Determination of Membrane Cholesterol Partition Coefficient Using a Lipid Vesicle–Cyclodextrin Binary System: Effect of Phospholipid Acyl Chain Unsaturation and Headgroup Composition

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ABSTRACT Lateral domain or raft formation in biological membranes is often discussed in terms of cholesterol–lipid interactions. Preferential interactions of cholesterol with lipids, varying in headgroup and acyl chain unsaturation, were studied by measuring the partition coefficient for cholesterol in unilamellar vesicles. A novel vesicle–cyclodextrin system was used, which precludes the possibility of cross-contamination between donor–acceptor vesicles or the need to modify one of the vesicle populations. Variation in phospholipid headgroup resulted in cholesterol partitioning in the order of sphingomyelin (SM) > phosphatidylserine > phosphatidylcholine (PC) > phosphatidylenthanolamine (PE), spanning a range of partition ΔG of −1181 cal/mol to +683 cal/mol for SM and PE, respectively. Among the acyl chains examined, the order of cholesterol partitioning was 18:0(stearic acid),18:1n-9(oleic acid) PC > di18:1n-9PC > di18:1n-12(petroselenic acid) PC > di18:2n-6(linoleic acid) PC > 16:0(palmitic acid),22:6n-3(DHA) PC > di18:3n-3(α-linolenic acid) PC > di22:6n-3PC with a range in partition ΔG of 913 cal/mol. Our results suggest that the large differences observed in cholesterol–lipid interactions contribute to the forces responsible for lateral domain formation in plasma membranes. These differences may also be responsible for the heterogeneous cholesterol distribution in cellular membranes, where cholesterol is highly enriched in plasma membranes and relatively depleted in intracellular membranes.

INTRODUCTION

Rafts are lateral microdomains in plasma membranes that are characterized by an enrichment with cholesterol and sphingolipids (Simons and Ikonen, 1997; Kurzchalia and Parton, 1999; Brown and London, 2000; Fielding and Fielding, 2000). The physical forces leading to raft formation are not yet clear, though they are likely to include the high affinity of sphingolipids for cholesterol. A variety of techniques indicate that cholesterol interacts differentially with different types of lipids (van Dijck et al., 1976; Demel et al., 1977; Fugler et al., 1985; Stillwell et al., 1994; Mitchell and Litman, 1998; Polozova and Litman, 2000). However, quantitative measurements of the differential lipid–cholesterol interactions, as measured by membrane partitioning, are scarce due to the difficulty of making such measurements (Nakagawa et al., 1979; Wattenberg and Silbert, 1983; Yeagle and Young, 1986; Leventis and Silvius, 2001). The typical experimental approach to quantify lipid–cholesterol affinity is to determine the cholesterol partitioning between two lipid vesicles of different lipid compositions (Nakagawa et al., 1979; Wattenberg and Silbert, 1983; Yeagle and Young, 1986; Leventis and Silvius, 2001). The characteristics of this method are a low rate of cholesterol exchange (Nakagawa et al., 1979; Wattenberg and Silbert, 1983; Yeagle and Young, 1986) and a difficulty in separating donor from acceptor vesicles due to potential vesicle adhesion (Wattenberg and Silbert, 1983). Varying vesicle size (Yeagle and Young, 1986) or vesicle surface charge (Nakagawa et al., 1979; Wattenberg and Silbert, 1983; Leventis and Silvius, 2001) are common tactics used to facilitate the separation of donor from acceptor vesicles. However, complete separation of donor from acceptor vesicles remains a challenging issue. Recently, it was shown that the rate of cholesterol exchange between donor and acceptor vesicles was greatly improved by inclusion of methyl-β-cyclodextrin (CD) as an exchange catalyst (Leventis and Silvius, 2001). However, CD accelerates the rate of phospholipid exchange between the donor and acceptor vesicles, as well as cholesterol, which imposes a limitation on this method.

We have developed an alternative method, which both enhances the rate of cholesterol exchange and eliminates the need to separate donor from acceptor vesicles. This method relies on thermodynamic principle to determine the cholesterol exchange reaction between two vesicles using CD as a common reference state (see Scheme 1). CD is widely used to manipulate membrane cholesterol levels because of the high rate of cholesterol exchange between CD (Ohvo-Rekila et al., 2000; Leventis and Silvius, 2001; Niu et al., 2002) and membranes, and the ability to quantitatively separate CD from membranes (Christian et al., 1997; Gimpl et al., 1997; Rodal et al., 1999; Sooksawate and Simmonds, 2001; Niu et al., 2002). Our previous study (Niu et al., 2002)

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Abbreviations used: 16:0, hexadecanoic acid or palmitic acid; 18:0, octadecanoic acid or stearic acid; 18:1n-9, 9-octadecenoic acid or oleic acid; 18:1n-12, 6-octadecenoic acid or petroselenic acid; 18:2n-6, 9,12-octadecadienoic acid or linoleic acid; 18:3n-3, 9,12,15-octadecatrienoic acid or α-linolenic acid; 22:6n-3, docosahexaenoic acid or DHA.

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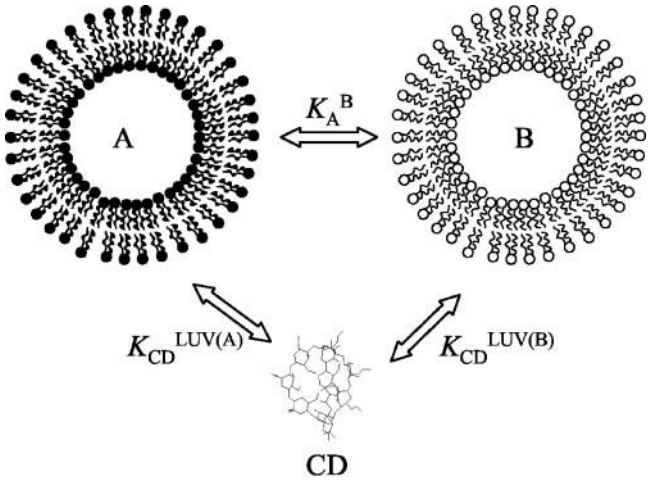

SCHEME 1 Scheme of the binary CD–LUV system used in this study for measuring cholesterol partition coefficients. A and B represent two large unilamellar vesicle populations, LUV(A) and LUV(B), and CD represents the CD-cholesterol complex. K_A^B is the equilibrium partition coefficient for cholesterol between LUV(A) and LUV(B), while $K_{CD}^{LUV(A)}$ and $K_{CD}^{LUV(B)}$ are the equilibrium partition coefficients for cholesterol between LUV(A) and CD and LUV(B) and CD, respectively. The usual method of measuring K_a^B involves mixing LUV(A) with LUV(B), allowing cholesterol to reach equilibration between vesicles, separating LUV(A) from LUV(B), and measuring the equilibrium concentrations of cholesterol in the two LUV populations. The binary CD–LUV system introduced here used CD as a reference state and reports the relative cholesterol partition coefficient of a given phospholipid to CD, K_{CD}^{LUV}. The K_A^B is then derived from the individual $K_{CD}^{LUV(A)}$ and $K_{CD}^{LUV(B)}$ using thermodynamic relationships.

and the results from this study show that cholesterol exchange between CD and membranes follows an equilibrium partition model. By determining the partition coefficients for cholesterol (K_{CD}^{LUV}) in systems composed of CD-vesicle A ($K_{CD}^{LUV(A)}$) and CD-vesicle B ($K_{CD}^{LUV(B)}$), the partition coefficient of cholesterol between vesicle A and vesicle B (K_A^B) can be calculated from the ratio of $K_{CD}^{LUV(B)}$ to $K_{CD}^{LUV(A)}$.

In this study, the cholesterol partition coefficient and partition free energy were determined for a variety of phospholipids with varying headgroup or acyl chain composition. Our results show that both phospholipid headgroup and acyl chain modulate phospholipid–cholesterol interaction. Among the phospholipids examined in this study, 16:0-SM (sphingomyelin) had the highest cholesterol partitioning, whereas 16:0,18:1n-9PE (phosphatidylethanolamine) had the lowest cholesterol partitioning. Such differences in lipid–cholesterol partitioning are expected to play an important role in regulating membrane cholesterol distribution and may explain the nonhomogenous distribution of cholesterol in cell membranes or the formation of cholesterol-rich microdomains in plasma membranes.

MATERIALS AND METHODS

Materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) with purity > 99%. *N*-palmitoyl-*d*-sphingomyelin (16:0-SM), cholesterol and CD were from Sigma (St. Louis, MO). CD–cholesterol complex was prepared as previously described (Niu et al., 2002). Microcon YM-30 filters were from Millipore (Millipore Co., Bedford, MA). Cholesterol CII kit and phospholipids B kit were from Wako (Wako Chemicals U.S.A., Inc., Richmond, VA).

Large unilamellar vesicles (LUV) preparation

All lipid samples were prepared in an argon-filled glove box. Large unilamellar vesicles (LUVs) were prepared as described (Mayer et al., 1986) with slight modification. Briefly, chloroform was removed from phospholipids under a stream of argon, and cyclohexane containing 25 μM butylated hydroxytoluene (BHT) was added to the dried phospholipids to yield a lipid-to-BHT ratio of 500:1 to prevent lipid oxidation. Samples were frozen on dry ice and lyophilized under vacuum for 4 h to remove solvent. The fluffy white powder formed after lyophilization was dissolved in pH 7.0 PIPES buffer containing 10 mM PIPES and 50 μM DTPA. The phospholipid solutions were then extruded by 11 passes each through 0.4- and 0.1-μm polycarbonate filters, using a Lipex extruder (Vancouver, BC, Canada), forming LUVs with a mean diameter of 100 nm.

Cholesterol and phospholipid assay

All assays were modified to be run in a 96-well microplate format. Cholesterol was determined by an enzymatic colorimetric method (Allain et al., 1974) using Cholesterol CII kit (Wako Chemicals U.S.A., Inc., Richmond, VA). The microplate-adapted method has a resolution on the order of 0.1 μg cholesterol per well. Total phospholipids were determined by the method of Barlett (1959). Choline was determined using Phospholipids B kit from Wako.

Time-course of cholesterol exchange between LUVs and CD

The time course of cholesterol exchange between LUVs and CD was carried out to determine the minimal time required for the system to reach equilibration. Briefly, 1 mM LUVs were mixed with 10 mM CD containing 0.5 mM cholesterol and incubated at 37°C on a shaker. Aliquots of 200 μl in triplet were removed from the sample mixture on an hourly basis and filtered through Microcon YM-30 membrane in a temperature-regulated microcentrifuge (Eppendorf 5417R, Hamburg, Germany) at 1300 × *g* for 15 min, at 37°C. This separates cholesterol-donor CD (2 nm in diameter) from acceptor LUVs (~ 100 nm in diameter) based on size difference. The filter was washed with PIPES buffer three times and the LUVs on the filter were resuspended in 200 μl buffer. The initial filtrate and the resuspended sample were assayed for cholesterol and phospholipid as described above. Control experiments were conducted to determine whether the LUVs go through the YM-30 membrane or CD–cholesterol is retained on the membrane. When 200 μl of either an LUV or CD–cholesterol sample was filtered through a Microcon YM-30 membrane, the filtrate of the LUV sample contained no phospholipid, demonstrating that LUVs do not pass through a YM-30 membrane, whereas no CD–cholesterol was retained on YM-30 membrane, as demonstrated by the identical concentration of cholesterol detected in the filtrate of the CD–cholesterol sample and in the original sample (data not shown).

Phospholipid extraction by CD

To determine whether any phospholipids were extracted by CD as reported in our previous study (Niu et al., 2002), we incubated the LUVs with various concentrations of CD at 37°C for 2 hr and separated the LUVs from CD by membrane filtration as described above. The

filtrate was assayed for phospholipid and the amount of phospholipid extracted by CD was determined.

Cholesterol partition coefficient measurement

Cholesterol partition coefficients (K_{CD}^{LUV}) were determined in the LUV–CD binary system, which contained 1 mM LUVs and 10 mM CD loaded with varying amounts of cholesterol (0 to 1.0 mM). Briefly, LUVs and CD–cholesterol complex were incubated at 37°C on a shaker for 2 h to allow cholesterol to equilibrate between donor and acceptor. LUVs and CD were then separated using the filtration method described above, and the cholesterol concentration in the filtrate, which is the equilibrium concentration of cholesterol complexed to CD, was determined by the cholesterol assay. The partition coefficient of cholesterol between LUV and CD, K_{CD}^{LUV}, was derived according to the following analysis.

Data analysis

K_{CD}^{LUV} was calculated using the equation,

$$K_{CD}^{LUV} = \frac{[ch]_{LUV}/[LUV]}{[ch]_{CD}/[CD]} = \frac{[ch]_{LUV}}{[ch]_{CD}} * \frac{[CD]}{[LUV]}, \quad (1)$$

where $[ch]_{CD}$ is the equilibrium concentration of cholesterol in CD, $[ch]_{LUV}$ is the equilibrium concentration of cholesterol in the LUVs, [LUV] is the concentration of phospholipids in LUVs, and [CD] is the concentration of CD. Eq. 1 can be rearranged as

$$[ch]_{LUV} = \frac{K_{CD}^{LUV} * [LUV]/[CD]}{1 + K_{CD}^{LUV} * [LUV]/[CD]} * [ch]_{tot}, \quad (2)$$

where $[ch]_{tot}$ is the initial donor cholesterol concentration that is the sum of $[ch]_{LUV}$ and $[ch]_{CD}$. K_{CD}^{LUV} is then derived from the plot of $[ch]_{LUV}$ versus $[ch]_{tot}$.

The partition coefficient (K_A^B) of cholesterol between two LUVs was derived from $K_{CD}^{LUV(A)}$ and $K_{CD}^{LUV(B)}$ as

$$K_A^B = \frac{K_{CD}^{LUV(B)}}{K_{CD}^{LUV(A)}}. \quad (3)$$

The partition free energy of cholesterol between LUV(A) and LUV(B), ΔG, was derived accordingly, which is $\Delta G = -RT \ln K_A^B$, where R is the gas constant, and T is the temperature of the measurement in Kelvin.

RESULTS

Cholesterol equilibration between LUVs and CD

The time course of cholesterol transfer from CD to LUVs is shown in Fig. 1, where the CD–cholesterol complex served as cholesterol donor and LUVs, consisting of 16:0,18:1n-9PC (phosphatidylcholine), as cholesterol acceptors. The initial cholesterol concentration in donor and acceptor were 0.5 and 0 mM, respectively. Incubation of CD–cholesterol with LUVs resulted in cholesterol transfer from CD to LUVs as shown by the reduced cholesterol concentration in CD and increased cholesterol concentration in the LUVs. Equilibrium of cholesterol exchange between CD and LUVs was established within the first hour of incubation, as demonstrated by the constant levels of cholesterol in CD and LUVs at extended incubation times. The sum of the cholesterol concentrations measured in CD and LUVs was equal to the initial total cholesterol concentration in the donor, indicating that cholesterol only partitioned between LUVs and CD and that no cholesterol loss due to precipitation occurred.

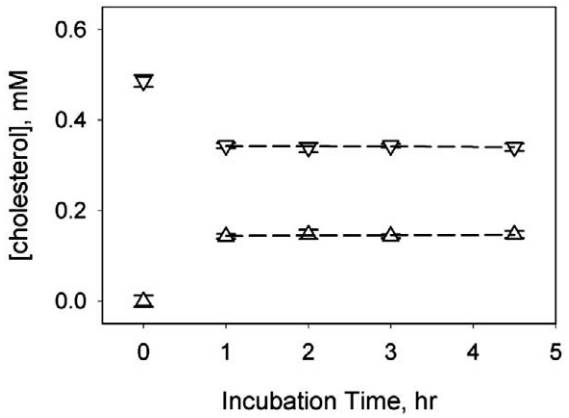

FIGURE 1 Time course of cholesterol exchange between CD and LUVs. LUVs (1 mM), consisting of 16:0,18:1n-9 PC, were mixed with 0.5 mM cholesterol/10 mM CD complex at 37°C on a shaker. Aliquots of 200-μl samples in triplet were removed on an hourly basis and filtered, using a Microcon YM-30 membrane. Cholesterol concentrations were determined in CD (∇) and LUVs (Δ) at various times. Concentrations shown at time 0 were the initial concentrations of cholesterol in CD and LUVs, which were 0.5 mM and 0 mM, respectively.

Phospholipid extraction by CD

Our previous study (Niu et al., 2002) showed that, at high concentrations, CD could extract a significant amount of phospholipid from rod outer-segment disk membranes. This suggests that using high concentrations of CD may perturb membrane structure due to the loss of phospholipid. Here, we have examined whether CD can extract phospholipids from LUVs. When the CD concentration is below 20 mM, the amount of phospholipids extracted by CD was less than 3% (Fig. 2). For example, in the presence of 10 mM CD, which was the selected concentration for partition measurements in this study, only 0.2% of phospholipids was extracted, thus the impact on membrane structure is minimal. However, at higher CD concentrations, significant amounts of phospholipids were extracted from LUVs, consistent with our previous observation (Niu et al., 2002). The percentage of phospholipid extracted by CD exhibited nonlinear behavior, increasing rapidly at higher CD concentrations. In the presence of 30 mM CD, 9.0% of phospholipids were found in CD, whereas the amount of phospholipids detected in CD increased to 44.8% at 50 mM CD.

Measurement of cholesterol partition coefficient

Because equilibrium of cholesterol transfer between CD and LUV was rapidly established, the partition coefficient K_{CD}^{LUV}

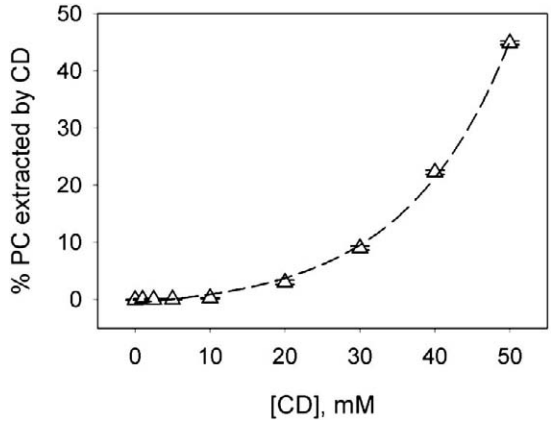

FIGURE 2 PC extraction by CD. LUVs (1 mM), consisting of 16:0,18:1n-9 PC, were incubated with various concentrations of CD at 37°C for 2 hr. LUVs and CD were separated by filtration through a Microcon YM-30 membrane. PC concentration was determined in the filtrate and the percentage of PC extracted by CD was calculated on the bases of the initial LUV concentration.

can be readily determined by measuring the equilibrium concentration of cholesterol in CD or in LUVs. An example of such a measurement using CD and LUVs consisting of 16:0,18:1n-9PC, at several donor cholesterol concentrations, is shown in Fig. 3. Varying the donor cholesterol concentration resulted in a linear increase of the equilibrium cholesterol concentration in LUVs, which is consistent with the prediction of the equilibrium partition model. The derived K_{CD}^{LUV} value was 6.7 ± 0.5 in favor of LUVs. The same method was applied to obtain K_{CD}^{LUV} for all phospholipids included in this study.

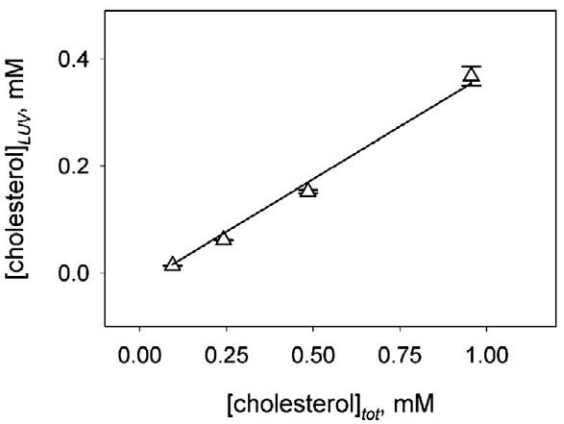

FIGURE 3 Cholesterol partition coefficient (K_{CD}^{LUV}) determination. LUVs (1 mM), consisting of 16:0,18:1n-9 PC, were incubated with 10 mM CD containing various amounts of cholesterol at 37°C for 2 hr. The equilibrium concentration of cholesterol in the LUVs was determined and plotted against the total cholesterol concentration in the sample mixture. The dashed line is the fitted line according to Eq. 2, and K_{CD}^{LUV} was determined accordingly.

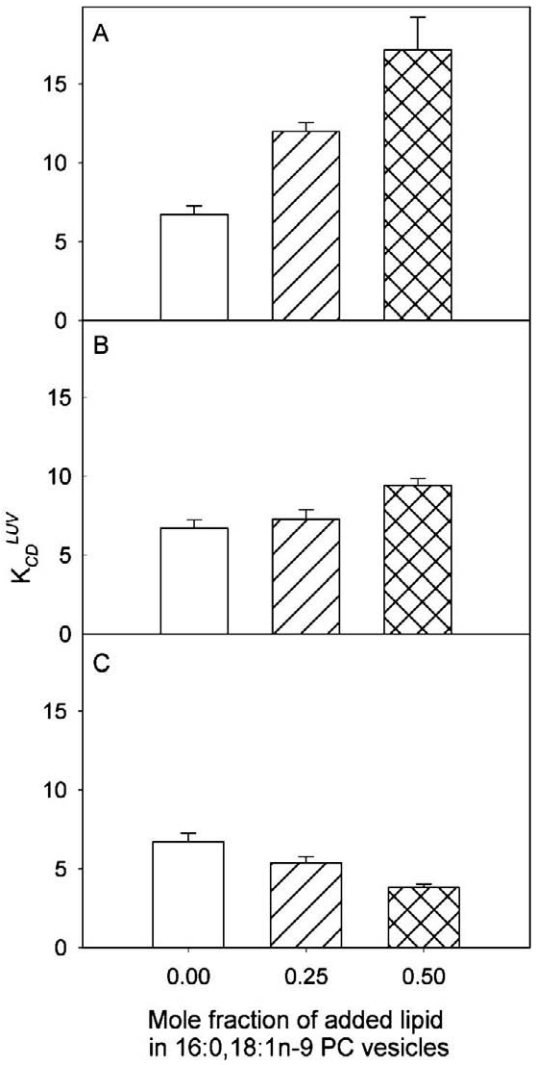

FIGURE 4 Effect of headgroup compositions on K_{CD}^{LUV}. K_{CD}^{LUV} values were measured for LUVs containing 16:0,18:1n-9 PC and 0 (*open bar*), 0.25 (*diagonal bar*), and 0.5 (*cross-hatched*) mole fraction of (*A*) 16:0-SM, (*B*) 16:0,18:1n-9 PS, and (*C*) 16:0,18:1n-9 PE under the same condition as described in Fig. 3.

Effect of lipid headgroup on membrane cholesterol partitioning

To examine the effect of lipid headgroup on membrane cholesterol partitioning, four major classes of phospholipids, PC, PE, phosphatidylserine (PS), and SM with identical or near-identical acyl chains were included in this study. The acyl chains in PC, PE, and PS are 16:0 in *sn*-1 and 18:1n-9 in *sn*-2 positions, whereas the amide-linked acyl chain in SM is 16:0. Inclusion of 0.25 and 0.5 mole fraction of 16:0-SM in 16:0,18:1n-9 PC LUVs resulted in ~80% and 160% increases of K_{CD}^{LUV} (Fig. 4 *A*), whereas inclusion of 0.25 and 0.5 mole fraction of 16:0,18:1n-9 PE in 16:0,18:1n-9 PC LUVs resulted in ~20% and 43% decreases of K_{CD}^{LUV} (Fig. 4 *C*). Inclusion of 16:0,18:1n-9 PS in 16:0,18:

TABLE 1 Summary of the effect of lipid composition on the cholesterol partition coefficient (K_A^B) and partition free energy (ΔG)*

LUV Composition	K_A^B	ΔG (cal/mol)	Published K_A^B
Head Group Composition			
16:0,18:1n-9 PC	1	0	
16:0-SM/16:0,18:1n-9 PC (25:75)	1.78 (±0.23)	−356 (±46)	
16:0-SM/16:0,18:1n-9 PC (50:50)	2.56 (±0.51)	−580 (±116)	
16:0-SM	6.78 (±0.88)	−1181 (±156)	
16:0,18:1n-9 PS/16:0,18:1n-9 PC (25:75)	1.09 (±0.18)	−53 (±9)	
16:0,18:1n-9 PS/16:0,18:1n-9 PC (50:50)	1.40 (±0.19)	−208 (±28)	
16:0,18:1n-9 PS	1.91 (±0.58)	−400 (±123)	
16:0,18:1n-9 PE/16:0,18:1n-9 PC (25:75)	0.80 (±0.13)	138 (±22)	
16:0,18:1n-9 PE/16:0,18:1n-9 PC (50:50)	0.57 (±0.08)	347 (±49)	0.83†
16:0,18:1n-9 PE	0.33 (±0.04)	683 (±88)	
Acyl Chain Composition			
18:0,18:1n-9 PC	1.40 (±0.11)	−207 (±19)	1.96‡
di18:1n-9 PC	1	0	
di18:1n-12 PC	0.67 (±0.13)	243 (±47)	
di18:2n-6 PC	0.55 (±0.04)	369 (±28)	0.61‡
di18:3n-3 PC	0.51 (±0.04)	416 (±30)	
16:0,22:6n-3 PC	0.54 (±0.10)	377 (±30)	
di22:6n-3 PC	0.32 (±0.039)	706 (±87)	

*All values were measured at 37°C. K_A^B and ΔG associated with headgroup compositions were referenced to 16:0,18:1n-9 PC, whereas those with acyl chain compositions were referenced to di18:1n-9 PC.
†Calculated from Yeagle and Young (1986). Donor: SUV 16:0,18:1n-9 PC; acceptor: LUV 16:0,18:1n-9 PC/16:0,18:1n-9 PE (50:50 mol%)
‡From Leventis and Silvius (2001) using donor vesicles consisting of 18:0,18:1n-9 PC/18:0,18:1n-9 PG (85:15 mol%).

1n-9 PC LUVs showed a slight enhancement in K_{CD}^{LUV} (Fig. 4 *B*). The corresponding partition free energies, ΔG, which are normalized to 16:0,18:1n-9 PC, are summarized in Table 1. Cholesterol preferred to partition into SM and PS relative to PC as shown by the negative ΔG values, whereas cholesterol association with PE was unfavorable compared to PC as shown by the positive ΔG value. The extrapolated value of ΔG for partitioning between 16:0-SM LUVs and 16:0,18:1n-9 PC LUVs was −1181 cal/mol in favor of the 16:0-SM LUVs. In contrast, ΔG for partitioning between 16:0,18:1n-9 PE LUVs and 16:0,18:1n-9 PC LUVs is +683 cal/mol against the 16:0,18:1n-9 PE LUVs. The extrapolated value of ΔG for exchange between 16:0,18:1n-9 PS LUVs and 16:0,18:1n-9 PC LUVs was −400 cal/mol in favor of PS and intermediate between with 16:0-SM and 16:0,18:1n-9 PE. K_A^B values calculated using the extrapolated values of ΔG, which correspond to LUVs consisting of pure SM, PS, PC, or PE, are shown in Fig. 5. K_A^B is 6.78-fold higher in 16:0-SM and 1.91-fold higher in 16:0,18:1n-9 PS relative to 16:0,18:1n-9 PC, whereas K_A^B is 3-fold lower in 16:0,18:1n-9 PE relative to 16:0,18:1n-9 PC.

Effect of acyl chain composition on membrane cholesterol partitioning

Phospholipids with identical PC headgroup and varying acyl chain composition were chosen to determine how acyl chain composition affects membrane cholesterol partitioning. The relative cholesterol partition coefficients are shown in Fig. 6. K_A^B values were referenced to di18:1n-9 PC, which has a normalized value of 1. The K_A^B value for LUVs consisting of 18:0,18:1n-9 PC was 1.40 ± 0.11, which means that cholesterol partitioning is 40% higher as a result of replacing the *sn*-1 chain of di18:1n-9 PC with a saturated 18:0 chain. Using the same level of acyl chain unsaturation while shifting the double bond location, affected cholesterol partitioning as well. The K_A^B value between di18:1n-12 PC LUVs and di18:1n-9 PC LUVs was 0.67 ± 0.13, showing a 33% reduction in cholesterol partitioning by moving the double bond from n-9 to n-12 position. The level of acyl chain unsaturation also demonstrated a large effect on mem-

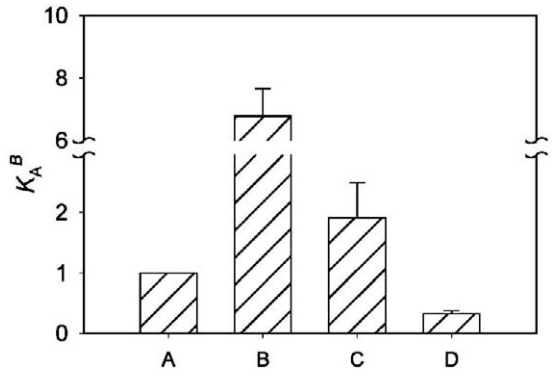

FIGURE 5 Summary of K_A^B as the function of headgroup composition. K_A^B values were reported relative to 16:0,18:1n-9 PC. The phospholipids contained in LUVs were (*A*) 16:0,18:1n-9 PC; (*B*) 16:0-SM; (*C*) 16:0,18:1n-9 PS; and (*D*) 16:0,18:1n-9 PE. The highest K_A^B value was observed in 16:0-SM, and the lowest in 16:0,18:1n-9 PE.

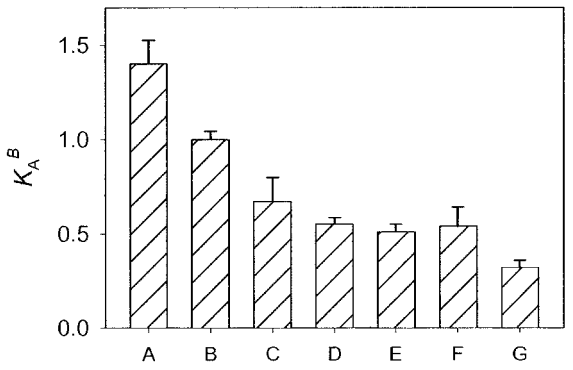

FIGURE 6 Summary of K_A^B as the function of acyl chain unsaturation. K_A^B values were reported relative to di18:1n-9 PC. The lipid compositions in the LUVs were: (*A*) 18:0,18:1n-9 PC; (*B*) di18:1n-9 PC; (*C*) di18:1n-12 PC; (*D*) di18:2n-6 PC; (*E*) di18:3n-3 PC; (*F*) 16:0,22:6n-3 PC; and (*G*) di22:6n-3 PC. Increasing the level of acyl chain unsaturation resulted in a reduction of K_A^B.

brane cholesterol partitioning. Addition of a second double bond at the n-6 position to both chains in di18:1n-9 PC to yield di18:2n-6 PC resulted in a 45% reduction in K_A^B. However, placement of a third double bond at the n-3 position in both chains of di18:2n-6 PC to yield 18:3n-3 PC only reduced K_A^B by 7%. The lowest K_A^B was observed in LUVs consisting of di22:6n-3 PC, the highest level of unsaturated lipid containing six double bonds in both chains. The difference of the corresponding ΔG values varied from -207 cal/mol for 18:0,18:1n-9 PC to $+706$ cal/mol for di22:6n-3 PC (Table 1). The addition of a saturated chain in the *sn*-1 position of di22:6n-3 to yield 16:0,22:6n-3 PC increased K_A^B by 69%.

DISCUSSION

Using LUV–CD pair to determine membrane cholesterol partitioning

This study introduces a novel method for measuring membrane cholesterol partition coefficients and associated partition free energy, which provides an alternative yet convenient way to probe cholesterol–phospholipid interactions. This method demonstrates several advantages over other methods currently in use.

1. Cholesterol exchange between CD and LUVs is rapid. Our results showed that cholesterol exchange between CD and LUV equilibrates within the first hour of incubation. A closer estimate of the halftime of cholesterol exchange between CD and LUVs is on the order of minutes (Ohvo-Rekila et al., 2000; Leventis and Silvius, 2001). This compares to the halftime of hours or longer for exchange between lipid membranes (Wattenberg and Silbert, 1983; Yeagle and Young, 1986). The enhanced rate of cholesterol exchange between CD and LUVs is associated with the reduced energy barrier for exchange (7–9 kcal/mol), which is much higher (20 kcal/mol) between lipid membranes (Yancey et al., 1996).
2. In the current method, separation of donor–acceptor pairs is convenient and complete. The size of LUVs and CD differ by more than 50 times, allowing separation of donor from acceptor by filtration. No alteration in vesicle property, such as varying vesicle size or inclusion of charged lipids in vesicles, is required for donor–acceptor separation.
3. Cross-contamination of lipids is eliminated. Because only one population of vesicles is present in the LUV–CD system, cross-contamination by vesicle adhesion or fusion between donor and acceptor vesicles is abolished.

In the few cases where there is overlap with the literature, K_A^B values derived from the LUV–CD system are comparable to published measurements (Table 1). However, subtle differences are expected considering the role of lipid charge and vesicle size, which were varied in the donor–acceptor vesicle methods previously used (Yeagle and Young, 1986; Leventis and Silvius, 2001) to study cholesterol partitioning. Such differences are evident in the data in Table 1. To be useful in explaining the nonhomogeneous distribution of cholesterol partitioning, ΔG needs to be accurate and consistent. Because of the simplicity of the current method, we expect that this method can be applied in a variety of systems to probe the differential lipid–cholesterol partitioning in a convenient and accurate manner. For example, this method can be applied to quantify the effect of acyl chain length or the effect of phase transition on cholesterol affinity where large effects are expected (Spink et al., 1996).

Effect of lipid composition on cholesterol–phospholipid partitioning

We examined the effect of phospholipid headgroup and acyl chain compositions on cholesterol partitioning in a variety of physiologically relevant phospholipids. The phospholipids selected in this study either contain identical or near-identical acyl chains with varying headgroup or an identical PC headgroup with varying acyl chain unsaturation, so the effects on cholesterol partitioning related to phospholipid headgroup and acyl chain composition can be isolated and quantified. Our results clearly differentiate the effects of phospholipid headgroup composition, level of unsaturation, and the location of double bond in phospholipid acyl chains on membrane cholesterol partitioning.

Among the lipid classes examined in this study, SM had the highest cholesterol affinity, characterized by a partition ΔG of -1181 cal/mol and a K_A^B of 6.78 relative to 16:0,18:1n-9 PC, Table 1. Among the various headgroups, 16:0,18:1n-9 PE exhibited the lowest cholesterol affinity with a partition ΔG of $+683$ cal/mol and a K_A^B of 0.33 relative to

16:0,18:1n-9 PC. Cholesterol partitioning in 16:0,18:1n-9 PS was found to be intermediate with a K_A^B of 1.91 relative to 16:0,18:1n-9 PC. This trend is in good agreement with qualitative observations using calorimetry and cholesterol exchange kinetics (Demel et al., 1977; Fugler et al., 1985; Bhuvaneswaran and Mitropoulos, 1986; Yeagle and Young, 1986; Leventis and Silvius, 2001) and limited K_A^B measurements (Yeagle and Young, 1986; Leventis and Silvius, 2001). The increased SM–cholesterol affinity is likely due to the hydrogen bond between the amide group in SM and the 3β-OH group in cholesterol, which is supported by the increased rate of cholesterol oxidation when the amide linkage in SM is replaced with a carbonyl ester, suggesting a reduction in the SM–cholesterol interaction (Bittman et al., 1994). The lower PE–cholesterol affinity could be explained by the intermolecular hydrogen bonds among PE headgroups (Sen et al., 1988; Shin et al., 1991), which would be disrupted by the inclusion of cholesterol and therefore energetically unfavored.

Among the PCs examined in this study, cholesterol partitioning followed the order of 18:0,18:1n-9 PC > di18:1n-9 PC > di18:1n-12 PC > di18:2n-6 PC > 16:0,22:6n-3PC > di18:3n-3 PC > di22:6n-3 PC, with a net difference in the partition ΔG of +913 cal/mol, corresponding to 4.4-fold difference in K_A^B. Phospholipid acyl chain–cholesterol interactions are dominated by van der Waals interactions, which are determined by acyl chain geometry and bond configuration. The presence of *cis*-double bond would distort the chain configuration, thus weaken the chain–cholesterol interaction. This is consistent with the observation of reduced cholesterol partitioning in phospholipids with increased unsaturation. A nuclear magnetic resonance (NMR) study (Stockton and Smith, 1976) and a structural model (Huang, 1977) suggest that the rigid sterol ring overlaps the first 9–10 acyl chain carbons, so the presence of *cis*-double bonds at the C9 position or closer to the carbonyl group would generate a kink in the acyl chain, thus weakening the chain–cholesterol interaction. This is consistent with the lower cholesterol partitioning in di18:1n-12 PC and di22:6n-3 PC, relative to di18:1n-9 PC. NMR (Huster et al., 1998) and fluorescence (Mitchell and Litman, 1998) studies suggest that cholesterol has the strongest interactions with saturated chains. This is borne out by the increased partitioning observed when the unsaturated *sn*-1 chain was substituted by a saturated chain in both di18:1n-9 PC and di22:6n-3 PC. However, the effect of the polyunsaturated chain is still present, as evidenced by the 2.6-fold higher partitioning of cholesterol in 16:0,18:1n-9 PC relative to 16:0,22:6n-3 PC.

Implications

Cholesterol distributes unevenly among cell membranes with enrichment in plasma membranes and depletion in intracellular membranes (Colbeau et al., 1971; Schroeder et al., 1976), across bilayer leaflets (Brasaemle et al., 1988; Casper and Kirschner, 1971), and laterally within plasma membranes forming microdomains or rafts (Simons and Ikonen, 1997; Brown and London, 2000). Paralleling the nonhomogeneous distribution of cholesterol is the asymmetric distribution of phospholipids in cell membranes (Colbeau et al., 1971; Keenan and Morre, 1970; Koval and Pagano, 1991). Our partition measurements, coupled with the high level of SM and saturated lipids in plasma membranes and relatively high level of PE and unsaturated lipids in intracellular membranes, are in accord with the observed enrichment of cholesterol in plasma membranes and are consistent with the higher cholesterol partition coefficients observed in plasma membranes relative to mitochondria membranes or endoplasmic reticulum membranes (Wattenberg and Silbert, 1983). The high affinity of cholesterol for SM is likely one of the driving forces for the formation of cholesterol-rich domains or rafts in plasma membranes. However, cholesterol-rich domains may also be formed in membranes containing lipids with large differences in cholesterol affinity. An example of this is the model membrane system consisting of cholesterol/di16:0 PC/di22:6n-3 PC/rhodopsin (Polozova and Litman, 2000). In this system, there is a large difference in the partition coefficient for cholesterol in di22:6n-3 PC and di16:0 PC, which manifests itself in the formation of di16:0 PC/cholesterol-rich and di22:6n-3PC/rhodopsin/cholesterol-depleted lateral domains. Similar considerations may be responsible of the reported domains in rod outer-segment disk membranes (Seno et al., 2001; Nair et al., 2002), where SM is a minor component. Several studies show an asymmetric transmembrane lipid distribution in plasma membranes, with a higher concentration of SM and PC in the outer leaflet and the more unsaturated PE and PS in the inner leaflets. Given that SM and PC are more saturated than PS and PE, one might expect that the lipid asymmetry will be accompanied by an asymmetry in the distribution of cholesterol, producing markedly different physical properties in the outer and inner plasma membrane leaflets.

In summary, the effect of phospholipid headgroup and acyl chain unsaturation on cholesterol partitioning was determined in a quantitative and highly reproducible manner, using a newly developed method, which is independent of any perturbation of the properties of the lipid bilayer being evaluated. Our findings of a strong lipid dependence of cholesterol partitioning suggests a thermodynamic role in cellular cholesterol distribution and the formation of cholesterol-rich domains.

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