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The Significance of Cytoskeleton in Exocytosis: Control of Apical Plasma Membrane-Evoked Amylase Release from Secretory Granules Isolated from the Rat Parotid Gland

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To investigate the significance of cytoskeletal proteins in the exocytotic in vitro interaction system, we observed the effects of two types of inhibitors, colchicine and cytochalasin D, which depolymerize the microtuble structure consisting of tubulin and microfilament consisting of actins, respectively. The apical plasma membrane-evoked amylase release was increased approximately two times by colchicine. These findings suggest that secretory granules could interact with microtubles coexist via microfilament in the apical plasma membranes and the exocytotic membrane fusion might be suppressed by microtubules. When R18-labeled secretory granules were incubated with apical plasma membranes under the same conditions, de-quenching clearly occurred. In addition, de-quenching was amplified by the addition of colchicine. In the intact cells, colchicine may induce disassembly of the microtubule resulting the inhibition of granule transport to the apical plasma membrane by motor proteins. Based on in vitro observation, it is suggested that the microtubles, which also facilitate cross talk with actin, may modulate access of secretory granules to the apical plasma membrane. On the other hands, cytochalasin D, an inhibitor of actin polymerization, in the cell-free reconstitution system did not affect apical plasma membrane-evoked amylase release. When fiber actin (F-actin) was added to the apical plasma membrane-evoked amylase release system, it decreased amylase release in a concentration-dependent manner. Isolated apical plasma membranes contain microfilaments but the concentration may be not sufficient to function as a barrier secretory granule access. These findings suggest that simplification of the exocytotic interaction system allows observation the direct effects of the cytoskeleton and that cytoskeletal protein inhibitors induce suppressive modulation of exocytotic membrane fusion.

Key words: amylase release, fluorescence de-quenching study, cytoskeleton, apical plasma membrane, secretory granule

INTRODUCTION

Parotid acinar cells are specialized for saliva secretion and strictly regulated by the autonomic nervous system[1]. On stimulation by various secretagogues, the secretory granules rapidly begin to fuse with apical plasma membranes to discharge the granular contents. In neuronal exocytosis, the membranes of synaptic granular vesicles have proteins like SNARE (the soluble *N*-ethylmaleimide- sensitive factor attachment protein receptors) which are thought to be the first driving force for the fusion to the target plasma membranes resulted in secretion[2]. The existence of those proteins are also reported and their importance in the membrane fusion are discussed in rat parotid gland[3~5]. Furthermore, the membrane properties of not only the composition but also its physical characteristics may play an important role in the modulation of membrane fusion[6~8].

The highly specialized storage organelle, the secretory granule, originates from the *trans*-Golgi network[9]. After condensation of a vacuole, it is matured and moves near the apical plasma membranes, which are fusion sites[1]. These processes, cytoskeletal elements, microtubles and microfilaments, control the movement of the secretory granules. Furthermore, in chromaffin cells, both elements are suggested to influence the recruitment of granule pools and control their mobility during secretion[10]. The secretory granules in parotid acinar cell, should move in a vectorial fashion to accomplish the process of exocytosis. Therefore, to understand the regulation of exocytosis, it is important to clarify how the access and fusion of secretory granules to the apical plasma membranes are controlled.

We have reported a cell-free interaction system consisting of secretory granules and apical plasma membranes isolated from the rat parotid gland[6]. In this interaction system, membrane fusion was monitored by fluorescence de-quenching study[7]. Thereafter, our system seems to be a functional model of exocytotic interaction and, compared with other artificial systems, conditions resemble those *in vivo*.

In this study, we investigated the significance of cytoskeleton, microtubles and microfilaments, using this cell-free reconstitution system and cytoskeletal inhibitors, and demonstrate the modulative effect of microfilaments during exocytosis.

MATERIALS AND METHODS

1. Chemicals

The lipid-soluble fluorescent probe, octadecylrhodamine B (R 18) was purchased from Molecular Probes (Oregon, OR). Cytochalasin D and actin from rabbit muscle, and colchicine were obtained from Sigma (St. Louis, MO) and Nacalai Tesque (Kyoto, Japan), respectively. 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

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the Animal Ethics Committee of Asahi University (No. 05-003)[11]. After fasting overnight, rats were killed by bleeding under light diethyl ether anaesthesia. The parotid glands were immediately isolated and connective and adipose tissues, and blood vessels trimmed away.

3. Preparation of secretory granules and plasma membranes-rich fraction

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 homogenized in ice-cold 2mM 4-morpholinepropanesulphonate buffer (2mM Mops-NaOH buffer), pH7.0, containing 0.3M sucrose and 0.2mM $MgCl_2$ with a Potter-Elvehjem Teflon-pestle homogenizer to give a 15% (w/v) tissue homogenate. The purifications of secretory granules and plasma membrane-rich fraction were carried out by differential and Percoll gradient centrifugations as described previously (Mizuno et al., 1992)[6].

4. Assay of interaction between secretory granules and plasma membranes

The interaction between secretory granules and plasma membranes has usually been estimated by measuring amylase release evoked by plasma membranes[6]. The standard procedure for assaying interactions between secretory granules and plasma membranes is as follows unless otherwise stated. Secretory granules (2.5 U of amylase/ml) was preincubated for 1 min at30°C in 20 mM Mops-NaOH, pH7.0, containing 0.3M sucrose, 1mM $MgCl_2$, 1mM ATP, 5mM EGTA, and 4.92mM $CaCl_2$, and the interaction was started by adding either plasma membranes (final protein concentration was 0.19mg/ml) or an equal volume of the incubation buffer as a control. In order to observe the effect of cytoskeleton, plasma membranes were preincubated with colchicine or cytochalasin D solved in ethanol for 30min at37°C and pretreated plasma membranes were replaced in the above assay mixture. To observe the effect of actin, fibular actin (F-actin), which was polymerized in 2mM Tris-HCl (pH7.4) buffer containing 0.14M KCl, 2mM $MgCl_2$, and 1mM ATP overnight at 4°C, was further added in the assay mixture. After incubation for an adequate period, 9 volumes of ice-cold buffer was quickly added to the assay mixture to stop the interaction, then intact granules were removed by centrifugation at 10,000×*g* for 3 min. The release of granular contents resulting from the interaction between secretory granules and plasma membranes was determined by measuring amylase activity in the supernatant. Increase of amylase release (%) evoked by the plasma membranes was calculated as the percentage of amylase activity present in the intact granules, using the following formula:

$$\text{Increased amylase release (\%)} = 100 \times (A_p - A_c)/(A_t - A_c)$$

A_t represents the total amylase activity, and A_p and A_c are the released amylase activities in the presence and absence of plasma membranes, respectively.

5. Fluorescence de-quenching assay for membrane fusion

Membrane fusion was monitored in terms of the relief of self quenching of the fluorescent probe R18, essentially using the method described by Maclean and Edwardson[12]. Assay was carried out as described previously[7]. The probe (1 μl of the 20mM solution in ethanol) was added to 300 μl of secretory granules (2 mg protein/ml). Secretory granules were incubated with the probe in 20mM Mops-NaOH, pH7.0, containing 0.3M sucrose, 1mM $MgCl_2$, 1mM ATP, 5mM EGTA and 4.92mM $CaCl_2$ at 30°C for 10min, then collected by centrifugation at 1,900×*g* for 10min. Labeled secretory granules were resuspended in 10 times the original volume of buffer. In the assay, 25 μl of secretory granules were added to 2ml of incubation buffer (20mM Mops-NaOH, pH7.0, containing 0.3M sucrose, 1mM $MgCl_2$, 1mM ATP, 5mM EGTA and 4.92mM $CaCl_2$) at 30°C. Fluorescence was measured continuously at 30°C in a spectrofluorophotometer (Model RF-540; Shimadzu, Kyoto) at an excitation wavelength of 560nm and an emission wavelength of 590nm. After 1-min preincubation, plasma membranes were added and the fluorescence was monitored for 2-4min. De-quenching was expressed as a percentage of that occurring after solubilization of the labeled secretory granules in 0.2% Triton X-100.

6. Biochemical analysis

Amylase 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[13]. SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed as described[14]. Protein concentration was determined using the method described by Lowry et al.[15] or the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) as described by Bradford (1976)[16] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

To investigate the significance of cytoskeletal proteins in the exocytotic *in vitro* interaction system, localizations of the microtuble by tubulin and the microfilament by actin were observed in our preparative fractions of organelle. On analysis by SDS-PAGE followed by immunoblotting, both actin and tubulin were present in the apical plasma membrane fraction, but not in the secretory granule fraction (data not shown). Microtubules in the nervous system function as molecular tracks along which granular vesicles are transported by the motor proteins, kinesin superfamily proteins (KIFs), in a vectorial fashion[17]. However, in the rat parotid glands, the role of microtubules and motor proteins in the secretory process are not yet known. The findings obtained here suggest that the specific interaction between the secretory granules and microtubles was broken during the isolation process. However, the purified apical plasma membranes were still attached by microfilament bundles that interact with microtubles.

Using the cell-free reconstitution system for the secretion, the effects of two types of inhibitors, colchicine and cytochalasin D which depolymerize the structure of microtuble consisting of tubulin and microfilament actin, respectively, were observed. The apical plasma membranes were pretreated with each inhibitor before the incubation with secretory granules. As shown in Fig.1, the effects of colchicine, which is an inhibitor of tubulin polymerization causing the deconstruction microtubules, was observed under optimal assay conditions[6,7]. The apical plasma membrane-evoked amylase release was increased approximately two-fold by colchicine at concentrations of 10^{-6}-10^{-5}M. At a higher colchicine concentration (10^{-3}M), the increase in apical plasma membrane-evoked amylase release was rather reduced. These findings suggest that secretory granules could interact with microtubles coexisting in the apical plasma membranes and the exocytotic membrane fusion might be suppressed by microtu-

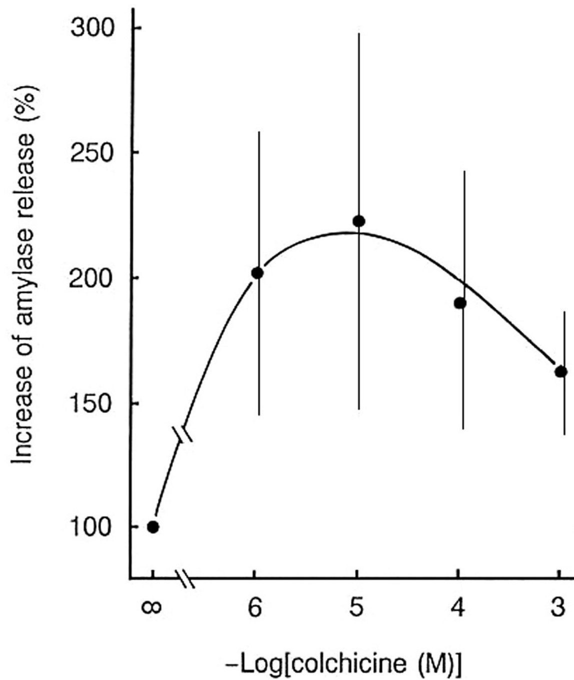

Fig. 1 Effect of colchicine on apical plasma membrane-evoked amylase release from secretary granules
Data are means±SD of 4-6 determinations.

bule. In order to clarify whether the increase in amylase release accompanies the membrane fusion, or not, the effects of colchicine on the fluorescence de-quenching caused by the membrane fusion[7] was observed (Fig.2). When R18-labeled secretory granules was incubated with the apical plasma membranes under the same conditions as those of the assay for amylase release, de-quenching clearly occurred. In addition, as well as amylase release, de-quenching was amplified by the addition of colchicine-treated apical plasma membranes. However, the rate of increased fluorescence de-quenching was not approximately two-fold like that of amylase release following treatment with colchicine. There were tendencies toward membrane interactions between secretory granules and apical plasma membranes, but the absolute values differed in the two assay systems. However, this discrepancy can be partially explained by the characteristics of both assay systems[7]. It was reported using parotid acinar cell, that treatment colchicine decreased the amylase release stimulated by carbamylcholine[18]. In the intact cells, colchicine may induce the disassembly of microtubules resulting in the inhibition of granule transport to the apical plasma membrane by motor proteins. Based on the *in vitro* observation, it is suggested that the microtubles, which also facilitate cross talk with actin[19], may modulate the access of secretory granules to the apical plasma membranes, the docking of these granules and suppressive exocytosis.

The effect of cytochalasin D, an inhibitor of actin polymerization, on the cell-free reconstitution system is shown in Fig.3. The apical plasma membrane-evoked amylase release was not affected by treatment with cytochalasin D at concentrations of 10^{-7}-10^{-5}M. Despite the common agreement that microfilament functions as a physical barrier inhibiting access of the secretory granules to the apical plasma membranes and its transient depolymerization enhances exocytosis, the result obtained here do not suggest any effect of the microfilament on the regulation of exocytosis. However, when fiber actin (F-actin) was added in the apical plasma membrane-evoked amylase release system, it decreased amylase release in a concentration-dependent manner (Fig.4). Furthermore, around 10^{-4} of F-actin completely inhibit the interaction. These findings suggest that the isolated apical plasma membranes contain microfilaments but their concentration may not be sufficient to function as a barrier to access to se-

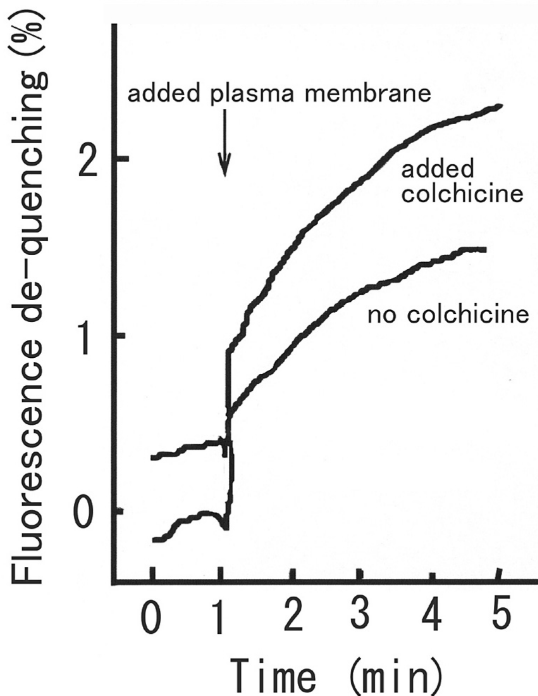

Fig. 2 Effect of colchicine on apical plasma membrane-evoked fluorescence de-quenching caused by membrane fusion

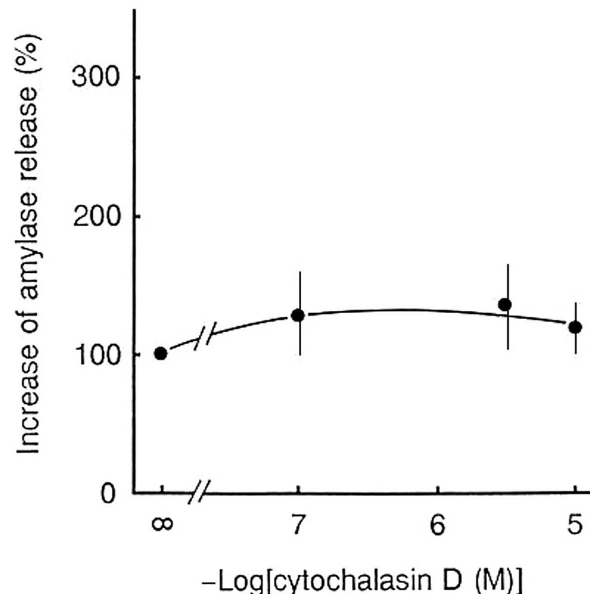

Fig. 3 Effect of cytochalasin D on apical plasma membrane-evoked amylase release from secretary granules
Data are means±SD of 4-6 determinations.

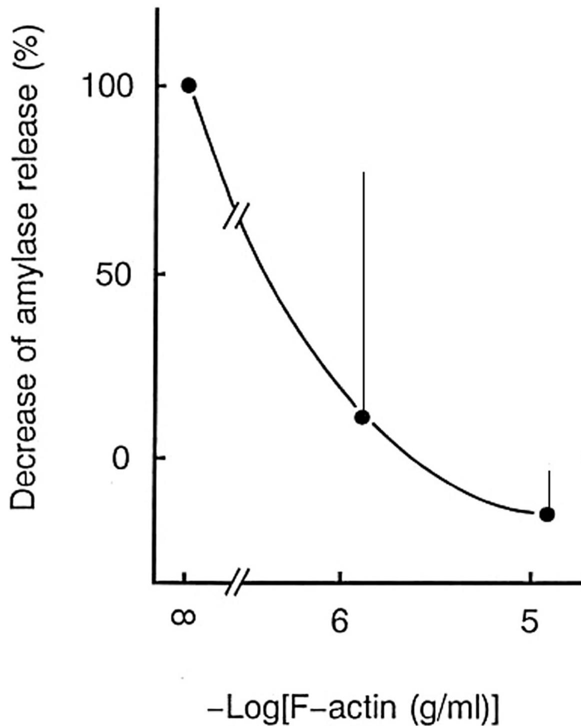

Fig. 4 Effect of F-actin on apical plasma membrane-evoked amylase release from secretary granules
Data are means±SD of 4-6 determinations.

cretory granules. However, in the intact parotid acinar cells, the treatment of cytochalasin D decreased the amylase release and affected the shape of the cells[18]. The microfilaments may have both these opposite functions caused directly or by the process of polymerization.

The intact cells rapidly alter their shape, movement and behavior in response to internal and external signals. All of these dynamic events require extensive remodeling of the microtubule and microfilament cytoskeletons. The final events of exocytosis occur in the apical plasma membranes, which are attached to the microfilaments. Recently, it has become clear that the assembly and polymerization of actin, which constructs the microfilaments, are widely regulated by specific proteins, formins, and play a pivotal role in many types of cell functions[19]. Furthermore, the attachment of microfilaments to the apical plasma membranes is controlled by the small GTP-binding protein, Rho, which is activated by Rho GTPases[19,20]. The formation of microfilaments by the polymerization of actin is also controlled by calcium signals[21] and phosphoinositides[22]. In the apical plasma membranes, the existences of Ca^{2+}-dependent deacylation from phosphoinositides as well as phosphplipase D are demonstrated and the contributions to the exocytotic fusion have been discussed[23].

In the present experiments, simplification of the exocytotic interaction system allows observation of the direct effects of the cytoskeleton on exocytotic membrane fusion. However, the physiological events occurring in the intact cells are, of course, more complicated as indicated above. In order to clarify each exocytotic function and the cross talk among the microfilament components, further studies will be necessary using this *in vitro* cell-free reconstitution system from rat parotid gland.

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References

1) Dowd F, Watson E and Suddick RP; Lazzari EP, ed. Handbook of Experimental Aspects of Oral Biochemistry. Boca Raton: CRC Press, Inc.; 1983: 285-306.
2) Südhof TC. The synaptic vesicle cycle. *Annu Rev Neurosci.* 2004; 27: 509-547.
3) Fujita-Yoshigaki J, Katsumata O, Matsuki M, Yoshigaki T, Furuyama S and Sugiya H. Difference in distribution of membrane proteins between low- and high-density secretory granules in parotid acinar cells. *Biochem Biophys Res Commun.* 2006; 344: 283-292.
4) Takuma T, Arakawa T and Tajima Y. Interaction of SNARE proteins in rat parotid acinar cells. *Arch Oral Biol.* 2000; 45: 369-375.
5) Imai A, Nashida T, Yoshie S and Shimomura H. Intracellular localization of SNARE proteins in rat parotid acinar cells: SNARE complexes on the apical plasma membrane. *Arch Oral Biol.* 2003; 48: 597-604.
6) Mizuno M, Kameyama Y, Yashiro K, Shin SO and Yokota Y. Properties of plasma membrane-induced amylase release from rat parotid secretory granules: effects of Ca^{2+} and Mg-ATP. *Biochim Biophys Acta.* 1992; 1116: 104-111.
7) Mizuno-Kamiya M, Inokuchi H, Kameyama Y, Yashiro K, Shin S-O and Fujita A. The significance of membrane lipids in Exocytosis: Control of liposome-evoked amylase release from secretory granules isolated from the rat parotid gland. *J Biochem.* 1995; 118: 693-699.
8) Kameyama Y, Kitamura K, Yashiro K, Mizuno-Kamiya M and Fujita A. Physical properties of subcellular membrane fractions isolated from rat salivary glands. *J Gifu Dent Soc.* 2008; 34: in press
9) Bard F and Malhotra V. The formation of TGN-to-plasma -membrane transport carriers. *Annu Rev Cell Dev Biol.* 2006; 22: 439-455.
10) Neco P, Giner D, del Mar Francés M, Viniegra S and Gutiérrez LM. Differential participation of actin- and tubulin-based vesicle transport systems during secretion in bovine chromaffin cells. *Eur J Neurosci.* 2003; 18: 733-742.
11) Mizuno-Kamiya M, Inokuchi H, Kameyama Y, Yashiro K and Fujita A. Ca^{2+}-independent phospholipase A_2 activity in apical plasma membranes from the rat parotid gland. *Arch Oral Biol.* 2001; 46: 789-799.
12) Maclean CM and Edwardson JM. Fusion between rat pancreatic zymogen granules and plasma membranes. Modulation by a GTP-binding protein. *Biochem J.* 1992; 286: 747-753.
13) Ceska M, Birath K and Brown B. New and rapid method for the clinical determination of α-amylase activities in human serum and urine. Optimal conditions. *Clin Chim Acta.* 1969; 26: 437-444.
14) Kameyama Y, Nagata K, Mizuno-Kamiya M, Yokota Y, Fujita A and Nozawa Y. Localization of a low Mr GTP-binding protein, *rap* 1 protein, in plasma membranes and secretory granule membranes of rat parotid gland. *Life Sci.* 1994; 55: 213-219.
15) Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem.* 1951; 193: 265-275.
16) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72: 248-254.
17) Hirokawa N and Takemura R. Kinesin superfamily proteins and

their various functions and dynamics. *Exp Cell Res.* 2004; 301: 50-51.
18) Nashida T, Yoshie S, Imai A and Shimomura H. Presence of cytoskeleton proteins in parotid glands and their roles during secretion. *Arch Oral Biol.* 2004; 49: 975-982.
19) Goode BL and Eck MJ. Mechanisms and function of Formins in the control of actin assembly. *Annu Rev Biochem.* 2007; 76: 593-627.
20) Lanzetti L. Actin in membrane trafficking. *Curr Opin Cell Biol.* 2007; 19: 453-458.
21) Malacombe M, Bader MF and Gasman S. Exocytosis in neuroendocrine cell: new tasks for actin. *Biochim Biophys Acta.* 2006; 1763: 1175-1183.
22) Takenawa T and Itoh T. Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. *Biochim Biophys Acta.* 2001; 1533: 190-206.
23) Fujita A, Mizuno-Kamiya M, Yashiro K and Kameyama Y. The role of phospholipase D associated with apical plasma membranes in post-docking steps of exocytosis. *Dentistry in Japan.* 2006; 42: 43-47.

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分泌時の細胞骨格系の役割：ラット耳下腺の頂端側形質膜によって惹起される分泌顆粒由来アミラーゼ分泌の調節

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ラット耳下腺から分離精製した頂端側形質膜と分泌顆粒からなる *in vitro* 開口分泌モデル系を用い，分泌時の細胞骨格系の役割を検討した。細胞分画で得られた両細胞内小器官の内，細胞骨格系タンパク質（チュブリン，アクチン）は頂端側形質膜に局在していた。開口分泌モデル系に微小管構造阻害剤のコルヒチンを添加するとアミラーゼ分泌活性が約２倍上昇した。この現象は蛍光プローブR18を用いた蛍光消光解消法による解析から膜融合の結果と一致していた。マイクロフィラメント構造阻害剤のサイトカラシンＤでは活性上昇傾向は認められなかった。しかし，F-アクチンを添加すると分泌活性はほぼ100%抑制された。これらの結果は，唾液分泌時に細胞骨格系（マイクロフィラメント，微小管）は頂端側形質膜と相互作用をし，膜融合をともなう唾液分泌に直接，抑制的に作用していることが示唆された。一方，分泌顆粒膜との直接相互作用はほとんど無いと考えられた。

キーワード：アミラーゼ，蛍光消光解消法，細胞骨格系，頂端側形質膜，分泌顆粒

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