Movement of Cholesterol between Vesicles Prepared with Different Phospholipids or Sizes*

The rates of exchange of [4-14C]cholesterol between lipid vesicles prepared with different phospholipids and with different sizes have been measured. The first-order rate constants were higher using vesicles prepared from phosphatidylcholines with highly branched or polyunsaturated fatty acyl chains than with saturated diacyl or di-O-alkyl chains. The rate measurements indicate that the affinity of cholesterol for phospholipid does not vary significantly on change of the type of linkage (ether or ester) in phosphatidylcholine (PC) or of the positions of the fatty acyl chains in 1,2-diacyl-PC bearing one saturated and one unsaturated chain; furthermore, egg phosphatidylglycerol and egg phosphatidylethanolamine appear to have comparable affinities for cholesterol. However, the molecular packing in the bilayer and nearest-neighbor interactions involving cholesterol appear tightened more by N-palmitoylphosphoglycerol than by dipalmitoyl-PC; on incorporation of 44 mol % of these phospholipids (which have the same fatty acyl chain composition) into either small or large unilamellar vesicles prepared with egg phosphatidylglycerol, the exchange rates were strikingly slower when the donor species contained sphingomyelin compared with PC. The rate of cholesterol exchange was 100% faster with small unilamellar vesicles than with large unilamellar vesicles as donors, suggesting that the looser packing in the highly curved small vesicles facilitates cholesterol desorption. The cholesterol exchange rate did not vary with the size of the acceptor vesicles, which indicates that desorption is the rate-limiting step in the exchange process in the presence of excess acceptors.

Unesterified cholesterol undergoes spontaneous exchange between membranes (reviewed in Refs. 1 and 2). Although unesterified cholesterol is distributed between the two leaflets of unilamellar vesicles and of plasma membranes of many cells (such as erythrocytes, fibroblasts, primary hepatocytes, and hepatoma cells), most of the cholesterol molecules are exchangeable in one kinetic pool (3–10). This observation suggests that cholesterol translocation from the inner to outer monolayer is rapid and, thus, is not the rate-limiting process for exchange with acceptors. When the acceptor species is present in excess, dissolution of the lipid from the surface of the donor particle appears to be rate-limiting for exchange (8, 11); under these conditions, movement of cholesterol from the aqueous phase into the acceptor membranes is rapid relative to the desorption step. One of the important determinants of the rate of desorption from cholesterol-containing PC clusters is the extent of close packing between the steroid nucleus and the PC fatty acyl chains (12). The extent to which phospholipid structural features affect the cholesterol exchange rate has not been fully described; several investigators have demonstrated that introduction of unsaturation into PC fatty acyl chains or shortening of saturated chains causes an enhancement in the cholesterol exchange rate (3, 5, 12–15).

We have studied the rate of [14C]cholesterol exchange between SUVs, using as the donor system vesicles from 1) phosphatidylcholines of different fatty acyl composition on positional distribution or 2) PG containing increasing amounts of either C16-SPM or DPPC. We have also compared the rates of [14C]cholesterol exchange from LUV to LUV, from LUV to SUV, and from SUV to LUV. The results indicate that cholesterol desorption from the vesicle interface is highly sensitive to the presence of SPM or to the introduction of branched chains and unsaturation into PC fatty acyl chains, but not to the substitution of ether linkages for ester linkages in PC or to the modification of the acyl chain position in sn-1 and sn-2 isomers of PC. Since we found that the cholesterol exchange rate from SUV to LUV is much slower than that from SUV, it is suggested that the degree of membrane curvature may have a physiologically significant role in the regulation of intermembrane cholesterol movement.

MATERIALS AND METHODS

RESULTS

Influence of C16-SPM and DPPC Incorporation into SUV on [14C]Cholesterol Exchange Kinetics—We recently found that the rate of [14C]cholesterol exchange from Mycoplasma gallisepticum cells to excess acceptor vesicles decreased dramatically when C16-SPM was incorporated into the cell membrane (16). The decrease in the rate of cholesterol desorption indicates an increase in cell membrane rigidity, which may arise because SPM interacts more strongly with membrane

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1 The abbreviations used are: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; C16-SPM, N-palmitoylsphingomyelin; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

2 Portions of this paper (including "Materials and Methods," parts of "Results" and "Discussion," Fig. 1, Table I, and additional references) are presented in mini-print at the end of this paper. Mini-print is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1653, cite the authors, and include a check or money order for $4.80 per set of photocopies. Full-size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

4 To whom reprint requests should be addressed.
proteins or with cholesterol compared with the interactions of PG and PC with these molecules. We used vesicles prepared with PG (which is the predominant phospholipid in the M. gallisepticum cell membrane) and varying contents of PC and SPM to examine the possibility that stronger SPM-cholesterol interactions are primarily responsible for the decrease in cholesterol exchange kinetics. The results of [14C]cholesterol exchange measurements at 37 °C from vesicles containing egg PG and C16-SPM or DPPC are shown in Table II. As expected, the half-time for [14C]cholesterol exchange increased as the proportion of saturated phospholipid in the donor vesicles increased. The t½ values were markedly different in bilayers containing DPPC and C16-SPM. When DPPC was added to a content of 44 mol %, t½ increased by 36%. The same amount of C16-SPM caused an increase of 100%. The influence of smaller SPM contents on t½ was also striking; for example, there was a 60% increase in the half-time when 20 mol % C16-SPM was incorporated into egg PG/cholesterol vesicles (Table II). Therefore, these kinetic studies suggest that SPM-cholesterol interactions are stronger than PC-cholesterol interactions in highly curved SUV.

**TABLE II**

Influence of PC and SPM on [14C]cholesterol exchange kinetics at 37 °C between SUV

<table>
<thead>
<tr>
<th>Donor vesicles contained egg PG and DPPC or C16-SPM, diacetyl phosphoric acid (15 mol %), and cholesterol (6 mol %). The total lipid concentration was 1.1 mM. The donor vesicles were incubated with a 10-fold excess of acceptor vesicles, which contained egg PG and cholesterol (6 mol %). The t½ values are the means ± S.E. of different vesicle preparations (n values in parentheses).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composition of donor SUV</strong></td>
</tr>
<tr>
<td>Egg PG</td>
</tr>
<tr>
<td>mol %</td>
</tr>
<tr>
<td>79</td>
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<tr>
<td>35</td>
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<td>35</td>
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</table>

**TABLE III**

Influence of DPPC and C16-SPM on [14C]cholesterol exchange kinetics at 49 °C between LUV donor species and SUV or LUV acceptor species

The acceptor SUV or LUV were prepared with egg PC and 6 mol % cholesterol. LUV were prepared by reverse-phase evaporation with 6 mol % cholesterol. The half-times are the average of two measurements with two different vesicle preparations; the error in t½ was <5%.

<table>
<thead>
<tr>
<th>Composition of LUV donor</th>
<th>Acceptor species</th>
<th>t½</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Egg PG</strong></td>
<td><strong>Egg PC</strong></td>
<td><strong>DPPC</strong></td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>44</td>
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<td>35</td>
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</tbody>
</table>

Half-times for [14C]cholesterol exchange at 37 °C between vesicles of different sizes

The vesicles were prepared with egg PC and 6 mol % cholesterol. The trapped aqueous volumes of SUV and LUV prepared by reverse-phase evaporation were 0.39 and 2.2 liters/mol of total lipid, respectively, as estimated using carboxyfluorescein. The results are reported as the mean ± S.E. The numbers in parentheses indicate the number of kinetic runs with different vesicle preparations.

<table>
<thead>
<tr>
<th>Exchange conditions</th>
<th><strong>t½</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>Acceptor</td>
</tr>
<tr>
<td>SUV</td>
<td>SUV</td>
</tr>
<tr>
<td>SUV</td>
<td>LUV</td>
</tr>
<tr>
<td>LUV</td>
<td>SUV</td>
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<tr>
<td>LUV</td>
<td>LUV</td>
</tr>
</tbody>
</table>

lipid order when SPM is present. Thus, the results obtained with both small and large vesicles as donors suggest that C16-SPM-cholesterol interactions in the liquid crystalline phase are stronger than DPPC-cholesterol interactions. Substitution of egg PC for egg PG did not affect the exchange rate. The half-times do not depend on the curvature of the acceptor species (present in 10-fold excess).

**Effect of Vesicle Size on [14C]Cholesterol Exchange Kinetics**—In order to examine in more detail whether the ease of cholesterol desorption varies with the surface curvature of donor and acceptor vesicles, we measured the exchange kinetics using SUV and LUV. Egg PC LUV prepared by reverse-phase evaporation have diameters above about 1600 Å and have 5-fold larger aqueous trapped volumes compared with SUV. LUV prepared by the octyl glucoside slow dilution-dialysis procedure are smaller than those made by reverse-phase evaporation, ranging from about 600 to 1600 Å in diameter (17), whereas most of the SUV have diameters of 250 Å or less. The rate of [14C]cholesterol exchange from donor SUV was independent of the size of the acceptor vesicles; Table IV shows that the half-time for exchange was the same when SUV and LUV (prepared by the reverse-phase evaporation method) were used as the acceptor. This finding is consistent with the conclusion that desorption of the lipid from the donor is the rate-limiting step in the exchange process when the acceptor is present in large excess (see details provided in the Miniprint Section). Similarly, when LUV prepared by the octyl glucoside slow dilution-dialysis method were used as the acceptor and SUV were the donor, the t½ value was 173 min. In contrast to the high extent of [14C]cholesterol exchange observed between donor SUV and acceptor vesicles, the extent of exchange of radiolabeled sterol from donor LUV was much more limited even at prolonged incubation times (24 h, 48 h) and with more than 10-fold excess of acceptor species. Table IV also shows that the rate of cholesterol removal from the less highly curved LUV was significantly slower (approximately 100%) than from SUV. We found the same trend when LUV prepared by the octyl glucoside method were used as the donor and SUV as the acceptor (t½ = 240 min). This implies that cholesterol (and PC) molecules are more loosely packed in SUV than in LUV. In confirmation of this suggestion, we found that 4Ca2+ efflux mediated by A23187 was faster from SUV than from LUV (t½ values of 41 and 52 min, respectively; see “Materials and Methods” for experimental details).

**DISCUSSION**

The hydrogen-bonding network that can form at the bilayer surface is more extensive with SPM than with PC, since SPM
can function as both a donor and acceptor of hydrogen bonds (18, 19). Preferential SPM-cholesterol interactions have been implicated in various studies (see Refs. 18–20 for reviews), including measurements of cholesterol transfer from cells or vesicles to acceptor species (5, 15, 21, 22). We found that mycoplasma cell membranes containing C_{16}-SPM gave decreased rates of spontaneous cholesterol exchange and protein-mediated phospholipid exchange compared with cell membranes containing DPPC (16). The results reported in Tables II and III indicate that there is a significant difference in cholesterol desorption relative to planar regions of the same membranes containing DPPC (16). The results reported in Tables II and III indicate that there is a significant difference in cholesterol desorption from SUV and LUV containing C_{16}-SPM relative to DPPC. Since these phospholipids have the same fatty acid composition, the difference in the half-time for cholesterol exchange is consistent with the postulated preference of cholesterol for SPM versus PC.

Table IV shows that compositionally identical donors of different sizes give rise to different cholesterol exchange rates. The biological significance of these studies with SUV and LUV is that cholesterol molecules located in highly curved regions of membranes or other particles may have enhanced rates of desorption relative to planar regions of the same composition. The enhanced cholesterol exchange rates from SUV as donors may be attributed to the looser packing of PC fatty acyl chains of highly curved SUV relative to large, essentially planar multibilayers (23, 24), or to the marked asymmetry of phospholipid packing within SUV (with much larger average area per PC head group on the outer surface of SUV compared with the interface between the two monolayers) (25). The cholesterol exchange rate is not dependent on the curvature of the acceptor (SUV or LUV) when the acceptor is present in 10-fold excess (Tables III and IV). Thus, the collision frequency between cholesterol in the aqueous pool and acceptor vesicles does not affect the exchange rate when acceptor vesicles are present in excess, since more collisions are expected with the larger LUV. The dependence of exchange rate on the size of the donor but not on that of the acceptor provides additional support for desorption as the rate-limiting step in cholesterol exchange between vesicles.

These kinetic studies show that nearest-neighbor phospholipid-cholesterol interactions are dependent on the SPM content, extent of branching or unsaturation in the fatty acyl chains, and degree of curvature of the bilayer.

REFERENCES


Additional references are found on p. 4102.
Cholesterol Exchange between Vesicles

Supplementary material to: Movement of Cholesterol between Vesicles Prepared with Different Phospholipids or Sizes

by

Lillemore Tugnay, Sandra Czajka, and Robert Strittmatter

MATERIALS AND METHODS

Chemicals. The following [1,2-3H]glycerophosphocholine and [1,2-3H]dipalmitoylphosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). [1,2-3H]dipalmitoylphosphatidylserine, [1,2-3H]dipalmitoylphosphatidylethanolamine, and [1,2-3H]dipalmitoylphosphatidylinositol were gifts from Dr. K. Daniel Kuo (Columbia University, New York). [1,2-3H]dipalmitoylphosphatidylcholine and [1,2-3H]dipalmitoylphosphatidylethanolamine were purchased from Amersham Corp. (Arlington Heights, IL). [1,2-3H]dipalmitoylphosphatidylserine was supplied by Dr. W. E. Nickerson (University of Colorado, Boulder, CO). [1,2-3H]dipalmitoylphosphatidylserine was a gift from Dr. M. J. Kuo (Columbia University, New York).

Preparation of Vesicles. The desired amount of [1,2-3H]PC (or PC, PE, and SM) and cholesterol (and dicyclophosphoryl donor lipid) was added to a solution of water (50 mL) and 100 mM NaCl. The mixture was then subjected to sonication for 10 min at 20 Hz, and the sonicated vesicles were then centrifuged and resuspended in 50 mM NaCl. The sonicated vesicles were then subjected to sonication for a second time, and the resulting suspension was used as the source of acceptor vesicles. The acceptor vesicles were then subjected to sonication for a third time, and the resulting suspension was used as the source of acceptor vesicles.

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Measurement of [1,2-3H]Cholesterol Exchange between Vesicles. The procedure for measuring vesicles differing in surface charge on short time-lapse volume was modified from the procedure of Nickerson et al. (15) and used for the desorption phase (16). The procedure relied on the use of a water-soluble fluorescent probe (21). The fluorescence was excited with a 585-nm waveband and measured at 595 nm. The fluorescence was measured at 37°C (unless noted otherwise) by incubating donor vesicles with 10-fold excess of acceptor vesicles. The same acceptor vesicles were then incubated with 10-fold excess of acceptor vesicles.

Results. The results were expressed as the percentage of acceptor vesicles labeled with [1,2-3H]PC.

Discussion. The effect of [1,2-3H]PC on the rate of desorption was studied by incubating donor vesicles with 10-fold excess of acceptor vesicles. The results were expressed as the percentage of acceptor vesicles labeled with [1,2-3H]PC.
The interaction between cholesterol and phospholipids bearing a 1-saturated chain is potentially different from that with phospholipids bearing a 1-unsaturated chain (39). A recent differential scanning calorimetry study of the gel to liquid-crystalline transition in 1-oleoyl-2-oleoyl- and 1-oleoyl-2-linoleoyl-PC bilayers showed smaller enthalpy changes in the range of 5 to 11 mol % cholesterol with the latter PC (40). This finding suggested that the 1-unsaturated-2-saturated species was associated with cholesterol to a greater extent or more tightly than the positional isomer. However, a monolayer study indicated that the packing behavior in the presence of cholesterol did not depend on the position of the fatty acid chains (41). It is therefore of interest to examine whether the positions of the saturated and unsaturated acyl chains result in a difference in [3H]cholesterol exchange kinetics. At 6 mol % cholesterol (Fig. 2B), the exchange rate of vesicles of the positional isomers, 1-16:0-18:1- and 1-16:0-18:0-PC, were similar. Table 1 also shows that the [3H]cholesterol exchange rate at 6°C was the same in SUV from the positional isomers of palmitoyl-oleoyl-PC with 6 and 6 mol % cholesterol. These results suggest that the molecule packing in the bilayer and interfacial behavior (interactions involving cholesterol and PC) are not highly sensitive to the positional distribution of the fatty acid chains. It thus appears that the normal positional distribution in the PC of the cholesterol-rich membranes (1-unsaturated-2-saturated) does not explain why membrane specificity results for cholesterol. Since the cholesterol-nonreducing phospholipase species have the usual 1-saturated-2-unsaturated positional distribution, it had been speculated that the unusual fatty acidomer distribution in PC could be implicated in the unique growth requirement of mycoplasma for cholesterol (42). It has also been observed that mycoplasmas have elevated content of cholesterol and 1-oleoyl-2-arachidoyl-PC species (and 1,2-dioleoyl-PC) (43,44). Our experiment suggests that the occurrence of phospholipids with unusual positional specificities does not result in a large change in the membrane properties.

### Table 1: Self-times for [3H]cholesterol exchange from SUV prepared from phosphatidylincholines with different fatty acyl-0-3H-acyl chains

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>SUV</th>
<th>Phospholipid</th>
<th>Exchange Rate (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0-18:0</td>
<td>SUV</td>
<td>1-16:0-18:0-PC</td>
<td>2.6</td>
</tr>
<tr>
<td>16:0-18:1</td>
<td>SUV</td>
<td>1-16:0-18:1-PC</td>
<td>2.8</td>
</tr>
<tr>
<td>18:0-18:1</td>
<td>SUV</td>
<td>1-18:0-18:1-PC</td>
<td>3.1</td>
</tr>
<tr>
<td>18:1-18:2</td>
<td>SUV</td>
<td>1-18:1-18:2-PC</td>
<td>3.4</td>
</tr>
</tbody>
</table>

### Discussion

The results presented demonstrate that the first-order rate constants for spontaneous [3H]cholesterol exchanges from SUV follow the order of multiply branched or polyunsaturated phosphatidylincholines (dipalmitoyl- or distearoyl-PC) 1 egg PC 1 saturated dipalmitoyl- or di-stearoyl-PC. Highly branched or unsaturated fatty acids in a phospholipid are required to result in looser packing of the lipid chains, so the rate-limiting description of cholesterol from dipalmitoyl- or di-stearoyl-PC is dependent on its ability to form bilayers. This finding that the [3H]cholesterol exchange rates of saturated dioleoyl- and dioleoyl-PC vesicles are similar at 4°C suggests the conclusion that cholesterol increases the packing density of dioleoyl and dioleoyl-PC bilayers to a similar extent (revised in ref. 35). Moreover, the effects of cholesterol on the hydrophobic portion of the membranes can be detected, and the type of linkage (ether or ester) (27,36,37), and no significant interaction of cholesterol molecules takes place in PC bilayers when the carbon group of the PC is replaced with dioleoyl-PC SUV, a slightly faster rate of cholesterol transfer between SUV and myelinosomes at 37°C was reported recently using SUV dioleoyl-PC and dioleoyl-PC SUV. The interaction between phospholipids bearing a 1-saturated chain is potentially different from that with phospholipids bearing a 1-unsaturated chain (39). A recent differential scanning calorimetry study of the gel to liquid-crystalline transition in 1-oleoyl-2-oleoyl- and 1-oleoyl-2-linoleoyl-PC bilayers showed smaller enthalpy changes in the range of 5 to 11 mol % cholesterol with the latter PC (40). This finding suggested that the 1-unsaturated-2-saturated species was associated with cholesterol to a greater extent or more tightly than the positional isomer. However, a monolayer study indicated that the packing behavior in the presence of cholesterol did not depend on the position of the fatty acid chains (41). It is therefore of interest to examine whether the positions of the saturated and unsaturated acyl chains result in a difference in [3H]cholesterol exchange kinetics. At 6 mol % cholesterol (Fig. 2B), the exchange rate in SUV from the positional isomers of palmitoyl-oleoyl-PC with 6 and 6 mol % cholesterol. These results suggest that the molecule packing in the bilayer and interfacial behavior (interactions involving cholesterol and PC) are not highly sensitive to the positional distribution of the fatty acid chains. It thus appears that the normal positional distribution in the PC of the cholesterol-rich membranes (1-unsaturated-2-saturated) does not explain why membrane specificity results for cholesterol. Since the cholesterol-nonreducing phospholipase species have the usual 1-saturated-2-unsaturated positional distribution, it had been speculated that the unusual fatty acidomer distribution in PC could be implicated in the unique growth requirement of mycoplasma for cholesterol (42). It has also been observed that mycoplasmas have elevated content of cholesterol and 1-oleoyl-2-arachidoyl-PC species (and 1,2-dioleoyl-PC) (43,44). Our experiment suggests that the occurrence of phospholipids with unusual positional specificities does not result in a large change in the membrane properties.

### Additional Supplemental Material References