Biochimica et Biophysica Acta, 1106 (1992) 40–44

BBAMEM 75590

Interaction of cholesterol with conformationally restricted phospholipids in vesicles

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(Received 4 November 1991)

Key words: Phosphatidylcholine; Phosphatidylglycerol; Cyclopentanoid lipid; Constrained phospholipid; Cholesterol–phospholipid interaction; Permeability; Cholesterol exchange

The interaction of cholesterol with conformationally restricted analogs of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) in the liquid-crystalline phase has been studied in vesicles. These analogs contain one of three cyclopentane triols in place of the glycerol moiety found in natural phospholipids and make possible an analysis of whether a limitation of the conformational mobility in the glycerol backbone region affects the interaction with cholesterol. When cholesterol was incorporated into vesicles from cyclopentanoid phospholipids in which the acyl group vicinal to the head group is *trans*, the first-order rate constant for Cl^- efflux is decreased similarly to that in vesicles from 'natural' DPPC or DPPG (about 50%). However, when the head group is in the unnatural 2 position, cholesterol has a much smaller effect on the rate of Cl^- efflux (a decrease of about 20%). Cholesterol decreased the rate constants for valinomycin-mediated $^{86}Rb^+$ efflux from vesicles of the cyclopentanoid PC analogs and of DPPC to a similar extent. The half-time values for spontaneous intervesicle cholesterol exchange were not markedly different using vesicles prepared with the natural glycerophospholipids and with the cyclopentanophospholipids, suggesting that the geometrical orientation of the acyl chains or the head group has little influence on cholesterol desorption from the lipid/water interface.

Introduction

The interaction between sterols and phospholipids is the most extensively investigated lipid–lipid interaction in membranes (for a review, see Refs. 1 and 2). The long axis of the cholesterol ring system is aligned parallel to the phospholipid hydrocarbon chains. The flat α face of cholesterol is considered to be accommodated between the hydrocarbon chains, maximizing the van der Waals interactions. Since conformational mobility within the phospholipid molecule may be a prerequisite for tight packing between the fatty acyl chains and rigid sterol nucleus, we have examined the possibility that an increase in rigidity in the glycerol backbone of glycerophospholipids may diminish the ability of the phospholipid to pack efficiently with cholesterol in bilayers. The cyclopentanoid analogs (Fig. 1) of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 3-phosphoglycerol (DPPG) have restricted intramolecular rotation about the backbone [3]. In previous work it was shown that cyclic analogs of DPPC form sealed vesicles [4], and that the unusual phase-transition properties of some of the stereoisomers may arise from closer packing of the chains below the main phase transition temperature relative to DPPC [5]. In a recent report the thermal properties of the cyclopentanoid phosphatidic acid analogs are described at acidic, neutral and alkaline pH [6]. Conformational attitudes in DPPC have also been analyzed by testing cyclopentanoid PC analogs as potential substrates for phospholipase A_2 [7–9]. These studies showed that the

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Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol. Cyclic compounds described in this paper are named according to the tentative rules for nomenclature of cyclitols (IUBAC-IUB Commission (1968) Arch. Biochem. Biophys. 128, 269–279).

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1,3/2-1P (all-*trans*) conformation was selectively preferred [7]. This finding suggests that such a conformation is most close to that obtained by the natural analog when in an aggregated state such as a micelle or a vesicle [9,10]. In the present study, we compare the ability of cholesterol to reduce the rates of spontaneous Cl^- efflux and valinomycin-mediated $^{86}Rb^+$ efflux from small unilamellar vesicles prepared from cyclopentanoid analogs and from the corresponding open-chain glycerophospholipids. These studies have been conducted at temperatures above the gel to liquid-crystalline transition temperature in order to be consistent with the physical state that exists in biomembranes. The effect of replacing the glycerol backbone region into a cyclopentanoid ring on the spontaneous desorption rate of radiolabeled cholesterol from vesicles was also examined. These studies show that conformational changes induced in cyclopentanoid analogs of PC and PG show varying effects on the rate of efflux of Cl^- and Rb^+ (via valinomycin) from vesicles but have relatively little effect on the cholesterol desorption rate.

Materials and Methods

The structures of the cyclopentanoid analogs of DPPC or DPPG used in these studies are shown in Fig. 1. The syntheses of these compounds have been reported previously [3,11]. DPPC, DPPG, dicetyl phosphoric acid, and cholesterol were purchased from Sigma (St. Louis, MO). The purities of the lipids were checked as described previously [12]. Phospholipid and cholesterol, when present, were dissolved in chloroform, then dried to a thin film under vacuum. The dry film was suspended in buffer preheated to 50–60°C; the buffer for studies of $^{86}Rb^+$ efflux was 10 mM imidazole, 150 mM RbCl (pH 7.8), whereas for studies of Cl^- efflux the buffer was 10 mM Tris, 100 mM NaCl (pH 8.0).

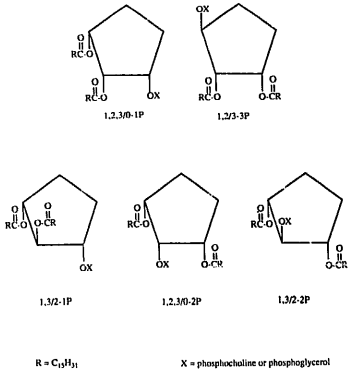

Fig. 1. Configurations of the cyclopentanoid analogs of phosphatidylcholine or phosphatidylglycerol that were used in these studies. Nomenclature is according to the IUBAC-IUB Commission.

The total lipid concentrations of the vesicles used in the $^{86}Rb^+$ and Cl^- efflux measurements were 1 and 2 mM, respectively. The aqueous dispersions were sonicated under nitrogen for 40–60 min in a Heat Systems Ultrasonics W375A sonicator equipped with a cup horn. Untrapped Cl^- and $^{86}Rb^+$ were removed by passing the vesicle suspensions through columns (1.5 × 40 cm) of Sephadex G-50 immediately before the kinetic experiments. This separation was performed at room temperature, which is below the gel to liquid-crystalline phase transition temperature of the phospholipid. To reduce nonspecific adsorption of the vesicles to Sephadex, the column was pretreated with egg PC vesicles followed by washing with several bed volumes of buffer immediately prior to removing the untrapped ions. The eluted vesicles containing trapped Cl^- or Rb^+ were placed in dialysis sacs which had been rinsed with the elution buffer. The dialysis sacs were placed in tubes containing the elution buffer (5 ml for Cl^- efflux, 4 ml for Rb^+ efflux) at 48°C (Cl^-) or 51°C (Rb^+), and shaken. The efflux of trapped $^{86}Rb^+$ was initiated by adding valinomycin (Sigma Chemical Co.) from a stock solution in dimethylformamide to a final ionophore concentration of 3.2 μM. Aliquots of 100 μl were removed in duplicate from the dialysate at intervals during the dialysis. Chloride ion was measured using an Orion 96-17 electrode. In most experiments, the leakage of Cl^- was rapid, e.g. about 90% efflux of the trapped Cl^- in 9–12 min; the vesicles were disrupted with 0.10% Triton X-100 to obtain the total Cl^- concentration trapped in the vesicles. The rate constants of Cl^- and Rb^+ efflux were estimated from first-order plots of $\log[(c_t - c_\infty)/(c_0 - c_\infty)]$ versus time, as described previously for other markers [12,13]; c_0, c_t, and c_∞ refer to the $^{86}Rb^+$ radioactivity or Cl^- concentration trapped in the vesicles at time zero, t, and at equilibrium, respectively. In the cholesterol exchange experiments, donor vesicles contained [4-^{14}C]cholesterol (New England Nuclear, Boston, MA) and dicetyl phosphoric acid (15 mol%) to confer negative surface charge; the acceptor vesicles contained a trace of [2-^{3}H]glycerol trioleate (ICN) as a nonexchangeable marker to monitor recovery after separation from the donor vesicles. The lipid films for the cholesterol exchange experiments were suspended in 20 mM sodium phosphate (pH 6.0) at total lipid concentrations of 1.1 mM and 11 mM in the donor and acceptor vesicles, respectively.

The exchange of [^{14}C]cholesterol from donor vesicles to a 10-fold excess of acceptor vesicles was mea-

sured at 46°C as described previously [14]. Aliquots (200 μl) of the donor-acceptor vesicle incubation mixture were applied at room temperature to small disposable columns of diethylaminoethyl (DEAE)-Sepharose CL-6B (Pharmacia) that had been pretreated with egg PC vesicles to minimize nonspecific lipid adsorption. The acceptor vesicles were eluted with 1.0 ml of 20 mM sodium phosphate buffer (pH 6.0) whereas the negatively charged donor vesicles were retained. The eluate was counted in 4 ml of Liquiscint scintillation fluid (National Diagnostics, Somerville, NJ) using a Beckman Model LS 7500 scintillation counter. The recovery of neutral acceptor vesicles in the eluate was approx. 85–90%, based on the cpm of [^{3}H]glycerol trioleate. The infinity value of [^{14}C]cholesterol exchanged was measured after 20–24 h of incubation. The fraction of [^{14}C]cholesterol exchanged at time t was calculated from the ratio of $((^{14}C/^{3}H)_t - (^{14}C/^{3}H)_0)/(^{14}C/^{3}H)_\infty$, where ^{14}C/^{3}H represents the ratio of [^{14}C]cholesterol/[^{3}H]glycerol trioleate at time zero, t, and ∞ in the acceptor vesicles. The pseudo-first-order rate constant was calculated by least-squares analysis of the slope of the logarithm of the fraction of [^{14}C]cholesterol exchanged vs time. The half-times for [^{14}C]cholesterol exchanged were calculated using the relationship $t_{1/2} = 0.693/k$.

Results and Discussion

The rate constants of spontaneous Cl^- efflux from vesicles prepared from cyclopentano-PC, cyclopentano-PG and their respective glycerol counterparts are presented in Table I along with the rate constants of vesicles in the presence of cholesterol. At temperatures above the phase transition, incorporation of cholesterol led to decrease in the rates of efflux, reflecting the well-known increase in lipid order caused by cholesterol incorporation into bilayers [13]. The net decreases of Cl^- release from vesicles prepared from 1,2/3-3P and 1,2/3-1P cyclopentano-PC and the respective PG analogs were analogous to those from the glycerophospholipids (≈ 50% decrease) and were significantly higher than those from vesicles prepared with the other cyclic phospholipids (1,2,3/0-1P, 1,2,3/0-2P, and 1,3/2-2P). This indicates that the 1,2/3-3P and 1,3/2-1P isomers have a geometry that permits a more favorable interaction between cholesterol and the phospholipid acyl chains than is possible with 1,2,3/0-1P and 1,3/2-2P isomers. The effect of the various ring conformations on head group and acyl chain orientation has been represented recently with space filling models of cyclopentano-phosphatidic acid [6].

Although the decrease in efflux rate caused by the presence of cholesterol was similar to that found with the natural phospholipids, the first-order rate constants were markedly higher for both the 1,2/3-3P and 1,3/2-1P isomers for both PC and PG analogs. This increased rate of 'leakiness' might be due to the necessity to accommodate the pentane ring into the phospholipid packing, or perhaps to the differences in head group orientation. The data might explain why no break at the transition temperature was observed for these two isomers in earlier studies when Na^+ efflux determinations were made after 180 min [4].

The various stereoisomers of the cyclopentano-PC's were tested as possible substrates for phospholipase A_2; one of the analogs, the 1,3/2-1P, was far superior to the other isomers [7]. Further studies supported the theory that this analog most closely possesses the conformation attained by phospholipids in the aggregated state [9,10]. In the efflux experiments presented here, the same analog was also found to closely behave similarly to the glycerophospholipid. It is probably not surprising that the 1,2/3-3P isomer which also possesses analogous efflux values would be the analog with the next closest arrangement; that is, the *trans* ar-

TABLE I

First-order rate constants for spontaneous Cl^- efflux from DPPC and cyclopentano-PC and the respective glycero- and cyclopentano-PG's

The efflux kinetics were measured at 48°C. Dicetyl phosphoric acid was incorporated into the vesicles at 10 mol%. Cholesterol, when present, was at 25 mol% in vesicles. The results represent the mean of measurements made with at least two (and generally three) preparations of vesicles from each phospholipid or phospholipid/cholesterol mixture. The error limits of the k values (standard errors of the mean) were 5% or less.

Vesicles	k (min^{-1})	% decrease with cholesterol
DPPC	0.26	
+ Cholesterol	0.12	56
1,2/3-3PC	0.46	
+ Cholesterol	0.23	51
1,3/2-1PC	0.45	
+ Cholesterol	0.22	50
1,2,3/0-1PC	0.25	
+ Cholesterol	0.20	21
1,2,3/0-2PC	0.25	
+ Cholesterol	0.19	24
1,3/2-2PC	0.24	
+ Cholesterol	0.19	21
DPPG	0.25	
+ Cholesterol	0.14	41
1,2/3-3PG	0.34	
+ Cholesterol	0.17	50
1,3/2-1PG	0.36	
+ Cholesterol	0.19	48
1,2,3/0-2PG	0.19	
+ Cholesterol	0.14	26

rangement of the head group with respect to the acyl chain whose linkage is closest to the vesicle surface.

The results of the valinomycin-mediated $^{86}Rb^+$ efflux experiments are summarized in Table II. The effect of cholesterol on vesicles prepared from 1,2/3-3P and 1,3/2-1P cyclopentano-PC was similar to that in DPPC vesicles, in agreement with the results described above for the Cl^- efflux. However, the vesicles prepared with each of the cyclopentano-PG analogs showed a greater decrease in Rb^+ efflux upon incorporation of cholesterol than did vesicles prepared with glycero-PG. Since the percent decrease in each case was about the same for each analog, the orientation of substituents was not a significant factor. It is interesting to note that the vesicles prepared from PG and its analogs gave lower rates of valinomycin-mediated $^{86}Rb^+$ efflux than vesicles prepared from PC and its analogs.

Desorption of [^{14}C]cholesterol is the rate-limiting step in the exchange from donor vesicles to an excess of acceptor vesicles [15–17]. Since the rate of cholesterol desorption from the vesicle interface is sensitive to the extent of phospholipid–cholesterol interactions [17], we have studied the rate of [^{14}C]cholesterol exchange between vesicles, using different phospholipids in the donor. The structure of the phospholipid in the acceptor does not influence the rate of exchange when acceptor vesicles are present in excess [14]. The results of radiolabeled cholesterol exchange kinetics from vesicles prepared with DPPC, DPPG, and cyclopentano-PC and -PG analogs are shown in Table III. The half-time values are similar. These kinetic studies indicate that the less flexible conformational orientation of cyclopentanoid analogs does not result in a significant difference in the ease of desorption of cholesterol with respect to glycero-PC and -PG. This result is consistent with previous results which indicated that changing the head groups (PC, PG) or type of hydrocarbon linkage (ester, ether, nitrogen or sulfur-linked chains [18]) in phospholipids caused only minor rate differences, whereas more striking rate differences were noted when the acyl structure (saturated vs. unsaturated) was varied [14].

TABLE II

First-order rate constants for valinomycin-mediated $^{86}Rb^+$ efflux from DPPC and cyclopentano-PC and the respective glycero- and cyclopentano-PG's

The efflux kinetics were measured at 51°C. Valinomycin was present at a final concentration of 3.2 μM in the vesicle suspension. Cholesterol, when present, was at 50 mol%. The results represent the mean of measurements made with at least two (and generally three) preparations of vesicles from each phospholipid or phospholipid/cholesterol mixture. The error limits of the k values (standard errors of the mean) were 5% or less.

Vesicles	$10^2 \times k$ (min^{-1})	% Decrease with cholesterol
DPPC	2.22	
+ Cholesterol	1.45	35
1,2/3-3PC	3.37	
+ Cholesterol	2.04	39
1,3/2-1PC	3.52	
+ Cholesterol	2.19	38
DPPG	1.56	
+ Cholesterol	1.02	35
1,2/3-3PG	1.42	
+ Cholesterol	0.68	52
1,3/2-1PG	1.55	
+ Cholesterol	0.66	57
1,2,3/0-2PG	1.79	
+ Cholesterol	0.84	53

TABLE III

Half-times for [^{14}C]cholesterol exchange from donor vesicles of different phospholipid composition to acceptor vesicles prepared from egg PC and cholesterol at 46°C

Donor vesicles contained 79 mol% PC or PG (as listed above), 6 mol% cholesterol, and 15 mol% dicetyl phosphoric acid. Acceptor vesicles contained 94 mol% egg PC and 6 mol% cholesterol. The total lipid concentrations of the donor and acceptor vesicles were 1.1 and 11 mM, respectively. The donor and acceptor species were separated rapidly using short DEAE-Sepharose CL-6B columns. The half-times for [^{14}C]cholesterol exchange are the mean ± S.E. (number of kinetic experiments performed with different vesicle preparations in parentheses).

Phospholipid	$t_{1/2}$ (min)
DPPC	133 ± 4 (3)
1,2,3/0-1PC	176 ± 7 (3)
1,2,3/0-2PC	165 ± 6 (6)
DPPG	148 ± 18 (2)
1,2/3-3PG	173 ± 7 (3)

Acknowledgement

This work was partially funded by a grant from the National Institutes of Health (HL-16660).

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