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INTERACTION OF CHOLESTEROL WITH PHOTOACTIVABLE PHOSPHOLIPIDS IN SONICATED VESICLES

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Summary

Photoactivable phospholipids containing either α-diazo-β-trifluoropropionyloxy or *m*-diazirinophenoxyl groups in the ω-positions of *sn*-2 fatty acyl chains were synthesized and incorporated into sonicated vesicles containing 33 mol% of cholesterol. Photolysis of the vesicles at 350 nm produced covalent cross-links between the synthetic phospholipids and cholesterol. The cross-linked products obtained using [^{14}C]cholesterol were characterized by their chromatographic behavior, cleavage on phospholipase A_2 treatment, base-catalyzed transesterification and mass spectral measurements. The cross-linking was shown not to involve the 3-β-hydroxyl group of cholesterol, and it was concluded that the reactive carbene intermediates formed from the photolabels inserted into the hydrocarbon skeleton of cholesterol in the bilayer. The extent of cross-linking obtained was comparable to that observed previously using phospholipids alone, indicating that no lateral phase separation occurred. The present approach is promising for further precise studies of the molecular interactions between cholesterol and phospholipids in biological membranes.

Introduction

We have recently described a new approach to the study of molecular interactions within biological membranes, which involves covalent cross-linking by

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the use of phospholipids containing photoactivable carbene precursors [1,2]. A series of mixed acyl phospholipids containing palmitic or stearic acids in the *sn*-1 position and a photolabeled fatty acid incorporating either trifluorodiazopropionyloxy or diazirino-phenoxy groups (phospholipids I and II, Scheme I) as integral parts of the fatty acyl chains were synthesized [1,2]. On sonication, these phospholipids formed sealed vesicles, and the latter, on irradiation at 350 nm, formed intermolecularly cross-linked phospholipids (3—5). It was of interest to investigate the usefulness of this approach in studies of cholesterol packing in biological membranes. In this paper we describe our initial experiments which clearly show that the photoactivable groups in the above phospholipids (Scheme I), which have been shown to be in the interior of the bilayer, undergo covalent cross-linking with cholesterol on photolysis.

Cholesterol is an indispensible component of biological membranes of higher organisms [6]. Although the molecular mechanism of its function is only poorly understood, extensive studies in model and biological membranes (for reviews, see Refs. 7, 18, 19) have indicated that cholesterol can affect the packing of lipid hydrocarbon chains in the bilayer. The interaction is envisaged to be such that above the thermal phase transition temperature (T_m), cholesterol inhibits flexing of the hydrocarbon chains whereas below T_m it prevents the chains from crystallizing into the rigid α-gel. A variety of studies using, for example, X-ray diffraction, ^{1}H- and ^{13}C-NMR, Raman spectroscopy, electron microscopy, ESR and DSC, have been carried out on the packing of cholesterol in the membrane, but the results have generated conflicting views. It is hoped that an extension of the present approach involving intermolecular cross-linking would lead to precise determination of the points of contact between cholesterol and phospholipids. This, in turn, would help delineate the configurational arrangement of cholesterol in membranes.

Materials and Methods

Materials

Cholesterol was purchased from J.T. Baker Chemical Co. and was used without further purification. [4-^{14}C]Cholesterol (54 mCi/mmol) was purchased from New England Nuclear. Crude rattlesnake venom (*Crotalus adamanteus*, Ross Allen Reptile Farm, FL) was used as the source of phopholipase A_2.

Sephadex LH-20 (25—100-mesh beads) was purchased from Pharmacia Fine Chemicals.

The phospholipids (Scheme I) used in this study were synthesized as described previously [2].

Methods

Vesicles containing phospholipids and cholesterol. A solution of 20 μmol of the synthetic phospholipid and 10 μmol of cholesterol (containing approximately $50 \cdot 10^6$ cpm [4-^{14}C]cholesterol) in chloroform was dried under a stream of nitrogen. The residue was suspended in 5 ml 0.15 M KCl in 0.01 M Tris-HCl buffer (pH 7.6), the tube was flushed with N_2, sealed, and sonicated using a bath-type sonicator (80 W, 80 kHz at 3.5 A) until an optically clear solution resulted (usually 30—45 min). The temperature of the bath was maintained between 25—35°C.

Photolysis of vesicles. Irradiation of the phospholipid was performed using a Rayonet photochemical reactor equipped with sixteen symmetrically placed RPR 3500A lamps. The solutions were contained in a quartz vessel, placed in the center of the reactor, with a jacket through which was circulated an aqueous (2% w/v) potassium hydrogen phthalate solution. The temperature of the latter was controlled, as desired, by a thermostat. Progress of photolysis was followed either by monitoring the disappearance of the characteristic infrared frequency of the diazo (2140 cm^{-1}) group or the ultraviolet absorption of the diazirine moiety.

Separation of photolysis products. The photolysis mixtures were extracted by the procedure of Bligh and Dyer [8]. The organic phase was evaporated and the residue, as a solution in a minimal volume of chloroform/methanol, 1 : 1 (vol./vol.), was applied to a Sephadex LH-20 column (2.5 cm × 100 cm) which had been previously equilibrated in the same solvent. The rate of elution was about 60 ml/h. Fractions (2 ml) were monitored by their phosphorus [9] content or radioactivity, if present.

Radioactivity was determined in a Beckman LS-250 liquid scintillation spectrometer using 2,5-diphenyloxazole (15.3 g) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.38 g) in toluene (3.78 l) as the scintillator.

Treatment with phospholipase A_2. Products of photolysis (about 1 mg) were treated with 100 μl of a stock solution of phospholipase A_2 (150 μg of phospholipase A_2 in 1 ml 0.05 M $CaCl_2$/0.02 M Tris-HCl, pH 9). Diethyl ether containing 2% methanol (500 μl) was added and sealed two-phase mixture was stirred at room temperature for 18 h. A control experiment with 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine showed that the cleavage was complete under these conditions.

Transesterification. To a solution of the cholesterol-phospholipid * (about 2 mg) in anhydrous methanol (1.0 ml) was added 0.5 M sodium methoxide in methanol (10 μl) and the mixture was stirred at room temperature for 10 h. After removal of solvent, the residue was dissolved in chloroform (500 μl). The aqueous layer was carefully removed and the organic phase which contained the cholesterol-fatty ester was washed two more times with water (500 μl). Removal of solvent from the organic phase yielded a crude mixture of fatty esters which was purified by preparative thin layer chromatography on 20 × 20 cm (0.25 mm thickness) silica gel plates (solvent system B).

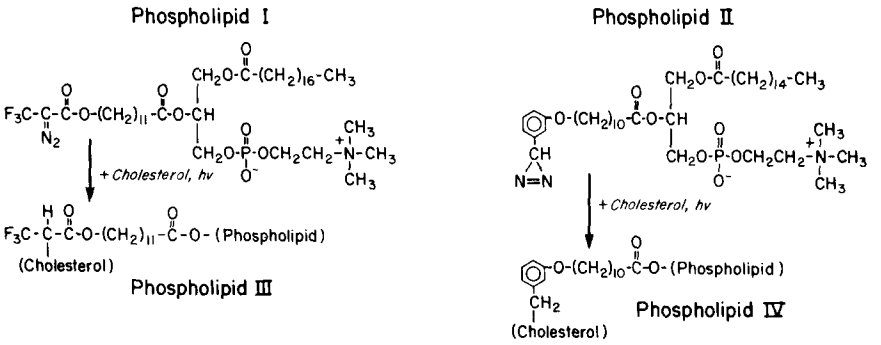
Thin-layer chromatography (TLC). Purity of the lipids was checked by TLC using Merck Silica Gel plates. The solvent systems used were: A, chloroform/methanol/water, 65 : 25 : 4 (v/v/v); B, ether/chloroform, 10 : 90 (v/v). Phospholipids were visualized by the molybdenum blue spray [10].

Results

(1) Cross-linking of phospholipid I (Scheme I) with [^{14}C]cholesterol and characterization of the products

A solution of 16 mg (20 μmol) of the phospholipid in chloroform was mixed

* The hyphenated words, cholesterol-phospholipid and cholesterol-fatty acid (ester), denote covalently cross-linked products containing cholesterol and phospholipid or fatty acid (ester), respectively.



Scheme I. Structures of the synthetic mixed acyl phospholipids incorporating α-diazo-β-trifluoro-propionyloxy (phospholipid I) and the diazirinophenoxy (phospholipid II) groups in ω-positions of the fatty acids at *sn*-2 positions. The cross-linked products obtained after irradiation of vesicles containing these phospholipids and cholesterol are indicated by general structures III and IV.

with 3.8 mg (10 μmol) of cholesterol and a benzene solution of [4-^{14}C]cholesterol ($50 \cdot 10^6$ cpm) and the mixture dried under nitrogen in a Pyrex glass tube. Vesicles were prepared and irradiated at 350 nm for 15 h at 4°C as described in Methods. The photolysis products obtained after a extraction using the method of Bligh and Dyer [8] were separated on a Sephadex LH-20 column (Fig. 1). The peak in fractions 58–65 contained the phospholipid-cholesterol (phospholipid III in Scheme I) and accounted for approximately 12% of the total radioactivity. A portion of this material, when rechromatographed on the same Sephadex LH-20 column, eluted in the same position as in Fig. 1. A second aliquot was treated with phospholipase A_2 as described in Methods, the products were isolated by extraction using the method of Bligh and Dyer [8] and then subjected to chromatography as above. The pattern now obtained is shown in Fig. 2. Thus, the entire radioactive peak now eluted from the column in a position consistent with the structure (V) (Scheme II).

Another portion of the phospholipid-cholesterol was treated with sodium methoxide in methanol as described in Methods. After neutralization of the reaction mixture and evaporation of solvents, the products were subjected to thin-layer chromatography as described in Methods. The major radioactive band corresponded to cholesterol-fatty ester (general structures (VI) * in Scheme II). After isolation, the ester was acetylated with acetic anhydride and dimethylamino pyridine in chloroform. The resulting product showed increased mobility on TLC (Fig. 3c) as expected for the acetylation of the 3-hydroxyl group. Further, the infrared spectrum of the acylation product showed the absence of a hydroxyl group. Thus the 3 β-OH group was not involved in the cross-linking reaction.

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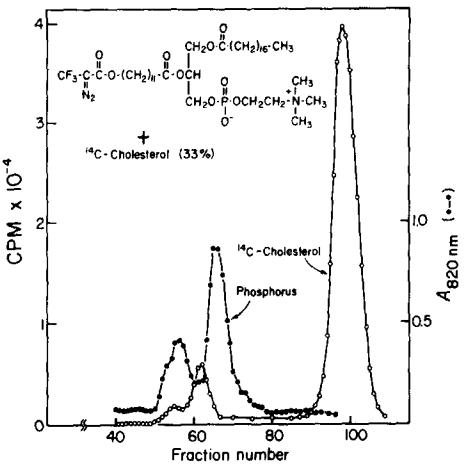
* As discussed earlier [3], the trifluoro ester linkage in Scheme II does not undergo transesterification, presumably because it is sterically hindered.



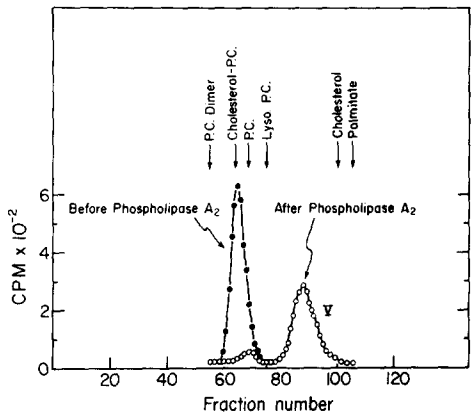
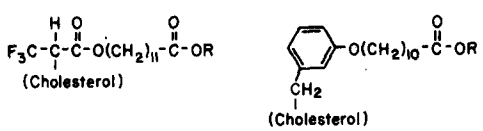


Fig. 1. Sephadex LH-20 elution profile of the products obtained after photolysis of diazophospholipid vesicles containing [4-^{14}C]cholesterol (33 mol% containing 50 · 10^6 cpm). The fractions were monitored for radioactivity in cholesterol and for their phosphorus content (A_{820}). Fractions 50—60 contained the oligomeric phospholipids while 62—75 contained the monomeric phospholipids. Fractions 58—65 represent cholesterol cross-linked to the phospholipid. This fraction contained 12% of the total radioactivity used in the vesicle preparation.

Fig. 2. Sephadex LH-20 elution profile of the cholesterol-phospholipid before and after phospholipase A_2 treatment. A portion of the cholesterol-phospholipid (fractions 58—65 of Fig. 1) was chromatographed on the same column as before and elution monitored by phosphorus determination. An equal portion was first subjected to phospholipase A_2 digestion, the products were isolated by extraction using the method Bligh and Dyer [8] and then chromatographed on Sephadex LH-20 as before. Radioactivity was monitored in different fractions. PC, phosphatidylcholine.

(2) Cross-linking of phospholipid II (Scheme I) with cholesterol and characterization of the products

A solution of 20 μmol of the phospholipid (Scheme I) in chloroform was mixed with 10 μmol cholesterol containing 50 · 10^6 cpm of radioactive cholesterol and the solution was dried under nitrogen. Vesicles were prepared and irradiated at 350 nm for 40 min at 4°C as described in Methods. The rate of appearance of the cross-linked products was monitored by autoradiography (Fig. 4). Thus, the photolysis was essentially complete in 10 min. The photolysis products were isolated by extraction using the method of Bligh and Dyer



V ; R=H
VI ; R=CH₃
VII ; R=H
VIII ; R=CH₃

Scheme II. General structures of the cholesterol-fatty acids and esters obtained after phospholipase A_2 treatment and transesterification of the cholesterol-phospholipids.

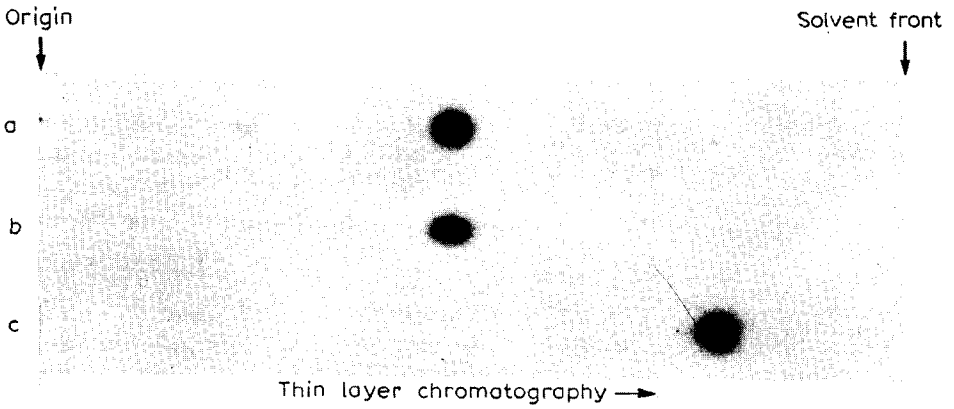


Fig. 3. Autoradiogram of a thin-layer chromatogram of an acetylation reaction of the cholesterol-fatty ester (solvent B). a, cholesterol; b, cholesterol-fatty ester; c, product obtained after acetylation of the cholesterol-fatty ester with acetic anhydride and dimethylamino pyridine.

[8] and were separated by chromatography on Sephadex LH-20 (Fig. 5). The first radioactive peak (fractions 45—50) presumably contained a dimeric phospholipid cross-linked to cholesterol while the major cross-linked product present in fractions 52—65 contained the phospholipid-cholesterol. A portion of the material in this peak was treated with phospholipase A_2. All the material

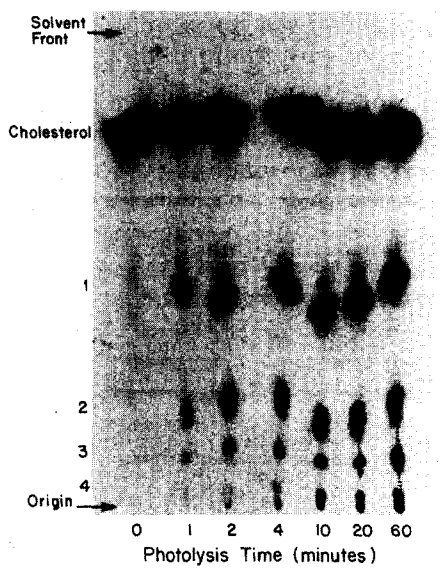


Fig. 4. Kinetics of photolysis of the vesicles prepared from diazirino phospholipids and [^{14}C]cholesterol. Vesicles containing 33 mol% [4-^{14}C]cholesterol were prepared and photolyzed as in Methods. Aliquots were withdrawn from the photolysis mixtures at the times indicated and the lipids were isolated by using the method of Bligh and Dyer [8] and subjected to thin-layer chromatography (Solvent A). Spot 1: cholesterol phospholipid that appeared in fractions 55—63 on Sephadex LH-20 column (Fig. 5). Spots 2, 3 and 4 represent the oligomeric phospholipid cross-linked to cholesterol (fractions 54—53 of Fig. 5).

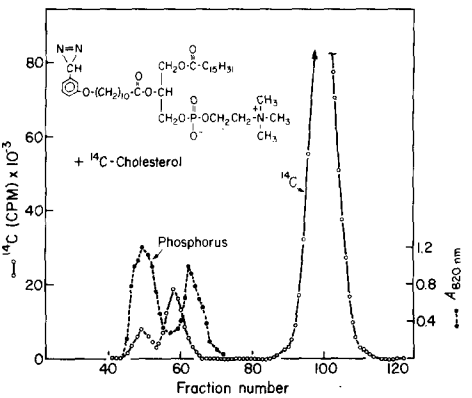


Fig. 5. Sephadex LH-20 elution profile of the products obtained after the photolysis of diazirino phospholipid vesicles containing [4-^{14}C]cholesterol (33 mol% containing 50 · 10^6 cpm). Fractions 45—55 contained oligomeric phospholipids while 60—70 contained monomeric phospholipids. Fractions 45—53 represent oligomeric phospholipids cross-linked to cholesterol. Fractions 55—63 contained cholesterol cross-linked to the phospholipid, and these were used for transesterification reactions.

was sensitive to this enzyme, and the product was eluted from the Sephadex LH-20 column in a position consistent with the assigned cholesterol-fatty acid structure (VII in Scheme II).

A second portion of this cholesterol-phospholipid was transesterified and subjected to TLC. The isolated cholesterol-fatty ester (VIII in Scheme II) was analyzed by field desorption mass spectrometry and high resolution mass spectrometry. The results showed that while the compound was not pure, the major molecular ion observed at m/e = 691 was consistent with the formula $C_{46}H_{74}O_4$ (calculated m/e = 691.0956). The product, therefore, was assigned the structure VIII (Scheme II).

Discussion

Cholesterol has previously been shown to have the dimensions 7.2 X 5 X 20 Å [11]. Structurally, the molecule is characterized by a 3-β-hydroxyl group which is hydrophilic, a backbone consisting of a tetracyclic ring 9 Å in length, and a mobile side chain approximately 11 Å in length. The steroid could thus span approximately one-half of the hydrocarbon portion of a phospholipid bilayer.

In the present paper, initial studies have been reported on the application of the photoaffinity labeling approach to the study of phospholipid-cholesterol interactions. The results show that cholesterol included in the synthetic phospholipid vesicles can undergo covalent cross-linking with the phospholipids. The extent of cross-linking observed is comparable to that obtained in related experiments with other mixed phospholipids [3]. It can, therefore, be concluded that the cholesterol remained in a mixed phase with the photoreactive phospholipid even at 4°C (well below the transition temperatures of the phospholipids; [17]). The sensitivity of all the cholesterol cross-linked products (Fig. 2) to phospholipase A_2 digestion, as well as the chemical characterization

described, firmly establish covalent cross-linking of the photoreactive phospholipids to cholesterol. The results also exclude the possibilities of free radical dimerization of cholesterol alone since such products would not be sensitive to the phospholipase. The sensitivity of the products to the enzyme also proves that only the *sn*-2 chain carrying the photolabel was involved in this reaction since this enzyme is specific for the cleavage of the *sn*-2 acyl chains. Furthermore, the phospholipase digestion products ran between the lysophospholipid and fatty acids on the Sephadex LH-20 column (Fig. 2), as would be expected for cholesterol-fatty acids (V and VII, Scheme II).

Transesterification and analysis of the cholesterol-fatty acid ester by field desorption and high resolution mass spectrometry showed that the molecular weight of the product was compatible with the structural formulation.

From earlier extensive studies, it is likely that the 3-β-hydroxyl group of cholesterol lies at the interface and the remainder of the molecule is embedded in the bilayer. On the basis of ESR studies and space-filling models, it has been postulated that the 3-β-hydroxyl group is involved in hydrogen bonding with the carbonyl oxygen of the fatty acyl groups [12,13]. However, in a recent study on the permeability properties of cholesterol-containing liposomes prepared from analogues of phosphatidylcholine with fatty ether instead of ester linkages, it was concluded that the above interaction is not essential for the properties characteristic of cholesterol [14]. Consistent with our previous results on phospholipid-phospholipid interactions, we have now shown that the hydroxyl group of cholesterol is not involved in the cross-linking reaction and, therefore, is outside the range of the photoreactive groups. These results are in accordance with the proposal that the hydroxyl group of the cholesterol is not embedded in the bilayer.

Darke et al. [15] graphically depicted the specific effects of cholesterol on each carbon atom of the acyl chain of lecithin. The solidifying effect of cholesterol, i.e. the prevention of gel crystalline-liquid crystalline transition was proposed to occur through interaction between cholesterol and the fatty acyl region around C4 to C6. On the other hand, the liquifying effect was concluded to be located more toward the core of the bilayer [16]. Further understanding at the molecular level of the cholesterol-fatty acyl chain interactions should be forthcoming from an extension of the present approach in which the sites of intermolecular cross-linking are determined following systematic variation of the photolabels along the fatty acyl chain. By using the side chain in cholesterol in perdeuterated form, the cholesterol-fatty ester formed could be analyzed by mass spectrometry. Similarly, cholesterol specifically labeled with ^{13}C at specific atoms (a kind gift from Professor G. Popjak of the University of California) could be used in ^{13}C-NMR studies of the cross-linked products. Such studies could clearly be of aid in elucidating the exact disposition of cholesterol in the phospholipid matrix.

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