Monolayer interaction of cholesterol with phosphatidylcholines: effects of phospholipid acyl chain length

Peter Mattjus, Gun Hedström, J. Peter Slotte*

Department of Biochemistry and Pharmacy, BioCity, Åbo Akademi University, P.O. Box 66, 20521 Turku, Finland

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Abstract

The degree of association of cholesterol with phosphatidylcholines having symmetric acyl chains from 10 to 20 carbons in length have been examined in monolayers at the air/water interface using cholesterol oxidase as a probe. Mixed monolayers having three different cholesterol/phospholipid (C/PL) molar ratios (0.9:1.0, 1.0:1.0, and 1.5:1.0 C/PL) were prepared. In these monolayers (at a lateral surface pressure of 20 mN/m), cholesterol was most readily available for oxidation in monolayers having phosphatidylcholines with short (di-10-PC and di-12-PC) or long (di-18-PC and di-20-PC) acyl chains, whereas the oxidation susceptibility was lower in monolayers having phosphatidylcholines with intermediate length acyl chains (di-14-PC, di-15-PC, di-16-PC and di-17-PC). Mixed monolayers having a C/PL of 0.9:1.0 were prepared to include 0.5 mol% NBD-cholesterol, and the monolayer surface texture at 20 mN/m was examined using epifluorescence microscopy. It was clearly revealed that monolayers containing di-10-PC and di-12-PC were laterally heterogeneous (containing both liquid-expanded and liquid-condensed lateral domains). With intermediate chain phosphatidylcholines (14–17 carbons), there was no surface texture (dominantly dark monolayer areas), whereas occasional bright NBD-cholesterol-rich inclusions again began to appear in di-18-PC and di-20-PC monolayers. The increased oxidation susceptibility of cholesterol in di-18-PC and di-20-PC could possibly result from a partial lateral phase separation of cholesterol-rich phases in these monolayers, since cholesterol can be expected to be less readily soluble in a long chain than in intermediate chain phosphatidylcholine matrix, and since cholesterol-rich phases are expected to be oxidized more readily than cholesterol-poor phases. We conclude that the susceptibility of cholesterol to oxidation by cholesterol oxidase was most pronounced in monolayers containing phosphatidylcholines with acyl chain lengths that did not match the length of the sterol molecule.

Keywords: Monolayer membranes; Cholesterol oxidase; Cholesterol; Phosphatidylcholine; Acyl chain length; Lipid interaction; Phase separation; Fluorescence microscopy

Abbreviations: C/PL, cholesterol phospholipid molar ratio; di-10-PC, didecanoyl-phosphatidylcholine; di-12-PC, didecanoyl-phosphatidylcholine; di-14-PC, ditetradecanoyl-phosphatidylcholine; di-15-PC, dipentadecanoyl-phosphatidylcholine; di-16-PC, dihexadecanoyl-phosphatidylcholine; di-17-PC, diheptadecanoyl-phosphatidylcholine; di-18-PC, dioctadecanoyl-phosphatidylcholine; di-20-PC, dioctadecanoyl-phosphatidylcholine; NBD-cholesterol, 22-{N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino}-23,24-bisnor-5-chole-3-ol.
* Corresponding author.

1. Introduction

The flexibility of phospholipid acyl chains in membrane structures is to a great extent influenced by the degree of acyl chain unsaturation, and cholesterol [1]. Cholesterol molecules have a disordering effect on model membranes containing saturated long chain phospholipids, whereas cho-
olesterol appears to bring more order to phospholipid membranes having unsaturated acyl chains [2,3]. The phospholipid acyl chains near the center of the membrane bilayer experience considerable motional freedom, because the hydrophobic tail of cholesterol is short and will not pack as well in the bilayer center as the hydrophobic tails of phospholipids [4]. The isooctyl side chain of the cholesterol molecule appears to be more ordered than the phospholipid acyl chains, and is therefore not free to move and fill up the free space inside the bilayer structure [5].

Molecular interactions in membranes is mostly influenced by hydrogen bonding at the polar/apolar interface [6], and by intermolecular van der Waals' forces operating at short distances between adjacent acyl chains or between cholesterol and phospholipid acyl chains [7]. Whereas the hydrogen bonding between cholesterol and phospholipids in membranes appears to be weak [8], the contribution of van der Waals' forces in stabilizing cholesterol/phospholipid interaction is more important [7,9]. Cholesterol exchange kinetics between donor and acceptor membranes has been shown to depend on the rate of cholesterol desorption from the donor membrane [9,10]. Further, the interaction of cholesterol (or other sterols) with phospholipids appears to provide the principal physical-chemical basis for determining the rate of spontaneous steryl desorption from a membrane [9]. Cholesterol interacts more favorably with phosphatidylcholines having long saturated acyl chains (giving more opportunities for van der Waals' interactions) than it does with a monounsaturated phosphatidylcholine (lesser van der Waals' interactions). Consequently, cholesterol desorbs more slowly from a membrane containing saturated long chain phosphatidylcholines as compared to a more fluid membrane containing unsaturated phosphatidylcholines [9,10].

The cholesterol oxidase susceptibility of membrane cholesterol also appears to depend on the strength of intermolecular interactions in the membranes [11–13]. Cholesterol in a fluid phosphatidylcholine membrane is oxidized more readily than it is in a saturated phosphatidylcholine membrane (either monolayer or bilayer) [14]. Cholesterol oxidase has therefore been used by us as a probe for determining strengths of cholesterol/phospholipid interactions in different model membrane systems. In the present study we have examined the strength of monolayer interaction between cholesterol and phosphatidylcholines having saturated acyl chains of different length, using cholesterol oxidase as a probe. We selected a series of chain length variable phosphatidylcholines (having symmetric acyl chain lengths from 10–20 carbons), since these should manifest different degrees of van der Waals' interactions with cholesterol.

2. Experimental procedures

2.1. Materials

Cholesterol (≥99%), and all the phosphatidylcholines were obtained from Sigma Chemicals (St. Louis, MO). The fluorescent probe 22-(N-(7-nitrobenz-2-oxa-1,3-diazol)-4-yl)amino-23,24-bisnor-5-cholesta-3-ol was purchased from Molecular Probes Inc. (Eugene, OR.) The phospholipids gave a single spot on thin-layer chromatography plates (Kieselgel 60, Merck) when eluted with chloroform/methanol/acetic acid/water (25:15:4:2 v/v) and stained with iodine. Cholesterol, the phosphatidylcholines and NBD-cholesterol were all diluted in hexane/isopropanol (3:2 v/v) and stored in the dark at −25°C and used without further purification. Cholesterol oxidase (Streptomyces cinnamomeus) was purchased from Calbiochem (San Diego, CA) and was used as delivered. Buffer salts were of pro-analysis grade; the water was purified with reverse osmosis and further purified with a Millipore (Milli-Q UF+) system to a resistivity of 18 MΩ/cm.

2.2. Lateral surface pressure versus mean molecular area isotherms

Force-area isotherms of the pure or mixed monolayers were obtained with a computer controlled KSV 3000 surface barostat (KSV Instruments, Helsinki). Data were collected by proprietary software from KSV Instruments. The isotherms were recorded at 22°C. Stock solutions of the lipids were made up in hexane/2-propanol and immediately used after mixing. The lipid solution was spread with a Hamilton syringe on a sub-phase of 140 mM NaCl in a rectangular teflon
tough. The monolayer was compressed at a barrier speed not exceeding 8 Å²/molecule min, and data were sampled every 2 s. At least three different runs were performed at each lipid composition and the error of reproducibility was less than 5%. The compressibilities of the pure or mixed lipid monolayers were calculated as described previously [15].

2.3. Cholesterol oxidation in mixed monolayers

The oxidation of monolayer cholesterol (in pure or mixed monolayers) was performed as described previously [12]. The assay temperature was 22°C and the enzyme concentration used was 30 mU/ml. The oxidation of monolayer cholesterol by cholesterol oxidase was carried out at a lateral surface pressure of 20 mN/m, since the catalytic reaction has been observed to be the optimal at this surface pressure [16]. The concentrations of phospholipid stock solutions were determined according to Bartlett [17].

2.4. Fluorescence visualization of monolayer films

Visualization of monolayers at the air/water interface was performed with a KSV Minisystems surface barostat mounted on an Olympus IMT-2 epifluorescence microscope. The surface barostat had a rectangular teflon trough equipped with a centrally located 50 mm diameter quartz window. Extra long-working distance objectives (Olympus 10× and 40×) were used to view the monolayer through the quartz window. The mixed monolayers (having a 0.9:1.0 molar ratio of cholesterol to phospholipid) also included 0.5 mol% of NBD-cholesterol as a fluorophore. The monolayers were compressed (8 Å²/molecule/min) to 20 mN/m at which pressure micrographs were taken using Kodak TMax 3200 ASA b/w film (1/4 s shutter speed). The film sensitivity was further pressed to 25 000 ASA during development.

3. Results

3.1. Pure phosphatidylcholine monolayers

Force-area isotherms of pure phosphatidylcholine at the air/water interface showed that shorter chain phosphatidylcholines were more expanded (at 22°C) than longer chain phosphatidylcholines (Fig. 1A), in good agreement with previously reported isotherms [18]. Because the isotherms of the short chain phosphatidylcholines were liquid expanded, whereas the long chain phosphatidylcholines were liquid condensed, there was also a difference in monolayer compressibility as a function of phosphatidylcholine chain length (Fig. 1B). Di-15-PC had a very high compressibility at 20 mN/m, because its lateral phase transition (expanded to condensed) ended near this lateral pressure. Phosphatidylcholines with acyl chains shorter than 15 carbons had compressibilities near 0.013 mN/m, whereas phosphatidylcholine longer than 15 carbons had calculated compressibilities between 0.0025 and 0.005 mN/m (Fig. 1B).

3.2. Mixed cholesterol/phosphatidylcholine monolayers

To examine how the length of the phosphatidylcholine acyl chains affected the mean molecular
areas in mixed cholesterol/phosphatidylcholine monolayers, force-area isotherms of the monolayers at three different compositions (0.9:1, 1:1 and 1.5:1 C/PL) were obtained. The observed mean molecular areas at a lateral surface pressure of 20 mN/m are shown in Fig. 2 as a function of phosphatidylcholine acyl chain length. The mean molecular area decreased with increasing phosphatidylcholine acyl chain length, at all C/PL ratios examined. Surprisingly, the mean molecular area at any C/PL differed only by about 2 Å² even though the phosphatidylcholine acyl chain length varied between 12 and 20 carbons. Only di-10-PC showed a markedly higher mean molecular area at 0.9 and 1:1 C/PL.

From calculations of mixed monolayer compressibilities at 20 mN/m, it is evident that the short chain phosphatidylcholines again gave more expanded force-area isotherms compared to the longer chain analogues (Fig. 3). The compressibil-
ty decreased dramatically with phosphatidylcholine acyl chain lengths going from 10 to 14, whereafter the change in compressibility as a function of phosphatidylcholine acyl chain length diminished. The calculated compressibilities also decreased with increasing cholesterol concentrations in the mixed monolayer.

3.3. Availability for enzyme-catalyzed oxidation of cholesterol

In order to examine the availability for oxidation of cholesterol in mixed monolayers as a function of phosphatidylcholine acyl chain length, we exposed the monolayers to cholesterol oxidase at 22°C. The average oxidation rate in a given mixed monolayer increased as a function of the C/PL ratio (Fig. 4). However, the average oxidation rate also differed as a function of phosphatidylcholine acyl chain length. Cholesterol was more readily susceptible to oxidation in mixed monolayers containing very short or very long acyl chain phosphatidylcholines (10–12 and 18–20 carbons), whereas cholesterol was not readily susceptible to oxidation (with the cholesterol oxidase concentration used!) in monolayers containing phosphatidylcholine with 14–17 carbon acyl chains (at 0.9:1 C/PL; Fig. 4). This oxidation trend was seen with all three C/PL ratios examined.

3.4. Surface texture of mixed monolayers

In order to assess whether separate lateral domains existed in the various phosphatidylcholine mixed monolayers, epifluorescence microscopy examination of the membranes was undertaken. This technique of epifluorescence microscopy has successfully been used to directly visualize the existence of lateral domains in pure and mixed monolayers [19–21]. Mixed monolayers containing cholesterol and different phosphatidylcholines (at 0.9:1 C/PL) were prepared to include 0.5 mol% of NBD-cholesterol, and observed with epifluorescence microscopy at a lateral surface pressure of 20 mN/m. If there is (macroscopic) lateral heterogeneity in the plane of the monolayer, a suitable lipid fluorophore will partition differently into condensed and expanded lateral phases, thus giving the monolayer a heterogeneous texture [22]. The micrographs shown in Fig. 5 are representative of the dominating surface texture observed in different monolayers at 20 mN/m. Mixed monolayers of di-10-PC and di-12-PC contained numerous bright circular (liquid) drops (Fig. 5, panels a and b). Monolayers of cholesterol and di-14-PC also contained bright drops (panel c), but to a much lesser extent as compared with the shorter phosphatidylcholines. The monolayers containing di-15-PC, di-16-PC, and di-17-PC (panels d, e, and
Fig. 5. Surface morphology of mixed monolayers containing cholesterol and phosphatidylycerine at 20 mN/m. The mixed monolayers were prepared to contain 46.9 mol% cholesterol, 0.5 mol% NBD-cholesterol and 52.6 mol% of phosphatidylycerine. The monolayer was compressed to 20 mN/m and photographed. The panels are as follows: a, di-10-PC; b, di-12-PC; c, di-14-PC; d, di-15-PC; e, di-16-PC; f, di-17-PC; g, di-18-PC and h, di-20-PC. The bar represents 150 μm.
f) were more or less homogeneously dark. With di-18-PC and di-20-PC, the mixed monolayers again began to contain bright areas (drop-like in di-18-PC and both drop-like and rough-edged in di-20-PC) which were enriched in NBD-cholesterol.

4. Discussion

The objective of this monolayer study was to compare how well cholesterol interacted with phosphatidylcholines having symmetric but variable-length saturated acyl chains. The relative strength of the interaction between cholesterol and the various phosphatidylcholines was assessed using cholesterol oxidase as a probe. The assumption we have made, i.e. that a strong or tight interaction between cholesterol and a co-lipid will decrease the oxidation susceptibility of cholesterol in membranes, has been supported in many previous studies from this laboratory [11–14]. We believe that intermolecular forces, which prevent or retard cholesterol desorption from membranes also retard or prevent the oxidation of membrane-bound cholesterol by cholesterol oxidase at the water/lipid interface. Such intermolecular forces include the van der Waals' interactions, which are stronger between cholesterol and saturated (phospholipid) acyl chains than between the sterol and unsaturated acyl chains [7]. Hydrogen bonding between molecules may also stabilize intermolecular interactions in membranes [6,8].

The oxidation results of this study show that cholesterol appeared to interact most favorably with phosphatidylcholines having acyl chain length between 14 and 17. With these monolayers (at different C/PL molar ratios) the average oxidation rate was lowest. This acyl chain dependence of cholesterol oxidation, and hence cholesterol/acyl chain interaction, apparently relates to how well cholesterol can align with acyl chains of different lengths. Previous bilayer studies using X-ray diffraction methods have demonstrated that cholesterol, at a C/PL of 0.5:1 with saturated symmetric length phosphatidylcholines, increases the bilayer thickness for phosphatidylcholines containing 12–16 carbons per chain, whereas it reduces the bilayer thickness for di-18-PC [23]. This result was interpreted to show that cholesterol, by interacting with di-12-PC to di-16-PC either removed the chain tilt or increased the trans conformations of the chains. With the di-18-PC bilayer, cholesterol apparently decreased the bilayer thickness because the long acyl chains of di-18-PC extended beyond the entire length of cholesterol, and therefore had to deform or kink in order to accommodate the shorter cholesterol molecule [23]. In that same study it was observed that in di-16-PC bilayers, the terminal methyl of the cholesterol side chain apparently was localized near the center of the bilayer, indicating that the length of di-16-PC best matched the length of cholesterol. Although bilayer membranes markedly differ from monolayer membranes, because the latter lack an opposite membrane leaflet and therefore the acyl chains have to project into the gas phase, similarities in cholesterol/phospholipid interactions probably exist between the two systems.

It is likely that the length of cholesterol matches the length of di-16-PC also in the monolayer system. Therefore, maximal opportunities for van der Waals’ interactions between these two lipids should be possible. This in turn should decrease the oxidation susceptibility of cholesterol [12], which actually is observed. When cholesterol is mixed with shorter chain phosphatidylcholines (i.e. di-10-PC and di-12-PC) there is markedly increased susceptibility to cholesterol oxidase-catalyzed oxidation of membrane cholesterol. The higher oxidation susceptibility is probably due to reduced opportunities for van der Waals’ interactions between cholesterol and the shorter chain phosphatidylcholines, since the length of the cholesterol molecule (including its isooyl side chain) apparently extends beyond the length of the phosphatidylcholine acyl chains. The interpretation that there is less van der Waals’ interactions between short-chain acyl chains, and between cholesterol and short-chain acyl chains is supported by the observation that the monolayers of short chain phosphatidylcholine are more expanded and have larger compressibility values (with or without cholesterol at 0.9:1 C/PL) (see also [18] and [24]).

Interestingly, the susceptibility of cholesterol to oxidation by cholesterol oxidase was markedly higher in long chain phosphatidylcholine (di-18-PC and di-20-PC) monolayers than it was with in-
intermediate length phosphatidylecholine monolayers (i.e. di-14-PC to di-17-PC). It is possible that the solubility (or miscibility) of cholesterol in the long chain phosphatidylecholines was diminished relative to the case with shorter chain phosphatidylecholines. A lower solubility of cholesterol in the condensed phospholipid phase would be expected to result in the formation of cholesterol-rich domains, which would be consistent with the oxidation results and with the monolayer surface texture observations. The mismatch in phosphatidylecholine acyl chain length and the length of the cholesterol molecule could in part give rise to the formation of cholesterol-rich domains. In an analogous bilayer system with a mismatch between the lengths of phospholipid acyl chains and cholesterol, McIntosh [23], suggested that distal segments of the long acyl chains were forced to bend or kink in order to accommodate the shorter cholesterol molecule. Such unfavorable packing properties may influence the strength of interaction between cholesterol and adjacent acyl chain segments in the monolayer, and may lead to the partitioning of at least a fraction of the cholesterol molecules into thermodynamically more favorable lateral domains.

The amount of cholesterol that different phospholipid membranes can solubilize varies. Phosphatidylecholine model membranes have been shown to dissolve equimolar amounts of cholesterol [12,25,26], although metastable membranes having a C/PL molar ratio above 1 can be prepared [27–29]. Sphingomyelin appears to have a greater capacity to dissolve cholesterol as compared with phosphatidylecholines [12]. In the present study, one set of phosphatidylecholine mixed monolayers had a C/PL of 0.9. At this C/PL the monolayers were consequently almost saturated with respect to cholesterol. Using epifluorescence microscopy to examine the lateral heterogeneity (or homogeneity) of the monolayer, as deduced from the partitioning of NBD-cholesterol among possible lateral phases, clear signs of lateral heterogeneity were observed from one monolayer type to the next. A heterogeneity of the partitioning of NBD-cholesterol occurs if laterally condensed and expanded phases coexist, because the fluorophore is excluded from condensed phases and forced into more loosely packed domains [21,22,30]. Di-10-PC and di-12-PC mixed monolayers had numerous droplike, probe-including lateral domains. The existence of these loosely packed (i.e. expanded) domains in the short chain phosphatidylecholine monolayers is consistent with the observed higher compressibility in these membranes, and also with the finding that cholesterol in these membranes was readily accessible to oxidation by cholesterol oxidase.

The monolayer surface texture in membranes containing phosphatidylecholine with acyl chains longer than or equal to 14 carbons per chain was almost exclusively homogenous when examined with epifluorescence microscopy. The cholesterol molecules appeared to interact so tightly with the phosphatidylecholines that the NBD-cholesterol probe was excluded from the cholesterol-rich phase. The surface texture of our cholesterol/di-14-PC and di-16-PC mixed monolayers (at about 47 mol% cholesterol, and 20 mN/m) are very different from those reported by Subramaniam and McConnell for cholesterol/di-14-PC [31], and Rice and McConnell for cholesterol/di-16-PC [32], because our monolayers have a very high level of cholesterol saturation compared to the cholesterol concentration they examined (varied between 10 and 30 mol%), and because we visualize our monolayers at the fairly high surface pressure of 20 mN/m. In our mixed monolayers with phosphatidylecholine having still longer acyl chains, droplets containing NBD-cholesterol were occasionally observed, but the fields of view which included probe-rich droplets were few (maybe one field out of 10–15 examined). However, with di-18-PC and di-20-PC, the NBD-cholesterol-rich droplets became more numerous, and with di-20-PC occasional large rough-edged areas containing the fluorophore were seen. It is possible that these NBD-cholesterol containing lateral domains are indicative of lateral phase separation within these long chain phosphatidylecholine monolayers. Although NBD-cholesterol would not very likely be dissolved in a pure di-18-PC or di-20-PC phase, and does not dissolve in a pure cholesterol phase [33], it is possible that the probe would localize at the interface between two pure phases where lateral packing defects most likely would occur. The
formation of a sterol-enriched lateral phase would be in line with the observed increased cholesterol oxidation susceptibility in di-18-PC and di-20-PC monolayers, since sterol-enriched or pure sterol domains are good substrates for cholesterol oxidases [12,13].

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References