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Original Article

Physical Properties of Subcellular Membrane Fractions Isolated from Rat Salivary Glands

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In order to clarify differences in the physical properties of native membranes in the rat salivary glands (parotid, submandibular and sublingual glands), the fluidity of their microsomal membrane fractions was observed by electron spin resonance study. Microsomes from the parotid gland showed a higher membrane fluidity than those of the submandibular and sublingual glands. To distinguish the specific characteristics of subcellar membrane physical properties, mitochondria-, endoplasmic reticulum-, apical plasma membrane- and secretory granule-rich fractions were purified from parotid glands. The order parameter of the mitochondria-rich fraction is smaller than that of the endoplasmic reticulum-rich fraction, indicating that mitochondrial membranes are more fluid than endoplasmic reticular membranes. However, the secretory granule-rich fraction of native membranes also showed more fluid than those from endoplasmic reticulum-rich fraction. The physical property of the apical plasma membranes, which is the fusion site for the saliva secretion, were the opposite of those of the secretory granules and the fluidity of the apical plasma membrane-rich fraction was more rigid than that of endoplasmic reticulum-rich fraction. In polarized cells such as those of the parotid gland, the apical plasma membranes demonstrate lipid raft-like structures. These specific membrane domains may affect the physical properties of the membrane and control the stability of the apical plasma membranes. These findings suggest that in the steps of saliva secretion, the fluid membranes supplied from secretary granules may disturb the rigid apical plasma membranes after fusion and enhance exocytosis.

Key words: subcellar fractionation, membrane fluidity, apical plasma membranes, secretory granules, rat parotid gland

INTRODUCTION

Salivary glands are specialized for saliva secretion and strictly regulated by the autonomic nervous system[1]. In the events of secretion, the secretory granules rapidly initiate fusion with the apical plasma membranes of acinar cells[2]. Membrane fusion is a common phenomena for exocytosis, and not only the components of the organelle membrane but also its physical properties are very important[2,3] because membrane fusion requires a drastic structural reorganization of the membranes[4]. The membrane physical properties are closely associated with the composition of membrane lipids, especially phospholipids with various fatty acids, cholesterol, which interact and regulate membrane fluidity[5,6] and membrane bound proteins[7]. In neuronal exocytosis, membrane bound proteins like SNAREs (soluble *N*-ethylmaleimide- sensitive factor attachment protein receptors), are thought to be the first driving force for the exocytosis of synaptic vesicles[8]. Indeed, SNAREs are able to fuse liposomes, but the fusion rates are very slow, requiring hours for completion[9]. These observations suggest that the first trigger of the membranes fusion for exocytosis might be controlled by specific proteins like SNAREs and the specific membranes including the membrane proteins might accelerate the exocytotic fusion phenomenon. Furthermore, the specific alteration of membrane lipid composition upon cell activation is a modulater of the exocytotic membrane interaction[10].

In the intracellular organelle including apical and basolateral plasma membranes, the compositions of membrane lipids and their fatty acids are different[11]. However, there are few reports comparing the physical properties of organelle membranes from the parotid gland cells[12]. In this study, we observed the physical characteristics of the native membranes from various organelles in rat parotid gland by measuring electron spin resonance (ESR) using a spin probe.

MATERIALS AND METHODS

1. Chemicals and Reagents

Two spin probe, *N*-oxyl-4', 4'-dimethyloxazolidine derivative of 5-keto-stearic acid (5-SAL) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). All other reagents were of the highest reagent grade available.

2. Animals

Male Wistar rats (9-12 weeks old) were maintained ad libitum on Oriental MF solid chow (Oriental Yeast Co., Tokyo, Japan) and water. The present experimental protocol was approved by the Animal Ethics Committee of Asahi University (No. 05-003)[14]. After fasting overnight, rats were killed by bleeding under light diethyl ether anaesthesia. Immediately, the parotid glands were isolated and trimmed off connective and adipose tissues, and blood vessels. Salivary glands and liver were also obtained from another rat using the method described by Kameyama et al. [15].

3. Preparation of membrane fractions

All procedures were carried out at 0-4°C. The obtained tissues were cut into small pieces with a McIlwain Tissue Chopper (Mickel Laboratory Engineering Co., Ltd., Surrey, U.K.) and

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homogenized with a Potter-Elvehjem Teflon-pestle homogenizer in 5-10 volumes of the buffer solution described below. For the preparation of microsomes, 10mM Tris-HCl buffer (pH7.2) containing 0.25M sucrose was used, and microsomes were obtained as a precipitate of centrifugation (105,000×*g*, 60min) as described previously[16]. To obtain organelle-rich fractions, mitochondria, lysosome and endoplasmic reticulum, step-wise centrifugation was performed[17]. A secretory granular fraction was purified from rat parotid glands by differential and Percoll gradient centrifugations as described previously (Mizuno et al., 1992)[18].

Apical and basolateral enriched plasma membrane fractions were prepared by Mn^{2+}-precipitation essentially using the process described by Hilden et al. (1989)[19] and slightly modified by Mizuno-Kamiya et al.[14,20]. The purified membrane fractions were stored at−80°C until use.

4. Biochemical analyses

Amylase was used as a marker enzyme of secretory granules and its activity was assayed with a commercially obtained assay kit using blue starch polymer (Pharmacia Diagnostics, Sweden) based on the method described by Ceska et al.[21]. Other marker enzymes were assayed as follows: γ-glutamyl transpeptidase for apical plasma membrane was measured with a commercially obtained assay kit (γ-GTP C-Test Wako, Wako Pure Chemical Ind., Ltd., Osaka) using γ-glutamyl-*p*-*N*-ethyl-*N*-hydroxyethyl aminoanilide as a substrate according to Tate and Meister (1974)[22]; β-*N*-acetyl glucosaminidase for lysosome according to the method described by Findlay et al. (1958)[23]; NADPH-cytochrome *c* reductase for endoplasmic reticulum as described by Sottocasa et al. (1967)[24]; succinate dehydrogenase for mitochondria as described by King (1967)[25]; and K^+-dependent *p*-nitrophenyl phosphatase for basolateral plasma membrane as described by Arvan and Castle (1982)[26].

Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) Bradford (1976)[27]. Bovine serum albumin was used as a standard.

5. Measurement of membrane physical properties by ESR

For the measurement of order parameter, S, in the subfractionated organellar membranes, 5-SAL spin probe was used and the spin probe-labeled membranes were prepared as described[6]. The spin probe in chloroform/methanol (6:1, v/v) was dried up by evaporation under a stream of nitrogen gas followed by evacuation for 20 min. The organellar membrane (about 0.3 μmol of phospholipids/ 1 mg of membrane protein) and then glass beads were added to the dried film (phospholipids/spin probe was about 100:1, by molar ratio), and mixed vigorously on a Vortex mixer. The labeled membranes were put into a glass capillary and ESR spectra were measured[7] and the order parameter, S, which represents membrane fluidity was obtained by the calculation as described[28]. A smaller value of the order parameter indicates a more 'fluid' membrane. ESR spectra were measured at various temperatures using a commercial X-band spectrometer (JES-FX 2XG, JEOL Ltd., Tokyo, Japan) equipped with a variable temperature controller (ES-DVT 1, JEOL Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

It is accepted that most biological functions are related to membranes, and that interaction between proteins and membrane lipids comprises the majority of the regulatory mechanisms governing membrane functions. Although the biological reactions in the membranes directly involve proteins such as membrane-bound enzymes and receptors, the physical state of the membrane lipid environment modulates their regulatory functions. In our previous study[6,28], membrane lipid fluidities, which are one of the parameters of membrane physical properties, were measured in the three salivary glands. In spite of the membrane liposomes being comprised of not native components but extracted lipids, the fluidities decreased in the order of parotid > submandibular > sublingual glands. Furthermore, the membrane fluidities of total phospholipids in these salivary glands were very similar. These results were also observed in both hydrophilic and hydrophobic core regions of the membrane phospholipid bilayer.

In order to clarify differences in physical properties in native membranes between rat salivary glands (parotid, submandibular, sublingual) and liver, the fluidity of their microsomal membrane fractions was measured with a 5-SAL spin probe (Fig. 1). Microsomes from the parotid gland and liver show similar order para-

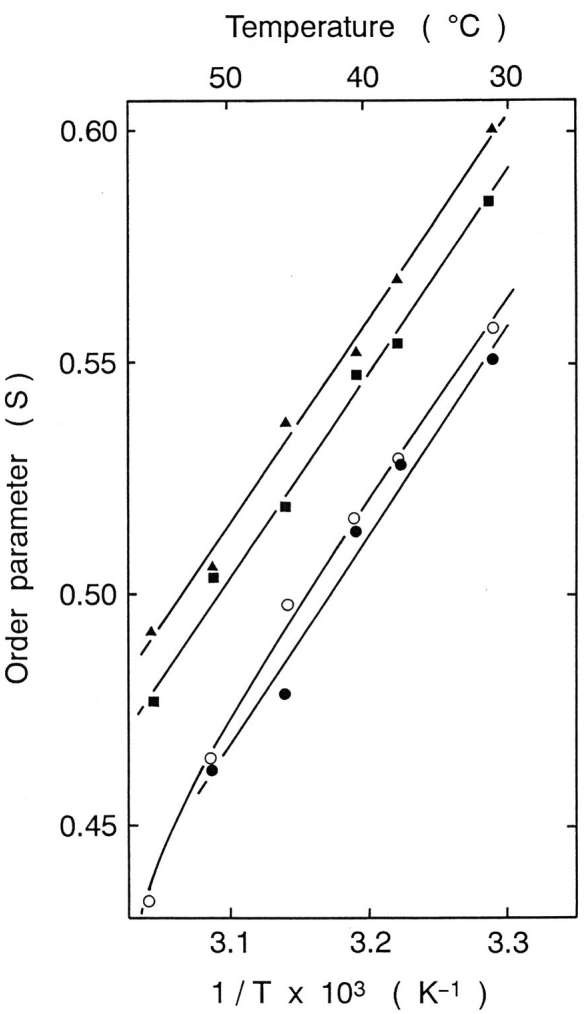

Fig. 1 Membrane fluidity of microsomes from rat salivary glands and liver measured with 5-SAL spin probe
●, Parotid; ■, Submandibular; ▲, Sublingual; ○, Liver

meters that are lower than those of the submandibular and sublingual glands. These observations suggested that native microsomal membranes from the parotid gland are more fluid than those from other salivery glands. During the isolation of organelles, the microsomal fraction usually contains endoplasmic reticulum and plasma membranes. The mitochondria-rich fraction also contain lysosomes. To distinguish the specific characteristics of subcellar membrane fractions, mitochondria- and endoplasmic reticulum-rich fractions were obtained by step-wise centrifugation[17]. The secretory granules and apical plasma membranes that are specific organelles for the saliva exocytosis, were further purified by Percoll gradient centrifugation[17] and Mn^{2+}-precipitation[20], respectively. Table 1 shows the distribution of marker enzymes in the subcellular organelle fractions from rat parotid gland. In the obtained endoplasmic reticulum- and mito chondria-rich fractions, slightly high contaminations by plasma membranes and lysosomes, respectively. Contamination of secretory granules by plasma membranes was estimated at less than 0.1% by the specific activity of the marker enzymes. By the same calculation, the maximum possible contamination with mitochondria, lysosomes and endoplasmic reticulum into secretory granules was judged to be less than 0.9%, 3.5% and 0.9%, respectively. Apical plasma membrane-rich fraction was purified from homogenate approximately at 60-fold. Although this fraction also contained basolateral plasma membranes (K^+-dependent *p*-nitrophenyl phosphatase), calculation of the distribution of marker enzymes and protein indicated that, the maximal contamination of basolateral plasma membranes in the apical plasma membranes-rich fraction was less than 20%. In this fraction, the contamination of all other organelles, measured by the marker enzymes, was negligible. These results suggested that the membrane physical properties obtained from those mem brane-rich fractions are reflected by the specific characteristics of both apical and basolateral plasma membranes.

In the eukaryote, biological functions for the maintenance of cell life are specialized in the organelles. In Fig. 2, the comparison of membrane order parameter, S, between endoplasmic reticulum and mitochondria-rich fractions was shown as a function of the reciprocal of absolute temperature. The order parameter of the mitochondria-rich fraction is smaller than that of the endoplasmic reticulum-rich fraction, indicating that mitochondrial membranes are more fluid than endoplasmic reticular membranes. It is well-known that mitochondria have their own typical membrane phospholipid, cardiolipin, in which unsaturated fatty acids are very abundant, as well as proteins for the generation of ATP. These observations suggest that mitochondrial membranes consisted of typical components, supporting more dynamic reorganization of membrane-bound enzymes than endoplasmic reticular membranes. The contamination of lysosomes in the mitochondria-rich fraction showed a decrease in fluidity (Fig. 2). It is suggested that lysosomal membranes might be more rigid than either mitochontrial or endoplasmic reticular membranes. In order to further clarify the physical property in lysosome, further purification will be necessary.

In our previous study[12], liposomes prepared from phospholipids extracted from the isolated secretory granules were more fluid than those from microsomes. As shown in Fig. 3, the order parameter measured in the native membranes from the secretory granule-rich fraction also showed more fluid than those from the endoplasmic reticulum-rich fraction. One of the characteristics of the phospholipids extracted from secretory granules was a high concentration of lysophospholipids and that was thought to be the reason for the higher fluidity of secretory granular membranes[12]. However, lysophospholipids are shown to exert a destabilizing effect on the membrane structure and inhibit membrane fusion by their specific structure[29]. Therefore, the higher membrane fluidity in secretory granules might be a characteristic depending not on the lysophospholipids but on a complex of its constituents. On the other hands, the physical property of the apical plasma membranes, which is the fusion site for saliva se-

Table 1 The distribution of marker enzymes in subcellular fractions from rat parotid gland.
The assay method for each marker enzyme is described in Materials and Methods. The relative activities are expressed as ratios of the specific enzyme activity to the values in homogenate fraction. The specific enzyme activities are obtained by the means of three to seven determinations.

Organelle-rich fractions	Relative activities of marker enzymes					
	Cytochrome *c* reductase (Endoplasmic Reticulum)	Succinate dehydrogenase (Mitochondria)	*β*-*N*-Acetyl glucosaminidase (Lysosomes)	Amylase (Secretory Granules)	K^+-dependent *p*-nitrophenyl phosphatase (Basolateral Plasma membaranes)	*γ*-Glutamyl transpeptidase (Apical Plasma membrane)
Homogenate	1	1	1	1	1	1
Mitochondria and lysosomes	2	6	4	1 >	1	1 >
Mitochondria	1	10	1	1 >	1 >	1 >
Endoplasmic Reticulum	5	1 >	1 >	1 >	4	4
Secretory Granules	1 >	1 >	1 >	3	1 >	1 >
Apical and Basolateral Plasma membranes	1	1 >	1 >	nd *	19	54
Apical Plasma membranes	1 >	1 >	1 >	nd *	14	57

* : not determined

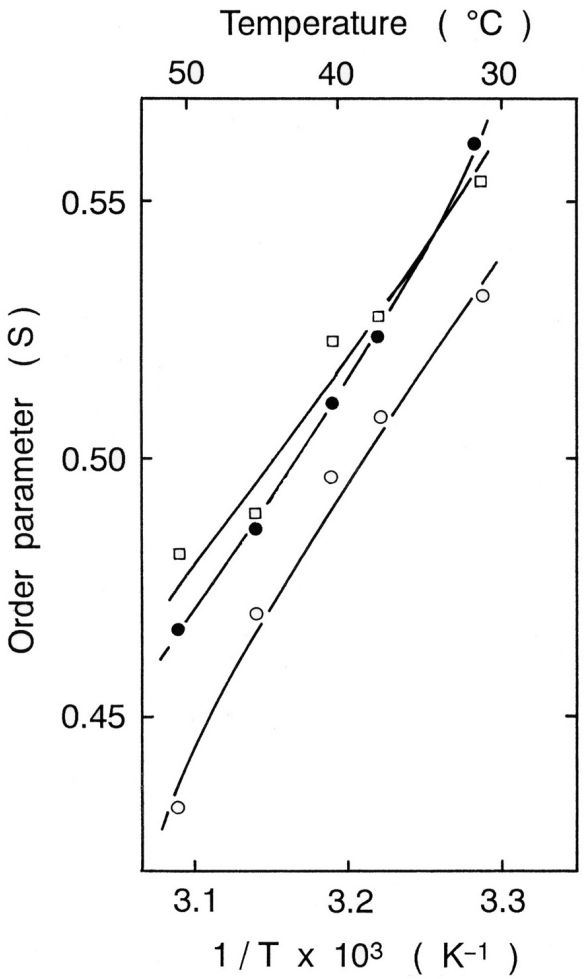

Fig. 2 Membrane fluidity of endoplasmic reticulum- and mitochondria-rich fractions measured with 5-SAL spin probe
●, Endplasmic Reticulum-rich fraction;
○, Mitochondria-rich fraction;
□, Mitochondria and lysosomes-rich fraction

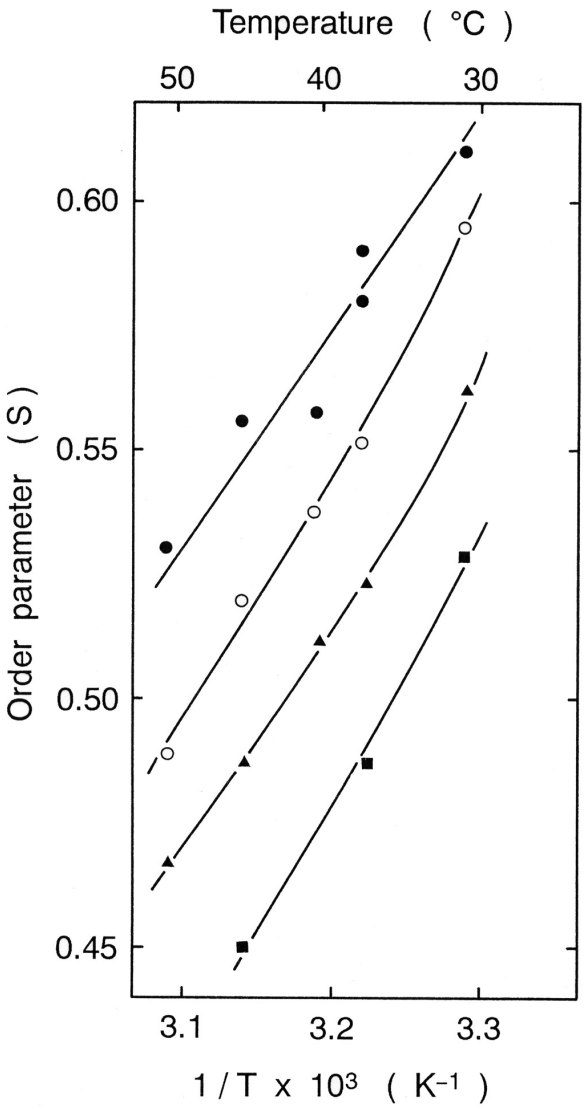

Fig. 3 Membrane fluidity of plasma membranes- and secretory granules-rich fractions compared with endoplasmic reticulum-rich fraction measured with 5-SAL spin probe
●, Apical Plasma membrane-rich fraction;
○, Apical and Basolateral Plasma membrane-rich fraction;
▲, Endplasmic Reticulum-rich fraction;
■, Secretory Granules-rich fraction

cretion, was the opposite of that of secretory granules and the fluidity of the apical plasma membrane-rich fraction was more rigid than that of endoplasmic reticulum-rich fraction. Furthermore, it was very interesting that apical plasma membranes containing basolateral membranes showed more fluid than the purified apical plasma membranes suggesting that the specification of plasma membranes in polarized cells leads to differences in physical properties of the membranes. The plasma membranes demonstrate unique constituents not only among membrane phospholipids but also among functional membrane-bound proteins. Especially, in the various polarized cells, the apical plasma membranes possess lipid raft-like structures that are very stable and rich in cholesterol, sphingomyelin and glycosylphosphatidylinositol (GPI)-anchored proteins[30,31]. These specific membrane domains may affect the physical properties of the membranes and regulate the stability of apical plasma membranes.

When the parotid gland cells are stimulated to secrete the saliva, the secretory granules move to the apical plasma membrane and dock with the membranes. Between secrerary granular membranes and apical plasma membranes, the membrane-bound proteins like SNAREs in the secretary granules are thought to be the initial driving force for membrane fusion[8]. The existence of those proteins has also been reported in rat parotid gland[32,33]. The fluid membranes supplied from secretary granules may disturb the rigid apical plasma membranes after fusion and enhance exocytosis. In order to clarify these steps in the saliva secretion phenomenon, further detail studies of each step in the rat parotid gland will be necessary.

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ラット唾液腺由来細胞内オルガネラの膜物性

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神谷真子　藤田　厚

ラット3大唾液腺(耳下腺, 顎下腺, 舌下腺)および肝由来生体膜の物理化学的性質を電子スピン共鳴(ESR)法で比較検討した。ミクロソーム画分では耳下腺，肝が顎下腺，舌下腺より膜流動性に富んでいた。耳下腺ホモジェネートから，遠心分画，$MnCl_2$処理，Percoll 密度勾配遠心分画の組合せで調製した細胞内小器官の生体膜の物理化学的性質を検討したところ，ミトコンドリア画分が小胞体画分，リソソーム含有ミトコンドリア画分より膜流動性に富んでいた。さらに，膜融合の結果惹起される唾液分泌に関与する細胞内小器官(分泌顆粒と頂端側形質膜）さらに小胞体の膜物性を流動性で検討すると，分泌顆粒＞小胞体＞頂端側形質膜の順に増加した。これらの物性は，細胞内小器官に特有の生体膜構成脂質のみならず，各小器官特有の機能を発揮する膜タンパク質の物理化学的性質を反映していると考えられる。さらに，耳下腺細胞のように細胞極性を持つ分泌細胞の頂端側形質膜にはラフト様構造の存在が指摘されている。これらの結果は，耳下腺唾液の分泌時には，膜流動性に富む分泌顆粒が流動性に乏しく，硬い頂端側形質膜と融合することによって部分的に形質膜を脆弱化し，膜融合後の外分泌を惹起していることが示唆された。

キーワード：細胞分画，膜流動性，頂端側形質膜，分泌顆粒，耳下腺

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