-NMR experiments employed here. When cholesterol is incorporated into bilayers from DPPC, DHPC, and the mixed ester/ether HPPC and PHPC molecules, the rate constants for efflux of $^{45}Ca^{2+}$ and carboxyfluorescein are markedly reduced. This observation indicates that cholesterol increases the bilayer packing density of these lipids and thus increases the permeability barrier to $^{45}Ca^{2+}$ and CF transport. It follows that there are no highly specific requirements for ester and ether linkages in the *sn*-1 or *sn*-2 position for cholesterol to reduce bilayer permeability. The $^{45}Ca^{2+}$ and carboxyfluorescein efflux data (Table III) suggest that there may be a smaller degree of interaction of cholesterol with 1-acyl-2-*O*-alkyl-3-PC bilayers than with 1-*O*-alkyl-2-acyl-3-PC bilayers. In fact,

monolayers of 1-acyl-2-*O*-alkyl-PC were condensed by cholesterol somewhat less than monolayers of diacyl-PC [5]. However, quantitative differences in the effect of cholesterol on ionophore-mediated Ca^{2+} flux and carboxyfluorescein unmediated diffusion across PHPC bilayers may reflect differences in the transport mechanism, and a better understanding of the role of the 1-acyl linkage in this regard requires more study.

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References

1 Paltauf, F., Hauser, H. and Phillips, M.C. (1971) Biochim. Biophys. Acta 249, 539
2 Bittman, R., Clejan, S., Jain, M.K., Deroo, P.W. and Rosenthal, A.F. (1981) Biochemistry 20, 2790
3 Paltauf, F. (1973) in Ether Lipids, Biochemical and Biomedical Aspects (Mangold, H.K. and Paltauf, F., eds), pp. 309–353, Academic Press, New York
4 Fong, J.W., Tirri, L.J., Deshmukh, D.S. and Brockerhoff, H. (1977) Lipids 12, 857
5 DeKruijff, B., Demel, R.A., Slotboom, A.J., van Deenen, L.L.M. and Rosenthal, A.F. (1973) Biochim. Biophys. Acta 307, 1
6 Yeagle, P.L., Hutton, W.C., Huang, C. and Martin, R.B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3477
7 Yeagle, P.L. and Martin, R.B. (1976) Biochem. Biophys. Res. Commun. 69, 775
8 Chatterjie, N. and Brockerhoff, H. (1978) Biochim. Biophys. Acta 511, 116
9 Bicknell-Brown, E. and Brown, K. (1980) Biochem. Biophys. Res. Commun. 94, 638
10 Bush, S.F., Levin, H. and Levin, I.W. (1980) Chem. Phys. Lipids 27, 101
11 Tirri, L.J., Schmidt, P.C., Pullarkat, R.K. and Brockerhoff, H. (1977) Lipids 12, 863
12 Schwarz, F.T. and Paltauf, F. (1977) Biochemistry 16, 4335
13 Brockerhoff, H. (1974) Lipids 9, 645
14 Huang, C. (1977) Lipids 12, 348
15 Clejan, S., Bittman, R., Deroo, P.W., Isaacson, Y.A. and Rosenthal, A.F. (1979) Biochemistry 18, 2118
16 Hermetter, A. and Paltauf, F. (1981) Chem. Phys. Lipids 29, 225
17 Presti, F.T., Pace, R.J. and Chan, S.I. (1982) Biochemistry 21, 3831
18 Vold, R.L., Waugh, J.S., Klein, M.P. and Phelps, D.E. (1968) J. Chem. Phys. 48, 3831
19 Lund-Katz, S. and Phillips, M.C. (1981) Biochem. Biophys. Res. Commun. 100, 1735
20 Weinstein, S., Wallace, B.A., Morrow, J.S. and Veatch, W.R. (1980) J. Mol. Biol. 143, 1
21 Raushel, F.M. and Villafranca, J.J. (1980) J. Am. Chem. Soc. 102, 6618
22 Hauser, H. and Phillips, M.C. (1979) Prog. Surface Membrane Sci. 13, 297
23 Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1976) Nature (London) 261, 390
24 Hauser, H. (1981) Biochim. Biophys. Acta 646, 203
25 Hauser, H., Guyer, W. and Paltauf, F. (1981) Chem. Phys. Lipids 29, 103
26 Worcester, D.L. and Franks, N.P. (1976) J. Mol. Biol. 100, 359
27 DeKruijff, B. (1978) Biochim. Biophys. Acta 506 (1978) 173
28 Levine, Y.K., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1972) Biochemistry 11, 1416
29 Godici, P.E. and Landsberger, F.R. (1975) Biochemistry 14, 3927
30 Yeagle, P.L. (1981) Biochim. Biophys. Acta 640, 263
31 Brainard, J.R. and Szabo, A. (1981) Biochemistry 20, 4618
32 Phillips, M.C. and Finer, E.G. (1974) Biochim. Biophys. Acta 356, 199
33 Lentz, B.R., Barrow, D.A. and Hoechli, M. (1980) Biochemistry 19, 1943
34 Brainard, J.R. and Cordes, E.H. (1981) Biochemistry 20, 4607
35 Taylor, M.G., Akiyama, T., Saito, H. and Smith, I.C.P. (1982) Chem. Phys. Lipids 31, 359
36 Burns, R.A., Jr., Friedman, J.M. and Roberts, M.F. (1981) Biochemistry 20, 5945
37 Hauser, H., Pascher, I., Pearson, R.H. and Sundell, S. (1981) Biochim. Biophys. Acta 650, 21
38 Hendee, W.R. (1973) Radioactive Isotopes in Biological Research, p. 242, Wiley, New York