Biochimica et Biophysica Acta 855 (1986) 265–270
Elsevier

BBA 72913

The effect of cholesterol on glycerophosphono- and glycerophosphinocholines. Permeability measurements in lipid vesicles

Robert Bittman [a,*], Sanda Clejan [a,**], Lillian Fugler [a] and Arthur F. Rosenthal [b]

[a] *Department of Chemistry, Queens College of The City University of New York, Flushing, NY 11367 and* [b] *Biochemistry Division, Long Island Jewish-Hillside Medical Center, New Hyde Park, NY 11042 (U.S.A.)*

(Received November 7th, 1985)

Key words: Cholesterol-phospholipid interaction; Phosphonolipid; Phosphinolipid; Membrane permeability

The kinetics of spontaneous chloride ion efflux and valinomycin-mediated rubidium-86 efflux from vesicles prepared from synthetic phospholipids with carbon-phosphorus linkages were investigated at temperatures above the gel-to-liquid-crystalline phase transition. The rate constants for the movement of chloride and rubidium ions were reduced by incorporation of cholesterol into bilayers of phosphono- and phosphinocholines. Nonisosteric phosphonolipids in which the oxygen was removed from the glycerol side of phosphorus without substitution by a methylene group interacted less with cholesterol than the analogous isosteric derivatives, as judged from the magnitude of the decrease in the rate constants for chloride and rubidium ion efflux. The experiments reported in this study suggest that steric factors in the glycerol side of the phosphorus function are important in phosphatidylcholine-cholesterol interaction. However, the oxygen atom on the choline side of the phosphorus in the phosphatidylcholine molecule is not required for strong phosphatidylcholine-cholesterol interaction, since isosteric glycerophosphinocholines interacted as well as the corresponding isosteric glycerophosphonocholines. Furthermore, steric requirements on the choline side of phosphorus are not important in this interaction since phosphinates whose head-group structures are $-P(O^-)CH_2CH_2N^+(CH_3)_3$ and $-P(O^-)CH_2CH_2CH_2N^+(CH_3)_3$ interacted equally well with cholesterol, as estimated by these permeability studies.

Introduction

The carbon-phosphorus bond is the unique structural feature of lipids that contain 2-aminoethylphosphonic acid, $H_2O_3PCH_2CH_2NH_2$, or its derivatives. The isolation, biosynthesis, and natural occurrence of lipids that contain a carbon-phosphorus bond (phosphonolipids) have been reviewed recently [1,2]. The biological function of phosphonolipids has attracted attention, since the relative resistance of the carbon-phosphorus bond to chemical and enzymatic degradation is well known; however, no specific role of phosphonolipids in membranes has been established yet. It is, therefore, of interest to examine the interactions between phosphonolipids and other membrane components.

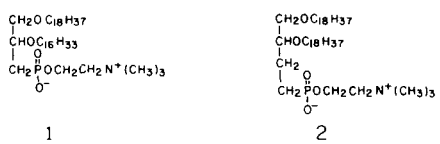
There has also been a great deal of interest in determining what structural features are important for the formation of the phospholipid-cholesterol complex. Compounds containing the carbon-phosphorus linkage(s) in place of the usual phosphate

* To whom correspondence should be addressed.
** Present address: Department of Pathology, City Hospital Center at Elmhurst, Mount Sinai School of Medicine of The City University of New York, NY 10029, U.S.A.

Abbreviation: PC, phosphatidylcholine.

ester linkage offer an approach for examining whether the oxygen atoms on either side of the phosphorus function affect the phosphatidylcholine-cholesterol interaction. Two carbon-phosphorus linkages are present in phosphinolipids, with the oxygen atoms removed from both the glycerol backbone and the head group. In the present paper, we report the kinetics of efflux of trapped markers from vesicles formed from synthetic glycerophosphono- and glycerophosphinocholines. These permeability measurements allow us to compare the ability of cholesterol to increase the packing density of bilayers containing these lipids above the gel-to-liquid-crystalline phase transition temperature. In order to analyze whether steric and electronic factors in the vicinity of the phosphorus function influence the interaction between cholesterol and phosphatidylcholine (PC), we have synthesized phosphono- and phosphinolipids containing dialkoxybutyl and dialkoxypropyl backbones. The dialkoxybutyl derivatives represent diether analogs of PC that are isosteric with respect to the phosphate-containing group of PC on the glycerol side of the phosphorus function (see compounds 2, 4 and 5, Fig. 1), since the oxygen atom connecting phosphorus with glycerol has been eliminated and a carbon atom has been substituted in its place.

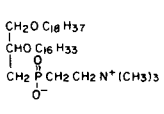
PHOSPHONOLIPIDS



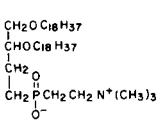
1

2

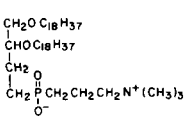
PHOSPHINOLIPIDS



3



4



5

Fig. 1. Structures of the synthetic diether glycerophosphono- and glycerophosphinocholines: 1, *rac*-2-hexadecoxy-3-octadecoxypropylphosphonylcholine; 2, *rac*-3,4-dioctadecoxybutylphosphonylcholine; 3, *rac*-2-hexadecoxy-3-octadecoxypropyl 2′-(trimethylammonium)ethyl phosphinate; 4, *rac*-3,4-dioctadecoxybutyl 2′-(trimethylammonium)ethyl phosphinate; 5, *rac*-3,4-dioctadecoxybutyl 3′-(trimethylammonium)propyl phosphinate.

Experimental procedures

Materials

The diether phosphonocholines, 1 and 2, which lack the oxygen atom on the glycerol side of the phosphorus function, were synthesized as described previously [3,4]. The dialkoxypropyl phosphinate, 3, and the dialkoxybutyl phosphinate, 4, were prepared according to Rosenthal et al. [5]. Compound 5, which is isosteric with respect to the usual PC on both the glycerol and choline sides of the phosphorus, was synthesized as described by Rosenthal and Chodsky [7]. All of the phospholipids migrated as single spots on silica gel G plates. The sources of cholesterol (recrystallized twice from absolute ethanol), $^{86}Rb^+$, carboxyfluorescein, and valinomycin were as cited elsewhere [6]. Carboxyfluorescein (recrystallized from methanol) was found to migrate as a single spot on cellulose MN plates (eluted with chloroform/methanol/water, 65 : 25 : 4 v/v).

Methods

Preparation of vesicles containing trapped $^{86}Rb^+$ and Cl^-. The preparation of vesicles containing trapped $^{86}Rb^+$ and the removal of untrapped Rb^+ were as described previously [8]. Ionophore-mediated efflux of $^{86}Rb^+$ was initiated by adding a 10-μl aliquot of valinomycin in dimethylformamide to 1 ml of Rb^+-loaded vesicles. The final concentration of valinomycin was 3.2 μM. The procedure used for measuring the time-course of Rb^+ efflux was as reported in Ref. 8. Samples were measured in triplicates at various times after the addition of valinomycin to a given preparation of vesicles maintained at 51°C. The results represent the average of at least two, and in general three, different vesicle preparations.

Efflux of Cl^-. Aqueous dispersions (2 μM total lipid) were prepared by suspending a thin lipid film of the desired glycerophosphono- or glycerophosphinocholines, dicetylphosphoric acid (10 mol%), and (when present) cholesterol (25 mol%) in 10 mM Tris buffer containing 100 mM NaCl (pH 8.0) at 55–60°C. The suspensions were vortexed at a temperature exceeding the lipid phase

transition *. The suspensions were then sonicated for 40 min under a stream of nitrogen in a Heat Systems Model W375A sonicator equipped with a cup horn through which cold water was circulated. Untrapped Cl^- was removed by passing the vesicles through a column (1.5 × 30 cm) of Sephadex G-50 at room temperature. The Sephadex was pretreated with a phospholipid suspension, and then washed with the elution buffer to reduce the nonspecific adsorption of Cl^--containing vesicles. The Cl^--loaded vesicles (1 ml) were placed in a Visking dialysis bag (1 cm diameter), which was then knotted with an air bubble trapped to ensure mixing of the contents. The bag was immersed in a jacketed vessel containing 10 ml of 10 mM Tris buffer containing 100 mM $NaNO_3$ (pH 8.0) which was maintained at 47°C with a Lauda temperature circulator. The solution was stirred magnetically with a 1-cm teflon-coated stirring bar. After the dialysis bag had been immersed in the buffer for about 1 min, the Cl^- concentration of the dialysate was monitored in millivolts using a Cl^--sensitive electrode (Orion) connected to an ion analyzer (Orion Model 801) and a Honeywell Model 19 recorder. Complete efflux of trapped Cl^- was achieved by adding Triton X-100 to the dialysis bag to a final concentration of 0.2%. After three runs, the electorde was rinsed, dried, and calibrated with 10 mM Tris buffer (pH 8.0) containing 100 mM $NaNO_3$ and KCl in the range of 10 μM to 1 mM. The time-course of Cl^- efflux was measured in quadruplicate from each vesicle preparation. At least three different preparations of vesicles were made from each phospholipid and phospholipid/cholesterol mixture. The first-order rate constants obtained from the plots of log% Cl^- retained vs. time (Fig. 3) agreed within about 10% in most experiments with vesicles from a given phospholipid or phospholipid/cholesterol mixture.

Characterization of vesicle sizes. The volumes of trapped carboxyfluorescein or $^{86}Rb^+$ per total lipid were calculated as described previously [6]. Incorporation of cholesterol did not influence the vesicle sizes significantly. The sizes of vesicles prepared from compounds 1–4 (see Fig. 1) were similar to those found for vesicles prepared from dipalmitoyl- and dihexadecylPC [9]. However, vesicles prepared from compound 5 trapped about 40% more carboxyfluorescein and $^{86}Rb^+$ than those from compounds 1–4.

Results and Discussion

The rate of movement of water-soluble molecules across lipid membranes depends on the degree of packing and the order of the hydrocarbon chains of the phospholipids. It is well known from many types of physical studies that cholesterol reduces the flux of cations, anions and neutral solutes across membranes by restricting the motion of the phospholipid fatty-acyl chains in the liquid-crystalline phase (e.g., for recent reviews of molecular packing in PC/cholesterol bilayers, see Refs. 10 and 11). Thus, cholesterol increases the order of the hydrocarbon matrix of the membrane. Ionophore-mediated ion fluxes across bilayers are dependent on the phospholipid hydrocarbon chain structure and temperature [12–14]. Thus, the permeation of ionophore-cation complexes may be controlled by membrane fluidity.

To investigate the influence of the oxygen atoms that link phosphorus with glycerol and choline in PC on the interaction with cholesterol, we have prepared vesicles from a series of synthetic phosphono- and phosphinocholines. Fig. 2 shows that cholesterol suppresses the rates of valinomycin-mediated $^{86}Rb^+$ efflux from the vesicles at 51°C. Similar results were obtained at 57°C (data not shown). As shown previously (Fig. 2 of Ref. 6), the time-course of valinomycin-mediated Rb^+ efflux follows first-order kinetics. Table I presents the first-order rate constants for $^{86}Rb^+$ efflux from vesicles prepared from the five PC analogs. Incorporation of cholesterol (at 50 mol%) into bilayers prepared from phosphonates and phosphinates with dialkoxybutyl backbones causes a signifi-

* The following phospholipid gel to liquid-crystalline phase transition temperatures were determined by Dr. Ira W. Levin using Raman spectroscopy: 1: 48.8°C; 2, 55.8°C; 3, 50.4°C; 4, 55.7°C. The main transitions of the phospholipids were shifted to lower temperatures in the presence of cholesterol (manuscript in preparation) or dicetylphosphate. Incorporation of 18 mol% cholesterol lowered the transition of 2 by 6.2Cdeg, whereas the transition temperature of the nonisosteric analog, 1, was lowered by only 2.6Cdeg.

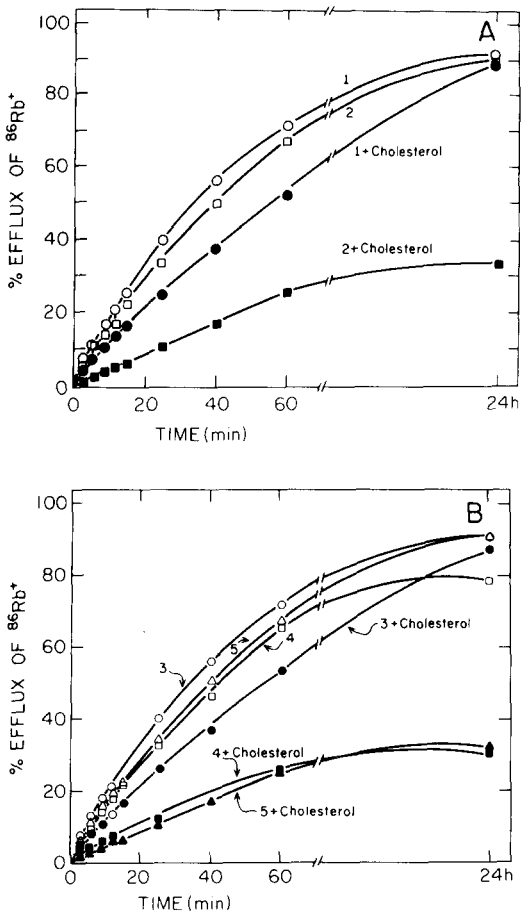


Fig. 2. Time-course of valinomycin-mediated $^{86}Rb^+$ release from vesicles at 51°C. Vesicles were prepared from (A) glycerophosphonocholines and (B) glycerophosphinocholines in the absence (open symbols) and presence (closed symbols) or 50 mol% cholesterol. The phospholipids used to prepare the vesicles have the following relationship with respect to the glycerol side of the phosphorus: nonisosteric (panel A, 1, and panel B, 3) and isosteric (panel A, 2, and panel B, 4 and 5). (See Fig. 1 for the structures corresponding to the compound numbers shown next to the curves.)

cantly larger percent decrease in the rate constant (65–73% decrease with compounds 2, 4 and 5) compared with bilayers from the analogous phospholipids with dialkoxypropyl backbones (40–46% decrease with compounds 1 and 3).

The passive diffusion of Cl^- across phosphono- and phosphinocholines was monitored using a Cl^--sensitive electrode. Cholesterol (at 25 mol%) decreases the rate of Cl^- efflux from vesicles prepared from both phosphono- (Fig. 3A) and phosphinolipids (Fig. 3B). Table I shows a similar trend in the rate constants for Cl^- efflux on cholesterol incorporation as noted above for $^{86}Rb^+$ efflux. The percent decrease is significantly lower in cholesterol-containing bilayers of the nonisosteric analogs 1 and 3) (33–35% decrease) than in cholesterol-containing bilayers of the isosteric lipids 2, 4 and 5 (53–61% decrease). These observations suggest that the packing density of bilayers from PC derivatives that are isosteric on the glycerol side of phosphorus are increased more by cholesterol than are bilayers from the nonisosteric PC derivatives.

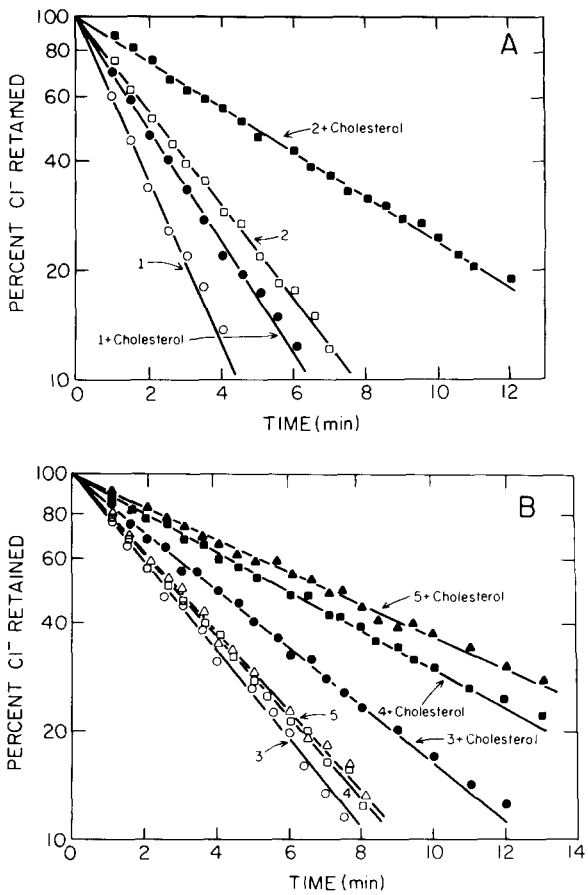


Fig. 3. First-order pltos fo Cl^- efflux from vesicles at 47°C. Vesicles were prepared from (A) glycerophosphonocholines and (B) glycerophosphinocholines in the absence (open symbols) and presence (closed symbols) of 25 mol% cholesterol. (A) ○), 1 and (●), 1 with cholesterol; (□), 2 and (■), 2 with cholesterol. (B) (○), 3 and (●), 3 with cholesterol; (□), 4 and (■), 4 with cholesterol; (△), 5 and (▲), 5 with cholesterol.

TABLE I

EFFECT OF CHOLESTEROL ON FIRST-ORDER RATE CONSTANTS (k) OF VALINOMYCIN-MEDIATED $^{86}Rb^+$ EFFLUX AND SPONTANEOUS Cl^- EFFLUX FROM VESICLES PREPARED FROM GLYCEROPHOSPHONO- AND GLYCEROPHOSPHINOLIPIDS

The vesicles contained 50 mol% cholesterol in the valinomycin-mediated $^{86}Rb^+$ efflux studies and 25 mol% cholesterol in Cl^- efflux studies. The total lipid concentration in the vesicles was 1 mM in the Rb^+ experiments and 2 mM in the Cl^- studies. Dicetylphosphoric acid was present at 10 mol% in the vesicles containing trapped Cl^-. The $^{86}Rb^+$ efflux measurements were conducted at 51°C, and the Cl^- efflux was monitored at 47°C. The rate constants were determined from first-order plots of $\log(C_t - C_\infty)/(C_0 - C_\infty)$ vs. time, as shown previously for $^{86}Rb^+$ efflux [6]. C_0, C_t and C_∞ represent the counts/min in the $^{86}Rb^+$ studies and the Cl^- content trapped in the vesicles at time zero, t and equilibrium, respectively. Time zero is the time of valinomycin addition in the $^{86}Rb^+$ efflux experiments, and the time of immersion of the dialysis bag into the bath above the phase transition temperature in the Cl^- efflux experiments. The value of C_∞ was determined from double-reciprocal plots of C_t^{-1} vs. time^{-1} at times approaching equilibrium (4.5, 5.5 and 6.5 h for $^{86}Rb^+$ efflux, and about 10–12 min for Cl^- efflux). The value of C_0 was obtained by releasing all of the trapped marker by adding Triton X-100 (0.2% final concentration). Although measurements of Cl^- concentration were generally begun at 1 min after immersion of the bag in the high-temperature bath, when the rate constants were high we approximated the time-course at times shorter than 1 min.

Compound number		$^{86}Rb^+$			Cl^-		
		$10^2 \cdot k$ (min^{-1})		% decrease with cholesterol	k (min^{-1})		% decrease with cholesterol
		without cholesterol	with cholesterol		without cholesterol	with cholesterol	
	Phosphonates						
1	Propyl (nonisosteric)	23.8	12.9	46	0.54	0.35	35
2	Butyl (isosteric)	20.7	7.1	66	0.30	0.14	53
	Phosphinates						
3	2C Propyl (nonisosteric)	22.1	13.3	40	0.28	0.18	33
4	2C Butyl (partially isosteric)	18.2	6.3	65	0.26	0.12	54
5	3C Butyl (fully isosteric)	18.7	5.0	73	0.25	0.10	61

The decreases in the rate constants for Cl^- efflux produced by incorporating 25 mol% cholesterol into dipalmitoylPC and dihexadecylPC bilayers (56 and 53%, respectively) are similar to those found with bilayers from the isosteric phosphono- and phosphinocholines. The observation that the substitution of a methylene group for the oxygen atom on the glycerol side of the phosphorus of PC does not alter the extent of the PC-cholesterol interaction suggests that the weaker interaction of the nonisosteric PC analogs 1 and 3 with cholesterol arises from steric rather than from polar factors. Changes in orientation that may result from steric changes in the glycerol moiety may also contribute to the differences in packing density. Furthermore, the presence of ether rather than ester linkages does not appear to influence the interaction with cholesterol, as estimated by the rates of Cl^- efflux. This finding is consistent with our recent report describing permeability and ^{13}C-NMR measurements in ether- and ester-linked PC bilayers [9]. Since ether groups in PC do not alter the average conformation or the segmental motion of the glycerophosphocholine group [15], the conclusions reached with our synthetic diether phosphonates and phosphinates are expected to apply to ester-linked lipids as well.

The effects of cholesterol on the properties of glycerophosphinocholine bilayers have been studied by de Kruyff et al. [16] and Bittman and Blau [17]. The mean molecular area of the nonisosteric phosphinate 3 was reduced when cholesterol

was incorporated into monolayers [17]. Although this condensation effect indicates a substantial degree of interaction between 3 and cholesterol, no comparisons were made with other synthetic phosphino- or phosphonolipids. It should be noted that the effect of cholesterol on 3 was less than on two phosphatidylcholines (18:1/16:0PC and 16:0/18:1PC). Evidence for interaction of 3 with cholesterol was also obtained from calorimetry measurements, which showed a large reduction in the endothermic phase transition in water/ethylene glycol [16]. In previous work from this laboratory, the effects of cholesterol on the rates of osmotic water permeability of multilamellar vesicles in the gel phase of phosphono- and phosphinocholines were reported [17]. Bilayers prepared from the isosteric 1,2-dialkoxybutyl-derived lipids were affected more by cholesterol than were bilayers prepared from the nonisosteric 1,2-dialkoxypropyl-derived lipids. The present study extends our previous work by examining the effects of cholesterol on phosphono- and phosphinocholines in unilamellar vesicles in the liquid-crystalline phase. We observed an interaction between cholesterol and the nonisosteric phospholipids 1 and 3, as judged by the reduction in the rate constants for Cl^- and valinomycin-mediated $^{86}Rb^+$ efflux. However, the extent of this interaction is appreciably higher with compounds 2, 4 and 5, which have the 1,2-dialkoxybutyl backbone. Table I shows that cholesterol decreases the permeability of bilayers from phosphinates 4 and 5 to a similar extent. This suggests that the steric and electronic requirements for interaction with cholesterol are not as high on the choline side of the phosphorus in PC compared with the glycerol side of the phosphorus function.

Acknowledgment

This work was supported in part by National Institutes of Health Grant HL 16660.

References

1 Hori, T. and Nozawa, Y. (1982) in Phospholipids (Hawthorne, J.N. and Ansell, J.B., eds.), pp. 95–128, Elsevier, Amsterdam
2 Vance, D.E. (1985) in Biochemistry of Lipids and Membranes (Vance, D.E. and Vance, J.E., eds.), pp. 242–270, Benjamin/Cummings Publishing Co., Menlo Park, CA
3 Rosenthal, A.F. (1966) J. Lipid Res. 7, 779–785
4 Rosenthal, A.F. and Pousada, M. (1969) Lipids 4, 37–40
5 Rosenthal, A.F. and Chodsky, S.V. (1971) J. Lipid Res. 12, 277–285
6 Rosenthal, A.F., Chodsky, S.V. and Han, S.C.H. (1969) Biochim. Biophys. Acta 187, 385–392
7 Rosenthal, A.F. and Chodsky, S.V. (1971) Biochim. Biophys. Acta 239, 248–251
8 Bittman, R., Clejan, S., Jain, M.K., Deroo, P.W. and Rosenthal, A.F. (1981) Biochemistry 20, 2790–2795
9 Bittman, R., Clejan, S., Lund-Katz, S. and Phillips, M.C. (1984) Biochim. Biophys. Acta 772, 117–126
10 Presti, F.T., Pace, R.J. and Chan, S.I. (1982) Biochemistry 21, 3831–3835
11 Hui, S.W. and He, N.-B. (1983) Biochemistry 22, 1159–1164
12 De Gier, J., Haest, C.W.M., Mandersloot, J.G. and Van Deenen, L.L.M. (1970) Biochim. Biophys. Acta 211, 373–375
13 Blok, M.D., De Gier, J. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 367, 210–224
14 Wun, T.-C. and Bittman, R. (1977) Biochemistry 16, 2080–2086
15 Hauser, H., Guyer, W. and Paltauf, F. (1981) Chem. Phys. Lipids 29, 103–120
16 De Kruyff, B., Demel, R.A., Slotboom, A.J., Van Deenen, L.L.M. and Rosenthal, A.F. (1973) Biochim. Biophys. Acta 307, 1–19
17 Bittman, R. and Blau, L. (1972) Biochemistry 11, 4831–4839