Physical properties of membrane lipids in rat salivary glands: involvement of cholesterol

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Abstract: The effects of cholesterol on the membrane fluidity of phospholipid liposomes from rat salivary glands were observed by the ESR technique using two kinds of fatty acid spin probes. When using egg phosphatidylcholine liposomes, the membrane fluidity decreased due to the increase of cholesterol (0-400 mmol/mol of phospholipid) and its rate was linear. These observations were similar in not only the hydrophilic but also the hydrophobic region of the membrane bilayer. On the other hand, the cholesterol contents in the three major rat salivary glands were 335, 284 and 286 mmol/mol of total gland phospholipids in the parotid, submandibular and sublingual glands, respectively. Although the membrane fluidities of total phospholipids in the three salivary glands were similar in spite of their differences in phospholipid and its fatty acid compositions, by the addition of cholesterol (300 mmol/mol of phospholipids), membrane fluidities decreased in the order of parotid>submandibular>sublingual glands. This decrease was larger in the hydrophobic region than in the hydrophilic one. These results suggest that cholesterol affects the membrane physical properties of each salivary gland in a different manner and membrane fluidity might be optimally provided for each unique membrane function in salivary glands.

Introduction

It has been accepted that most biological functions are related to the membrane, and also that interaction between proteins and membrane lipids occupies a major part of the regulation mechanism of membrane functions. In fact, the activities of many membrane-bound enzymes1) and the functions of hormone or neurotransmitter-dependent and membrane-bound receptors5,6) are affected by the physical state of surrounding lipid environments. For the most part, membrane lipids in mammalian cells consist not only of phospholipids with various fatty acids but also cholesterol, and their compositions interact naturally and adjust proper membrane fluidity4,5). On the other hand, three major rat salivary glands, parotid, submandibular and sublingual, which are continuously controlled by the autonomic nervous system through adrenergic receptors6), have unique characteristic compositions for not only phospholipids but also their fatty acids7,8). However, in recent observations9), the membrane fluidities of liposomes prepared from total extracted phospholipids from the three major rat salivary glands were very similar to each other. Therefore, in order to clarify the physical properties of the more native membrane
liposomes, the effect of cholesterol on the membrane fluidity of phospholipid liposomes from rat salivary glands by the ESR technique using two kinds of fatty acid spin probes has been studied. The results indicate that the interaction of cholesterol with membrane phospholipids is different in each salivary gland.

**Materials and Methods**

Male Sprague-Dawley rats, 9 weeks of age, were sacrificed by bleeding under light ether anesthesia after fasting overnight. The three major salivary glands (parotid, submandibular, sublingual) were removed with care to avoid their mingling with adipose and connective tissues. Each salivary gland was cut into small pieces and homogenized in 2–3 volumes of ice-cold 0.9% NaCl solution using a Potter-Elvehjem homogenizer.

**Lipid extraction and analysis.**

Total lipids were extracted from the homogenate according to the method of Bligh and Dyer. The first chloroform solution in the lipid extraction procedure contained 50 µg/ml of BHT (2,6-di-tert-butyl-p-cresol) to avoid oxidation of unsaturated fatty acids. The resulting lipids were stored in chloroform/methanol (6:1, v/v) under nitrogen at −60°C. Total phospholipids were separated from the neutral lipids on a silica gel thin-layer chromatography (TLC) plate (Merck, Darmstadt) and phospholipid phosphorus was determined by the procedure of Bartlett as modified by digestion with perchloric acid according to Marinetti. Cholesterol content in total lipids was measured by a commercially obtained assay kit (Wako Pure Chemical Ltd. Osaka) based on the method of Richmond.

**ESR spectroscopy.**

Two stearic acid spin probes, N-oxy-4′,4′-dimethyloxazolidine derivatives of 5-keto-stearic acid (5-SAL) and 12-keto-stearic acid (12-SAL), were purchased from Aldrich (Milwaukee). For preparation of spin labelled-liposomes, 1 µmol of egg yolk phosphatidylcholine (Sigma, St. Louis) or the phospholipid extract was dissolved in chloroform/methanol (6:1, v/v), then appropriate concentrations of cholesterol were mixed with the 5- or 12-keto-stearic acid spin probe in benzene (phospholipid/spin probe=100:1, by molar ratio) and the solvent was evaporated first under a stream of nitrogen gas followed by evacuation for 20 min. To this dried lipid film, 0.1 ml of 10 mM Tris-HCl buffer (pH 7.2) containing 150 mM NaCl and several small glass beads (0.17–0.18 mm, B. Braun, Melsungen) were added and mixed vigorously. These spin-labelled liposomes were used for measurement of the membrane fluidity.

The labelled liposomes were put into a glass capillary and ESR spectra were measured at various temperatures using a commercial X-band spectrometer (JES-FX 2XG, JEOL Ltd., Tokyo) equipped with a variable temperature controller (ES-DVT 1, JEOL Ltd., Tokyo). The parallel (T∥) and perpendicular (T⊥) principal values of the hyperfine tensor of an axially symmetrical spin Hamiltonian were estimated from the ESR spectra and the order parameter, S, was calculated using the following relation.

\[
S = \frac{a(\langle T_{11} \rangle - \langle T_{11} \rangle^0)}{a' \langle T_{zz} \rangle - \frac{1}{2}(\langle T_{xx} \rangle + \langle T_{yy} \rangle)}
\]

where \(\langle T_{xx} \rangle = \langle T_{yy} \rangle = 5.9 \text{ G}\) and \(\langle T_{zz} \rangle = 32.9 \text{ G}\) are the hyperfine principal values of the nitroxide radical. \(a/a'\) is the polarity correction factor, where \(a = (\langle T_{xx} \rangle + \langle T_{yy} \rangle + \langle T_{zz} \rangle)/3 = 14.9 \text{ G}\) and \(a' = (\langle T_{11} \rangle^0 + 2\langle T_{zz} \rangle)/3\).

**Results**

**Effect of cholesterol on the physical properties of membrane liposomes**

It is well known that cholesterol is one of the important membrane constituents and has a wide variety of effects on the physical properties of biological membranes. Using ESR spectra from the fatty acid spin probes, the effect of cholesterol on membrane fluidity was observed. Two kinds of spin probe, 5-SAL and 12-SAL which have a nitroxide radical at 5th and 12th carbon of stearic acid, respectively, provide different information. The motion of the former probe is reflected mainly by fluidity near the hydrophilic core region of membrane phospholipid bilayer and the latter is for the hydrophobic region. Figure 1 shows the effect of cholesterol on the order parameters of egg phosphatidylcholine liposomes plotted as a function of the reciprocal of absolute temperature using the 5-SAL spin probe.
Fig. 1 The effect of cholesterol on the order parameter of egg phosphatidylcholine liposomes measured with 5-SAL spin probe.

![Graph showing the effect of cholesterol on membrane fluidity](image1.png)

Fig. 2 The effect of cholesterol on the order parameter of egg phosphatidylcholine liposomes measured with 12-SAL spin probe.

![Graph showing the effect of cholesterol on membrane fluidity](image2.png)

With the increase in cholesterol, the order parameters increased within the measured temperature which is above the transition point. This observation shows that membrane fluidity is reduced by cholesterol. Figure 2 also shows the cholesterol effect in the same conditions as Fig. 1 except for the replacement of the 5-SAL spin probe with the 12-SAL. The order parameter also increased in proportion to added cholesterol. In order to clarify the quantitative effect of cholesterol on membrane fluidity, the difference (ΔS) of the order parameter at 25°C was plotted as a function of cholesterol concentration. As shown in Fig. 3, increase of the order parameter was proportional to the amount of cholesterol up to 400 mmol/mol of phospholipids in both 5- and 12-SAL spin probes. Furthermore, the increasing rate was steeper in 12-SAL than in 5-SAL.

Quantitative analysis of cholesterol in rat salivary glands

In order to compare the composition of cholesterol in rat salivary glands (parotid, submandibular, sublingual), the contents were measured after extraction of total lipids from tissue homogenates. Table 1 shows their cholesterol content expressed per mol of total phospholipids. Although the submandibular and sublingual glands had rather higher values per tissue weight than the parotid gland, the values per phospholipid were inversely the highest in the parotid followed by submandibular and sublingual glands. Average of the three glands was approximately 300 mmol/mol of total extracted phospholipids.

Characterization of physical properties of phospholipids extracted from rat salivary glands concerning cholesterol

In our previous observation⁹, the order parameters of total phospholipid liposomes prepared from major rat salivary glands were
almost the same in spite of the differences of their phospholipid compositions. Membrane lipids consist of not only phospholipids but also cholesterol which affects the membrane fluidity as shown in Figs. 1-3. Therefore, the effect of cholesterol on the order parameter of the extracted phospholipid liposomes from three major rat salivary glands was observed by the measurements of ESR spectra. Figure 4 shows the changes in the order parameter as a function of the reciprocal of absolute temperature using the 5-SAL spin probe. According to the values from Table 1, concentration of added cholesterol was used in 300 nmol per μmol of total phospholipids. The order parameters of liposomes from the three major glands were increased by the addition of cholesterol and, furthermore, their increasing rates were in the order of parotid > submandibular = sublingual in spite of the same cholesterol concentration.

The effect of cholesterol on the order parameters of phospholipid liposomes prepared from the three salivary glands was also observed using the 12-SAL spin probe. As shown in Fig. 5, the increase of order parameter was more drastic than with 5-SAL by the addition of cholesterol (300 mmol/mol of total phospholipids). Although the order parameters were very similar between the three salivary glands, they were separated in the order of parotid > submandibular > sublingual when cholesterol was present.

Discussion

Mammalian cells require cholesterol for various normal cell functions. Most cholesterol in the cell is located in membranes and affects not only membrane physical properties but also the cell’s functionality\(^5\). Therefore, the present study was focused on the effects of cholesterol on the fluidity of membrane liposomes which included phospholipids extracted from rat salivary glands by ESR studies using two different fatty acid spin probes. The increase of the order parameter, meaning a decrease of membrane fluidity, caused by added cholesterol (Fig. 1) has also been observed in \(^{2}\)H-NMR studies\(^{16}\). Although Stockton and Smith\(^{16}\) showed that cholesterol minimally affected the properties of the bi-

<table>
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<tr>
<th>Cholesterol</th>
<th>μmol/g of wet tissue</th>
<th>mmol/mol of total phospholipids</th>
</tr>
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<tbody>
<tr>
<td>Parotid gland</td>
<td>5.8 ± 0.4*(2.2)(^b)</td>
<td>335 ± 9.9</td>
</tr>
<tr>
<td>Submandibular gland</td>
<td>7.4 ± 0.1 (2.8)</td>
<td>284 ± 0.5</td>
</tr>
<tr>
<td>Sublingual gland</td>
<td>6.8 ± 0.3 (2.6)</td>
<td>286 ± 2.1</td>
</tr>
</tbody>
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\(^a\) Mean of three separate experiments ± SD.

\(^b\) Values in parentheses are represented as mg of cholesterol/g of wet tissue.
layer center, it decreased the membrane fluidity at least within the portion of 12th-carbon of stearic acid from the glycerol backbone of phosphatidylcholine liposomes (Fig. 2) and furthermore, its decreasing rates of fluidity were larger than at the portion near the hydrophilic domain (Figs. 1-3).

The amounts of cholesterol per tissue weight in rat salivary glands were very similar to those of mouse. Although there are many sterol derivatives in salivary glands, it was not possible to estimate the individual sterols from the present results. As most cholesterol in mammalian cells is located in the biomembrane, its content per phospholipid is thought to reflect the exact properties of cholesterol in the cells. From the present observations, the parotid gland, which has the highest cholesterol content within cell membranes of the three major salivary glands, might have different cholesterol functions.

Membrane fluidity is affected by various membrane components, such as phospholipid fatty acid and its head group, and proteins. In spite of the complex composition of phospholipid and its fatty acids in salivary glands, membrane phospholipid fluidity has been maintained constantly at not only hydrophobic but also hydrophilic regions. However, different reduced fluidities by cholesterol were observed between phospholipid liposomes from the three major salivary glands (Figs. 4, 5), although cholesterol decreases membrane fluidity linearly in egg phosphatidylcholine liposomes (Fig. 3). The cholesterol molecule interacts not only with fatty acids of the phospholipid but also with its

**Fig. 4** The effect of cholesterol on the order parameter measured with 5-SAL spin probe in total phospholipid liposomes from rat salivary glands. The dotted line is control and the solid one is added cholesterol (300 mmol/mol of total phospholipids). ●; parotid, △; submandibular, ■; sublingual

**Fig. 5** The effect of cholesterol on the order parameter measured with 12-SAL spin probe in total phospholipid liposomes from rat salivary glands. The dotted line is the control and the solid one is added cholesterol (300 mmol/mol of total phospholipids). ●; parotid, △; submandibular, ■; sublingual
head groups). Therefore, the effect of cholesterol on membrane fluidity is very complicated. However, it could be suggested that cholesterol affected the membrane physical properties from each salivary gland in a different manner and membrane fluidity may be optimal to provide each unique membrane function in salivary glands.

Although in this paper membrane physical properties were observed in total extracted lipids, each organelle in mammalian cells has its own characteristic membrane fluidity. Furthermore, the distribution of cholesterol among the membranes and most membrane functions of a cell are not uniform.

In order to clarify more precisely the functional effects of cholesterol on membrane physical properties in salivary glands, the purification of specific organelle membranes, the analyses of their constituents and functions and reconstitution will be required.

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References

11) Bartlett, G. R.: Phosphorus assay in col-


