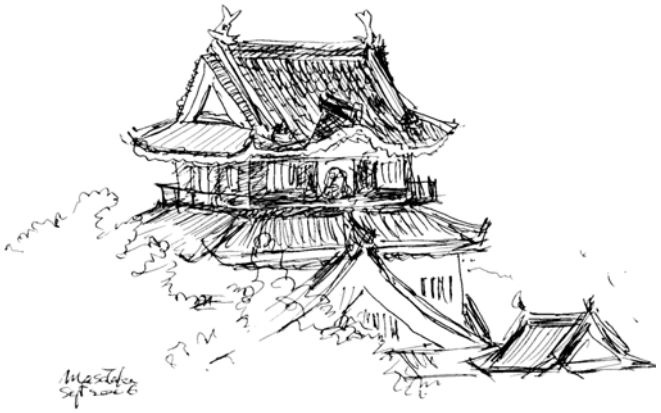
ABSTRACTS

3rd INTERNATIONAL SYMPOSIUM ON SALIVARY GLANDS IN HONOR OF NIELS STENSEN

Chairmen:

M. Murakami, H. Sugiya, A. Riva



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E. Functional morphology of secretory granules I.

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Preface

Niels Stensen (1638-1686) first recognized, in 1661, the organs that secretes saliva as salivary glands. Before then, saliva was believed as a waste product flown down from brain. This story reminded me of the importance of considering the relationship between morphology and function, in order to understand the reality of nature. Modern techniques have enabled the scientists to investigate in detail the secretory mechanisms at the levels of molecules and ions even *in vivo*. However, specialized techniques tend to accumulate one-sided knowledge under limited condition. In order to overcome the problem, we should, from time to time, gather to review and discuss, under a multi-disciplinary point of view, the scatterred data obtained by different techniques.

The First Symposium of this series was held in Florence 1995 and dedicated to Niels Stensen (1638-1686), whose grave is located in the Medici's Cathedral of San Lorenzo. The second symposium was held in Cagliari, Italy in 1997. Recent push from GRC (Gordon Research Conference) friends and the FAOPS (Federation of Asian and Oceanian Physiological Societies) in Seoul made me to organize, with the help of people listed below, the third Stensen Symposium at Okazaki in Japan. Fortunately JSPS (Japanese Society of Promotion of Science) approved our application as one of JSPS International meeting series and decided to support. The organizing committee started at the pub of Tokyo station September 2005. The symposium was officially sponsored by the meeting program of the National Institute for Physiological Sciences.

We organized a series of small international workshops on salivary secretion at Okazaki, Japan, regularly from 1992 to 2000, in relation to international collaborative research projects. After several year intervals, the system of my institute was changed and a new conference hall was built in addition to the several buildings of the three institutes (National Institute of Molecular Science, National Institute of Basic Biology, National Institute for Physiology). According to the policy that the National Institute should be open to public, we planned the citizen forum, which includes an historical lecture and a concert, in the new hall that provides not only satisfactory environment for meetings, but also space for other public functions.

The 3rd Stensen Symposium was especially planned to discuss how to apply the results of basic research to clinical field. This Basic Idea for program making is according to Dr Bruce Baum's suggestions. The talks will start from topics on Clinical applications in order to stress the practical importance of salivary research. Thereafter sessions will shift gradually to Basic researches.

This symposium could not have taken place without the support of numerous business and cultural organizations. A list of these appears on another page of this volume- we are truly grateful for their generosity and their faith in science. We bear also gratitude to all those who have agreed to contribute to this conference.

Masataka Murakami
President of the Acting Organizing Committee
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Demonstration of Japanese Classical Techniques and Entertainments:

Soba: Shoji Akahane and his wife (Shiojiri, Nagano).
Sushi: Harada Family from Daruma Sushi Tatusmigaoka Restaurant (Okazaki).
Japanese Tea: Ai-ya (Nishio).

Lute Concert: **Terrell Stone (Padova, Italy).**
Okinawa Drum Dance: **Hiroko Nakasone and Umikaji Eisah (Okazaki).**
Chainsaw Curving: **Hiroto Achiwa (Okazaki Yanase).**
Japanese Classic Dance "Sakura Emaki": **Ruriko Fujikage,Shizuhisa Fujikage,Miko Fujikage (Tokyo)**
Directed by **Shizue Fujikage (Tokyo)** co-ordinated by**Ayako Sugiya (Yokohama)**
Excursion: **Toyota Motor Company Co.Ltd, Okazaki City Museum, Hatcho Miso no Sato Co. Ltd.,**
Daiju-ji Temple, Takisanji Temple, Iga-Hachiman-Gu Shrine, restaurant SERENO.
co-ordinated by **Meitetsu-Tobu Kanko Co. Ltd, Hitomi Sugiura (ELP, Okazaki).**
Ladies' program: **Masako Murakami** and **Reiko Amano (Okazaki).**
Pre-and Post Symposium tours co-ordinated by **Toshio Sano** of Kinki Nippon Tourist Co.Ltd, Okazaki.
Accommodation: **Okazaki New Grand Hotel, Okazaki Grand Hotel, Okazaki Central Hotel,**
Green Hotel Rich Tokugawa-en, NINS Mishima Lodge, NINS Yamate Lodge.
Meals: **Sangria (Sunfood), Okazaki Bread.** Drinks: **Nomura Sake-ten Co. Ltd.**
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Technical Staffs and Secretaries of the National Institute for Physiological Sciences.
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Opening Talk

The work of Fabricius ab Aquapendente (Harvey's Teacher) in the light of the recently restored Tabulae Pictae: its influence in the development of modern anatomy in Europe and in Japan.

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In the dedication of his De Visione, Voce, Auditu, Fabricius ab Aquapendente (1533-1619) reports that he had in preparationg an atlas of human and animal anatomy of which, in 1600, he had ready more than 300 tables (the Tabulae Pictae) all in colour and life-size. The painted tables, lost after Fabricius's death, were traced in 1909 by Giuseppe Sterzi (1876-1919) in the Marciana, the former state library of the Venetian Republic. In the Marciana there are 169 large oil-painted illustrations collected in eight files, while the rest (43) are in 3 volumes containing 5 Fabricius's published works. Most of them are true masterpiece of figurative art. The Tabulae are unlabeled and are the work of many artists still unknown. The evaluation of the Tabulae Pictae under the anatomical profile, fully endorses both the great admiration of Fabricius's contemporaries, and Sterzi's statement that they represent the most important anatomical work of the XV-XVII centuries. There are, in fact, many priorities that will be reported here. Fabricius is the man who introduced Aristotelian anatomy at Padua. He was the first to describe, inter alia, the sensitivity of pupil to light, the disappearance of the ductus arteriosus and of the umbilical vessels, and the discoverer, in fowl, of the lymphatic organ which now bears his name (bursa of Fabricius). His research program greatly influenced his students, among who there were Julius Casserius (1552-1616), Adrianus Spigelius (1578-1625), Johannes Veslingius (1598-1646), William Harvey (1578-1657) and many others from all Europe. Even if Casserius became Fabricius's fierce academic rival, and Harvey, on the valves of the veins, reached conclusions opposite to those of his erstwhile teacher, all their published works are based on Aristotle's philosophy. Unlike Leonardo's drawings and Eustachius's engravings, that also were lost for centuries and, therefore, could not influence the development of the new anatomical science, many discoveries contained in Fabricius tables that during his lifetime were freely available (1) are incorporated in many books published by Anatomists that had been his pupils. In fact, it is through the "Syntagma Anatomicum", a book originally published in Venice by Veslingius (Johannnes Wesling), who belonged to Fabricius's school and was one of his successors in the chair of Padua, that Western Anatomy first entered Japan (2, 3). Moreover, it has been said that most of the illustrations of Kulmus's' "Ontleedkundige Tafelen", the book that gave rise to "the dawn of Western medicine in Japan", were taken just from the "Syntagma Anatomicum" (4).

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Talk-O1

Stensen and the early history of glands and exocrine secretion.

Riva A., Conti G.
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Niels Stensen (1638-1686), a truly versatile genius, gave, in his relatively short lifetime, fundamental contributions to disparate fields of human knowledge such as Anatomy, Embryology, Physiology, Pathology (he was the first to describe the cardiac malformation now known as Fallot's tetralogy), Paleontology, Geology, Mathematics and Theology.
His first discovery, the one which was soon linked to his name by Johannes van Horne, was made at the age of 22, while Stensen, a medical student in Amsterdam, was practicing dissection in the house of the anatomist Gerhard Blaes (Blasius). The circumstances of the discovery of the *ductus parotideus*, that was made on April 7, 1660 on the head of a sheep purchased with his personal money, are described in the letter that, on April 22, 1661, he wrote to his ersthswhile teacher Thomas Bartholin to defend the priority of his discovery against Blasius who had tried to laying claim to his discovery of the *ductus parotideus.* The letter was written when Stensen had moved to Leiden and the ductus had already been demonstrated in humans and even in a variety of animals by Frans de la Boe (Sylvius) and Johannes von Horne. Moreover, the latter had called the parotid duct "Stenonianus" in his honor. Stimulated by the dispute, Stensen proceeded with research on glands and in the same year published in 1661 his results documented by a table illustrating many of his discoveries. By adopting the terminology proposed by Sylvius, who, inter alia had been greatly impressed by Stensen s findings, he called the exocrine glands *glandulae conglomeratae*, i.e., made up of parts, in contrast with the lymph nodes named *glandulae conglobatae* (fused masses). The *glandulae conglomeratae* derive their secretion from blood and excrete it into a cavity of the body through their excretory ducts, under the influence of nerves. It must be remarked that, though other excretory ducts such as the pancreatic duct (Wirsung, 1642) and the submandibular duct (Wharton, 1656) had been discovered earlier, they had been related to the removal of dross either from chyle by Riolan (1643) or from nerves (Wharton, 1656). Thus, beyond its strictly anatomical relevance (Stensen himself reported that the parotid duct had been already described by Casserius who had mistaken it for a muscle), the importance of Stensen's discovery resides in the fact that, for the first time, he clearly outlines the physiology of exocrine secretions. He also states that the saliva contained in the long ducts (the one which is now called serous) is less viscous than that contained in the short ducts (the one coming from the minor glands that are mostly mucous). Moreover, not only saliva but tears, milk, sweat, and other organic fluids are products secreted by the relevant glands. Stensen discoveries greatly impressed his contemporaries. Malpighi, the father of microscopic anatomy and the discoverer of capillaries, put Stensen concept of glands at the basis of his general physiologic system. In addition, just two years after the publication of Stensen's data, Reiner de Graaf, another pupil of Sylvius, described the surgical technique for obtaining, in dogs, chronic salivary and pancreatic fistulae that allowed not only the chemical study of exocrine secretions but that of their stimulus-dependent variations as well. The era of exocrinology had begun.

Talk-A1.

Clinical examination using saliva: Influence of fluid secretion on the concentration of glucose in saliva: How to overcome the problem for *in vivo* application.

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Since a self-monitoring blood glucose (SMBG) was developed, the control of blood glucose has been more convenient for the patients with diabetic milletus. However, a blood sampling is essential for the method. Frequent blood sampling is not welcomed especially for the insulin-treated patients because of pain and threatening of infection. In the present study, we developed a high sensitivity procedure for glucometry and applied to assess the relationship of salivary glucose and perfusate glucose using isolated perfused submandibular glands of rats.
Methods To assess the relationship between salivary glucose and circulatory glucose the isolated vascularly perfused submandibular glands were used from Wistar strain, diabetic rats (GK/Crj) and those of 2 male rabbits. Under anesthesia, the glands were isolated surgically and vascularly perfused. Arbitrary concentration of glucose was added to the perfusate in the range from 0 mg/dL to 360 mg/dL. The gland was stimulated by addition of carbachol (CCh) and/or isoproterenol (ISP) to the perfusate. The secreted saliva was weighed from which the fluid secretion rate was caluculated as volume per one minute assuming the specific gravity of saliva is 1.0**. Salivary glucometry system** was developed using glucose dehydrogenase and tetrasolium salt. The glucose in saliva and NAD^+ react under glucose dehydrogenase (Amano Enzyme Co.) and produce NADH. Then, the system produces formazon from 2-(4-Iodophenyl) -3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-Tetrazolium (WST-1) through oxidation-reduction reaction by addition of 1-Methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS). The formazon can be detected by colorimetry at the wavelength of 450/630 nm.
The concentration of salivary glucose is ranged around 1/100 of blood sugar. Therefore the blood glucose at 70-90 mg/dL gives the salivary glucose in the range of 0.7-0.9 mg/dL. The calibration curve of the present glucometry was linear in the range of expected salivary glucose. The sensitivity of the procesure was 0.05 mg/dL.
Results. 1) The time course of salivary glucose and fluid secretion was measured at different dose. The fluid secretion increased dose-dependently and the salivary glucose concentration decreased dose-dependently. The relationship between salivary glucose and fluid secretion was reciprocal. Even if the glucose concentration in the perfusate were changed, above reciprocal relationship was same. 2) No modulation was observed both for fluid secretion and salivary glucose concentration during carbachol stimulation at 1.0mM with/without added isoproterenol. 3) To normalize the relationship of salivary glucose to perfusate glucose concentration, we calculated S/P-G values. Then we examined the all S/P-G data against the fluid secretion in the same graph, with no regard of experimental conditions; 1) perfusate glucose concentration, 2) dose of secretagogue, 3) kind of secretagogue, 4) repeatness of stimulation. Figure 5 shows the data from the isolated perfused submandibular glands of. The relations from three different animals (normal rats, diabetus rats, rabbits) were all reciprocal; the S/P-G was higher as the rate of fluid secretion was lower, whereas the S/P-G was lower and tended to be constant as higher fluid secretion. The constancy of S/P-G indicates that the salivary glucose has a linear relation with blood glucose, which enables estimation of blood glucose from salivary glucose concentration. In another words we could use salivary glucose as an indicator of blood glucose when the fluid secretion rate is enough high.

Talk-A2
EXTRAORAL FUNCTIONS OF SALIVARY PROTEINS.

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Salivary proteins play many roles in the mouth. Among these are digestive functions, maintaining mineral and calcium homeostasis, protection of soft tissues and antimicrobial activities. Typically salivary proteins have more than one function and the same function may be shared by different proteins.
In contrast to great efforts in determining the function of salivary proteins in the mouth, little is known about possible roles of salivary proteins in other parts of the organism such as the intestines, which is the site of uptake of many foods from animals and plants.
Tannins are polyphenolic compounds widely found in plant based foods. There are many examples of harmful consequences that tannins in feed can have on animals and tannin gaining access to the Human organism can have toxic effects.
The possible existence of a tannin defensive system was raised in a series of experiments by Carlson and coworkers [1)]. They were able to show in Rats that the harmful effects seen upon feeding tannin were overcome by an onset of synthesis of proline-rich proteins (PRPs) in Rat salivary glands. Thus interaction of PRPs with tannins may have neutralized the effects of tannin.
In Humans it was found that salivary basic PRPs and Histatins were highly effective in precipitating tannins. Such tannin-salivary protein complexes remained insoluble under conditions similar to those in the stomach and intestines and their formation could possibly diminish uptake of tannin.
Using a Human intestinal cell line (Caco-2) the transport of a hydrolysable tannin, pentagalloyl glucose (PGG) was characterized. In the presence of a basic PRP or Histatin the transport of PGG was drastically reduced. This reduction in PGG transport paralleled the formation of insoluble PGG-salivary protein complexes, indicating that formation of such complexes diminished uptake of tannin.
Beside tannins plant foods contain another group of polyphenols known as flavonoids which are closely related to condensed tannins. There is considerable evidence that flavonoids can lower the incidence of heart disease and may also have an anti-carcinogenic effect. Using Quercetin, a representative flavonoid, it was found that its transport across Caco-2 cells was unimpeded in the presence of both basic PRP and Histatin. Thus these salivary proteins can inhibit uptake of tannin with their associated harmful effects, but they do not interfere in the absorption of the related flavonoids and exploitation of their beneficial roles.
Basic PRPs account for 23% of parotid protein, but there is little evidence for functions other than tannin binding. Thus it appears that these proteins, in contrast to other salivary proteins, have a major role outside the oral cavity by acting as scavenger molecules in the intestines.
In view of the ever increasing ability to identify salivary proteins and their breakdown products it is worth keeping in mind that these molecules may have functions outside the oral cavity.
(Supported by grants from the Medical Research Council of Canada and Canadian Institutes for Health Research).
Reference 1. Mehansho H. et al, Annu Rev Nutr 1987;7: 423-440.

Talk-A3

HUMAN SALIVARY PROTEINS – A FASCINATING COMPLEX OF POLYMORPHIC AND POLYFUNCTIONAL PROTEINS

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Human saliva is secreted by three pairs of major glands which, together with minor gland secretions and crevicular fluid, constitute oral fluid or whole saliva.

When secreted, parotid saliva is a watery fluid in which 90% or more of the proteins consist of polymorphic families of α-amylase, proline-rich proteins (PRPs) and histatins. Their phenotypes are genetically determined and are the result of post translational modification of a limited number of gene products. The familial inheritance of α-amylase genes has been characterised, and the phenotypes for PRPs are subject specific. There are two groups of PRPs, acidic and basic, which may comprise up to two thirds of the duct secretion. Other key proteins in parotid saliva, including statherin, lysozyme, carbonic anhydrase, lactoferrin, sIgA, secretory component, lactoperoxidase and cystatins may constitute as little as 10% of the total protein.

Submandibular and sublingual glands differ from parotid saliva in being viscous secretions, this property being attributed to families of mucins. These include a multimeric highmolecular weight gel forming mucin family, MUC5B and the membrane associated mucins, MUC1 and MUC4. In addition, basic PRP genes are not expressed in the submandibular / sublingual glands.

The proteins in saliva have a variety of properties which are essential in the maintenance of oral health. α-amylase is digestive, mucins act as lubricants, PRPs protect against the toxicity of dietary tannins and carbonic anhydrase maintains the bicarbonate essential for buffering. Proline rich proteins are involved in bacterial binding and together with lactoferrin, lysozyme, lactoperoxidase and sIgA, modulate the oral flora. Saliva has anti-fungal properties largely attributable to histatins. It also contains anti-viral factors and is a poor vehicle of transmission of HIV, probably resulting from the binding of basic PRPs Ps1 & Ps2 to GP120 on HIV. Saliva is a potent fluid for the remineralisation of dental enamel despite the sparing solubility of hydroxyapatite; this is facilitated by the calcium binding properties of acidic PRPs and proteins such as statherin which inhibit precipitation of hydroxyapatite.

Mixed (whole) saliva differs from duct saliva in that it not only contains proteins arising from minor glands and crevicular fluid, but in that PRPs are rapidly lost, probably by binding to bacteria and epithelia. Some proteins are also degraded. It is the interaction of these proteins, together with their many functions, which maintains the integrity of the oral tissues, oral function and oral health.

Poster-A1

A PROTEOMIC STUDY OF SALIVA FROM CELIAC PATIENTS.

Tiziana Cabras[1], Elisabetta Pisano[2], Andrea Mastinu[2], Caterina Montaldo[2], Gloria Denotti[2], Vincenzo Piras[2], Chiara Fanali[3], Rosanna Inzitari[3], Massimo Castagnola[3], Irene Messana[1].

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Celiac disease is a gastrointestinal disorder characterized by inflammation, leading to injury to the mucosal lining of the small intestine. Type 2 transglutaminase (TG2) is an important component of the disease, both as deamidating enzyme and as target autoantigen in the immune response. Insulin-dependent diabetes mellitus, connective tissue diseases and Sjogren's syndrome, that affects salivary secretion, are more frequent among celiac patients, than in general population [1]. Moreover, several studies showed that the celiac children are affected by dental enamel defects and oral mucosa diseases [2]. The oral cavity represents a unique environment where peptides and proteins play a key role in maintaining the health status. The aim of this study was to investigate saliva of celiac patients in order to perform a semi-quantitative characterization of selected proteins and peptides and compare the results with those of a control group. In particular, the study was focused on statherin, which is a good substrate of TG2, cystatins S, S1, S2, SN and SA, which are inhibitors of cysteine proteinases and could play a protective and regulatory role under inflammatory conditions, defensins, which are involved in the innate immune system of mammals, and PC and PB peptide, as representative of basic proline-rich proteins. Saliva was collected from 20 healthy subjects and 19 celiac patients matched for sex and age (mean age 10 ± 6 yars). The acidic soluble fraction of salivary samples was analysed by RP-HPLC-ESI ion-trap mass spectrometry and peptides of interest were revealed using the extracted ion current strategy. Areas of the ion current peaks were used to perform the quantitative comparison. Differences between patients and controls with P values ≤0.05 (Student's test) were considered statistically significant. The results show that statherin concentration is significantly reduced in celiac patients with respect to the control group. The reduction of statherin concentration may be connected to an increased activity of oral transglutaminase in celiac patients [3]. Interestingly, cystatin S1 (and its oxidized product) was significantly higher in celiac patients than in the control group, differently from the three α-defensins, which were significantly reduced.

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Poster-A2

SALIVARY ACIDIC PROLINE-RICH PROTEINS IN PRE-TERM NEWBORNS

Rosanna Inzitari[1], Giovanni Vento[2], Ettore Capoluogo[1], Simona Boccacci[2], Chiara Fanali[1], Tiziana Cabras[3], Costantino Romagnoli[2], Bruno Giardina[1], Irene Messana[3], Massimo Castagnola[1]

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This study was devoted to establish the development of secretion of the different aPRPs isoforms and their post-translational modification (PTM) derivatives during the first months of human life. To this aim, the aPRPs complex was analyzed in whole saliva of pre-term and at-term newborns from the first week of life with a follow-up of more than one year. Human salivary acidic proline-rich proteins (aPRPs) are specific salivary proteins and they represent one of the major fractions of the protein salivary content [1,2]. aPRPs are good markers for the study of the development of different enzymatic activities effective during the secretion process. They are submitted to different post-translational modifications: i) phosphorylation, due to a Golgi kinase, with a casein-kinase-like activity [3,4]; ii) a not-complete enzymatic cleavage at arginine residues due to the action of a pro-protein convertase, generating different truncated isoforms [5] and iii), in a minor quantity, the removal of the C-terminal residue under the action of unknown carboxypeptidase/s [6]. Very low phosphorylation levels of aPRPs in pre-term newborns with respect to adults were found. These results suggested a delay in the development of the Golgi casein-like kinase activity. The mean phosphorylation level of at-term newborns was not-significantly different from that one of pre-term newborns at 285 day from conception, suggesting that the maturation of secretory kinase activity of pre-term newborns is synchronous with that of at-term newborns. The trend of the phosphorylation levels as a function of age indicated that the adult phosphorylation level is achieved at about 450-550 days from the conception (from six to eight months of age), corresponding to the beginning of deciduous dentition. Slightly higher amounts of truncated aPRPs isoforms were measured in pre-term newborns with respect to at-term newborns and adults. Higher percentages of P-C peptide missing C-terminal residues in pre-term newborns with respect to at-term newborns and adults were found, suggesting the activity of different carboxy-peptidases in the last phases of human embryonic development.

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Poster-A3

Oxidative modification of serum albumin via paracellular route of rat submandibular gland.

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Human serum albumin has one reactive sulfhydryl group which has been reported to be highly oxidized in aging[1)], exercise[2)] and renal dysfunction[3)]. However, the oxidization mechanism including site within organ still is not clear. The isolated rat salivary gland[4)] allow us to sample intercellular fluids, venous effluent and saliva, thus to understand the site of oxidization through capillary, across vascular wall or through paracellular route. In this study, to clarify the oxidization mechanism of serum albumin in living system, we isolated the rat submandibular gland and perfused with the perfusate containing commercial human albumin. The collected samples were analyzed by a special system of high-performance liquid chromatography[1-3)].

The findings indicated that: 1) despite the evidence that the salivary gland has no intrinsic secretion system of human albumin, small amount of the albumin could be detected in the saliva at 1.0 μM carbachol stimulation and its concentration was approximately 0.05-0.7% of albumin in the perfusate; 2) secretion-perfusate ratio showed a tendency to increase exponentially according to decline of flow rate at 0.5 μM carbachol-0.5 μM isoproterenol stimulation; 3) a distinguishing feature was existence of S-nitrosoalbumin fraction only in saliva, and its concentration was approximately 10-30% of that in the salivary albumin; 4) the S-nitrosoalbumin was hardly able to be detected by addition of an inhibitor on synthesis of nitric oxide (*l*-NAME) to perfusate, but the inhibitor hardly affected secretion volume of saliva; 5) in the salivary gland, the intercellular passage tended to increase an irreversible albumin fraction which was directly oxidized by reactive oxygen species. These facts suggested that the human albumin in saliva might be passed through the paracellular pathway such as tight junction in rat submandibular gland and oxidative modification of the albumin might be occurred during the pathway.

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Poster-A4

A preliminary study on salivary protein expression in diabetic patients.

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Salivary dysfunction and several oral disorders have been documented in patients affected by Diabetes Mellitus. Bacterial infections, periodontal disease and wound healing alterations, as well as xerostomia were found more prevalent in diabetic patients (Genco et al., 2005; Twetman et al., 2002). The correlation between oral symptoms and systemic diseases has been proposed as a valuable tool in discovering and in the early diagnosis of occult diabetic conditions. The purpose of this investigation was to study, by means of transmission electron microscopic procedures, the morphologic and functional changes that may occur in salivary glands of diabetic patients.
Biopsy samples of salivary glands excised from both normal and diabetic consenting patients were processed by standard methods for both routine morphology and EM immunogold labelling. For morphological studies 1-2 mm pieces of the glands were fixed for 2 hr in 1% paraformaldehyde/1.25% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.2, postfixed in osmium tetroxide and embedded in Epon resin, whereas when immunohistochemical procedures were used, samples were fixed for 2 hr in 3% paraformaldehyde/0.1% glutaraldehyde and embedded in epon resin, osmium postfixation being omitted. Ultrathin sections were collected on nickel grids and processed for immunostaining using a post-embedding immunogold staining method. Primary antiserum was a rabbit polyclonal antibody specific for amylase (Sigma). Control grids were incubated in media devoid of primary antibodies or with non-immune serum.
All tissue samples, of both normal and diabetic glands treated with primary antisera, showed specific reactivity for amylase in the cytoplasm of acinar cells. However, within their secretory granules, the main site of labelling deposition, they displayed substantial differences in the distribution of the amylase.
These preliminary studies on amylase distribution suggest that diabetic disease appears to affect secretory processes of glandular parenchyma that may be related to the progressive alteration of salivary functions described by clinicians in diabetic conditions.

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Poster-A5

Clinical examination using saliva: an *in vivo* application to human adults.

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In the experiments using the isolated and vascularly perfused submandibular glands from the rats, the constancy of the ratio of salivary glucose to perfusate glucose (S/P-G) indicates that the salivary glucose has a linear relation with perfusate glucose, which enables estimation of blood glucose from salivary glucose concentration. In another words we could use salivary glucose as an indicator of blood glucose when the fluid secretion rate is enough high.

Methods: For practical application, the saliva was sampled from ductal openings of the sublingual/submandibular glands and the parotid gland of 42 adult volunteer (20-28years old) under resting and salivary stimulation with oral application of sweet tablet. The ratio of salivary glucose to blood glucose (S/B-G) was examined among different individuals. The salivary flow rates increased by stimulation with sweat tablet. It was shown that salivary glucose concentration showed well correlation with blood glucose under this stimulatory condition.

Results: In the non-stimulated parotid saliva, the S/B-G showed a reciprocal relation with the salivary flow rate, as observed in the isolated perfused submandibular glands from the rats and rabbits. When the flow rate was lower than 50μL/min, the S/B-G increased to 0.02. When the stimulation was applied, the salivary flow rate increased, all the S/B-G decreased less than 0.02. Under this condition the salivary glucose level was proportional to the blood glucose in the parotid saliva. Because the salivary flow rate was high in the sublingual/submandibular saliva, all the S/B-G value was less than 0.02.

When stimulation added, the S/B-G value of sublingual/submandibular saliva tended to be stable rather than that without stimulation.

Conclusion

Can the salivary glucose monitor blood glucose?
Yes. We can estimate blood glucose from salivary glucose if the salivary fluid secretion were enough high.

Talk-A4

Salivary glands and saliva composition: a proteomic approach.

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Proteomics has emerged in the last few years as a multidisciplinary and technology-driven science focused on the proteome analysis, including: the complex of proteins expressed in a biological system, in a given moment, their structures, interactions, and post-translational modifications. The result of the application of this methodological approach on the study of saliva was the identification of several hundred proteins/peptides and the promise that this number will quickly increase in the near future, particularly when the actual major limiting problems of protein identification in saliva, such as the high contents in mucins, debris and bacteria, as well as its high proteolytic activity are overcome. The main actual goals are the assignment of the functional role of the identified species and the comparison of the salivary composition in different pathological conditions and to correlate them with morphologic and biochemical disturbances for future clinical applications and to a better fundamental knowledge. A short review, based on results obtained in our laboratory, will be presented.

In general, it is considered that the most prominent proteins present in and secreted by the salivary glands are very well characterized. However, a more careful analysis of the specialized literature shows that the composition of the salivary granules matrix/membranes is far of been establish and will be of interest the application of new analytical approaches such as proteomics. Using parotid and submandibular salivary glands of Charles River rats we proceed to the isolation of granules and acinar cells and to the subcellular fractionation of the acinar cells into nuclear and cytoplasmic fractions. Preliminary results will be presented.

Talk-A5

SALIVARY PEPTIDES AS POTENTIAL SUBSTRATES OF TYPE 2 TRANSGLUTAMINASE.

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Tissue transglutaminases (TG2, type 2 TG) are enzymes of the transglutaminase family ubiquitously expressed. Previous studies reported that selected components of saliva are enzymatically cross-linked by an epithelial transglutaminase during mucosal pellicle formation [1]. Moreover, it was shown that acidic proline-rich proteins, statherin, and the major histatins are capable of undergoing cross-link reactions catalyzed by oral TG2 *in vitro* [2,3]. The purpose of this study was to investigate reactivity of purified statherin and basic PRPs in the presence of type 2 TG (guinea pig liver). Enzymatic reaction products were analyzed by SDS-PAGE and HPLC-ESI-IT-MS. Analysis of the data indicated that the enzyme shows high affinity for statherin *in vitro* ($K_m = 0.65 \pm 0.06$ μM) and it catalyses the specific transformation of statherin into a cyclic derivative, that was named cyclo-statherin Q-37 due to the almost specific involvement of Gln-37 in cycle formation. Even after prolonged incubation other cross-linked products were not observed. Interestingly, in the presence of the competitive amino-donor dansylcadaverine (DC) the main reaction products were cyclo-statherin Q-37 and cyclo-statherin Q-37 linked to one molecule of DC. Only negligible amounts of statherin bound to one DC molecule were detected, suggesting that DC does not inhibit cycle formation, cycle formation cooperatively activates another glutamine residue and Lys-6 of statherin is a TG2 preferred substrate. In spite of structural similarities, basic PRPs showed different reactivity also with respect to statherin. PC, PH and PD peptides formed cyclic structures, but they were less reactive than statherin. In the presence of DC cycle formation was inhibited and linear derivatives cross-linked to one or two molecules of DC were detected. This finding suggests that at least two glutamine residues of the peptides are good substrates of TG2, while lysine residues are poor ones. II-2 and IB-1 peptides were transformed into cyclic derivatives and linked one molecule of DC, but at very low percentages. IB-7, PE, PF and PJ did form neither cyclic structures, nor linked derivatives with DC. Therefore, their lysine residues seem to be not substrates of TG2 at all. PB, which has not lysine residues, did not undergo cross-link reactions both in the presence of statherin and DC. On the whole our data suggest that further than statherin, aPRP's and histatins, several basic PRPs (i.e. PC, PH, PD, IB-7, PE, PF and PJ) may be involved in the formation of the oral pellicle. Moreover, among the different studied peptides, reactivity of statherin seems really specific.

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Talk-A6

PRE-SECRETORY POST-TRANSLATIONAL MODIFICATIONS COMMON TO DIFFERENT FAMILIES OF HUMAN SALIVARY PROTEINS

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RP-HPLC-ESI MS analysis of whole human saliva [1,2] allowed the detection of salivary components belonging to the classes of acidic and basic proline-rich proteins [3,4], histatins [5], statherin and P-B peptide, S cystatins [6] and α-defensins. Like in many other exocrine and endocrine secretions, glycosylation, phosphorylation [7] and different proteolytic cleavages [8,9] are the principal post-translational modifications (PTM) responsible for the generation of the mature products detected in the oral cavity. These processes occur in specific compartments and definite time periods, along a route that crosses the ER and the Golgi apparatus and comprehends the storage into secretory granules and their stimulated or constitutive secretion into the duct lumen. The route terminates with variable fluid stream into the oral cavity [10]. Whole saliva analysis can offer only a picture of the mature secretion products, where the action of exogenous enzymatic activities (i.e. from oral flora) cannot be excluded. This study describes the comparative analysis of the protein content of whole saliva with respect to that one of selected gland secretions (SGS; parotid and submandibular/sublingual) and enriched granule preparations (EGP). This comparison provided information about the events occurring either before secretion (SGS analysis) or before storage into the granules (EGP analysis). Results indicated that the majority of the PTMs (phosphorylation, glycosylation, convertase and carboxypeptidase cleavages) occurred before or during the storage into the granules, some continued during the secretion and only few may be ascribed to enzymatic activities operating in the oral cavity. Furthermore, despite great structural differences but due to the presence of similar consensus sequences, the various classes of salivary peptides show some common PTMs, suggesting that they can share enzymes and routes during maturation.

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Talk-A7

Tissue kallikrein mK13 is a candidate of the processing enzyme for pro-IL-1β in the mouse submandibular gland

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Members of the interleukin-1 (IL-1) family are thought to be the key mediator when the host responds to microbial invasion, inflammation, tissue injury, and immunological reactions. The previous study showed that IL-1β is induced in the submandibular gland (SMG) and secreted into the saliva in response to the endotoxin (lipopolysaccharide, LPS) injected intraperitoneally. In the present study, we report here that kallikrein mK13 is a candidate processing enzyme of pro-IL-1β.

By Western blot analysis, high levels of 17.5- and 20-kDa IL-1β proteins were detected in the SMG and saliva sample from LPS-injected C3H/HeN mouse. Despite this fact, the amount of pro-IL-1β protein, a precursor of IL-1β, with a molecular size of 35 kDa in this tissue was below the detectable level; although strong expression of pro-IL-1β mRNA was observed. The protein for IL-1β converting enzyme (ICE), a processing enzyme for pro-IL-1β, was expressed only at a low level in the SMG as compared with its level in various epithelial tissues or LPS-stimulated macrophages. On the other hand, mK1, mK9, mK13, and mK22, members of the kallikrein family, were detected strongly in the SMG but not in other tissues. By incubation with mK13, but not with mK1, mK9 or mK22, the 35-kDa pro-IL-1β was cleaved and gave 2 major products with molecular weights of 17.5 and 22 kDa, which production was inhibited by PMSF, a serine protease inhibitor, but not by ICE inhibitors. A peptide segment corresponding to amino acid numbers 107-121 of mouse pro-IL-1β (^{107}WDDDDNLLVCDVPIR) was cleaved by incubation with mK13, generating 2 peptides, ^{107}WDDDDNL and ^{114}LVCDVPIR. Therefore, kallikrein mK13 would appear to hydrolyze pro-IL-1β between its Leu 113 and Leu 114 residues. The results of immunohistochemistry and an autonomic therapy experiment showed that IL-1β and kallikrein mK13 were co-localized in the secretory granules of granular convoluted tubular (GCT) cells. Our present results thus suggest kallikrein mK13 is a plausible candidate for the processing enzyme for pro-IL-1β in the SMG of mice.

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Poster-A6

Protein content comparison of mouse submandibular and parotid salivary glands

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A subcellular fractionation (nucleous and cytosol) was performed for acinar cells isolated from parotid and submandibular glands from Charles River rats. Protein analysis was performed using two-dimensional gel electrophoresis (2-DE) in the pH range of 3-10 and mass spectrometry using a MALDI-Tof/Tof. Both fractions (nuclear and cytosol) showed more than 300 spots each when silver stained. Parotid gland cytosol presents an enrichment of polypeptides above 30KDa while submandibular gland cytosol showed enrichment on low molecular weight components. Mass spectrometry analysis lead to the identification of cytoplasmic proteins and salivary proteins such as parotid secretory protein, kallikreins, amylase, and albumin. Preliminary obtained data already allowed comparing the two studied glands concerning their proteins relative abundances and composition.

Poster-A7

CHARACTERIZATION OF PROLINE-RICH PEPTIDES FROM PIG PAROTID GLANDS

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Basic proline-rich proteins (bPRPs) are a class of proteins largely represented in human and mammal saliva [1]. They are usually synthesized as pre-pro-proteins and enzymatically cleaved in small peptides before secretion. Recently, we characterized two proline-rich peptides (SP-A and SP-B) in parotid secretory granules of pig (*Sus Scrofa*) [2] deriving from an unusual cleavage at Pro↓Ala bond occurring on three pre-pro-proteins, which sequences were already determined by cDNA analysis [3] (Swiss Prot Data Bank: **Q95JC9**, **Q95JD0** and **Q95JD1)**. SP-A (diphosphorylated on Ser-12 and 14) corresponds to the 1-56 N-terminal fragment of **Q95JD0** and **Q95JD1**, while SP-B corresponds to a sequence repeated ten times in **Q95JD0**, eleven times in **Q95JD1** and twenty two times in **Q95JC9**. The quantity of SP-B peptide in pig saliva largely overcomes that of any other peptide detected. SP-A and SP-B peptides cover cDNAs for 53.7% (**Q95JD0**), 52.2% (**Q95JD1**) and 70.0% (**Q95JC9**). This not complete covering induced us to search for other peptides deriving from the protein precursors. Enriched granule preparations (EGP) were obtained from pig parotid glands according to described procedures by differential centrifugation of the homogenate tissue samples [4]. EGP's were dissolved in 0.2% trifluoracetic acid and analyzed by HPLC-ESI-IT-MS apparatus. Two different strategies were followed: a) the search of masses corresponding to possible peptides deriving from Pro↓Ala bond cleavages of the pro-proteins excluding those generating SP-A and SP-B peptides; b) an evaluation of which masses detected in the TIC chromatogram could potentially correspond to fragments of the precursors using the FindPept software (http://us.expasy.org/tools/findpept.html). The correct sequence, among the different possibilities, was established comparing the experimental fragmentation pattern obtained by SIM MS-MS with the theoretical ones (ProteinProspector: MS Products). By the two strategies eight new fragments deriving from **Q95JD0** and **Q95JD1** were identified, reaching a covering of 67.7% for **Q95JD0** and of 64.7% for **Q95JD1**. Further studies are required to completely define the processing of the three pro-proteins. However, the identification of these peptides indicates that specific unknown pro-protein convertases are acting during the pig salivary secretory process. The function of these small proline-rich peptides is still unknown.

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Poster-A8

TYROSINE SULFATION OF HISTATIN 1. A POST-TRANSLATIONAL MODIFICATION SPECIFIC OF SUBMANDIBULAR GLANDS

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Histatins are low molecular weight histidine-rich peptides specific of human saliva secreted by major and minor salivary glands [1,2]. The parent histatins, namely His-1 and His-3, are coded by HTN1 and HTN2 genes, located on chromosome 4q13 [3]. Histatin 3 is submitted to a pre-secretory fragmentation [4] and some of its fragments, mainly His-5 ($histatin_3$ 1/24), show powerful antifungal and antimicrobial properties. Conversely, His-1, a peptide of 38 amino acid residues, phosphorylated on Ser-2, largely sharing its sequence with His-3, is not submitted to fragmentation, probably due to the lack of convertase consensus sequence. Specific function of His-1 in the oral cavity was not clarified yet.

In whole saliva HPLC-ESI-MS analyses, just before the peak of His-1, minor amounts of its derivatives with serial mass increases of 80 Da (His-1: 4929 Da; His-1 derivatives: 5009, 5089, 5169, 5249 Da) were detected. These His-1 derivatives were detected in submandibular (SmG) secretions, but not in parotid ones. An enriched preparation (EDP) of these His-1 derivatives was digested by pronase and submitted to CE-MS amino acid analysis. In the digestion mixture sulfo-tyrosine was detected as the amino acid derivative justifying the observed mass increases. Treatment with alkaline phosphatase of SmG secretions and EDPs provided a mass decrease of 80 Da for His-1 and sulfo-derivatives, indicating the presence of only one phosphate group in the molecules (phospo-Ser-2). By HCl 1 M treatment at 100° C (4-6 min) His-1 derivatives were almost completely transformed into His-1, in agreement with the properties of sulfo-Tyr, more labile to acid hydrolysis than phospho-Tyr. Enzymatic digestion of EDP by trypsin, proteinase K and protease V8 originated 87 fragments, which were analysed by HPLC-ESI-IT-MS-MS and MALDI-TOF-MS. Results located sulfation on the last four tyrosines of the sequence (on five total), namely Tyr-27,-30,-34,-36. MS/MS spectra of the His-1 derivatives and its sulfated proteolytic fragments allowed detecting serial neutral losses of 80 Da, distinctive of SO_3 loss, while phosphate of serine provided the characteristic 98 Da neutral loss. Positive and negative MALDI-TOF-MS spectra of EDP did not reveal significant differences. Poly-sulfated His-1 may reach more than 10% of total His-1, this value obviously depending on the submandibular contribution to whole human saliva.

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Poster-A9

Different roles of salivary mucins in viscosity and spinnbarkeit of human saliva

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Objectives: It has been reported that the two rheological properties of saliva, viscosity and spinnbarkeit, are associated with dental caries[1)] and periodontitis[2)]. Mucin glycoproteins are thought to be involved in the rheological properties of human saliva strongly. However, there is no report demonstrating directly correlations between rheological properties and levels of the two types of mucin in human saliva. Therefore, we investigated relationships of two rheological properties with two salivary mucins (MG1 and MG2) levels.

Methods: Unstimulated and chewing-stimulated whole saliva were collected by spitting method from healthy young adults, and the flow rate, total protein, amylase activity, viscosity and spinnbarkeit were measured. MG1 and MG2 levels in saliva were measured by Stains-all staining and relationship of them with viscosity and spinnbarkeit was investigated.

Results: The correlations of the two rheological properties with the other salivary parameters, the flow rate, total protein and amylase activity, were not entirely identical. The viscosity increased with MG1 levels and the spinnbarkeit with MG2 levels.

Conclusion: These results suggest that MG1 and MG2 respectively contribute to viscosity and spinnbarkeit.

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Poster-A10

MALDI based Mass Imaging revealed abnormal distribution of phospholipids in cancer.

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We will present results of matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) and direct molecular identification using tandem mass spectrometry (MS/MS) in cancer metastasis. In this study, we took colon cancer liver metastasis as a model, and the cancer tissue was removed from Japanese patient and the tissue was frozen immediately without any fixations. The sections were sliced at thickness of 3 mm. We used 2,6-dihydroxyacetophenone (DHA) as a matrix for lipid ionization. The matrix solution was applied with an air-brush in order to make a uniform thin matrix layer on the tissue surface. After two-dimensional scanning by the laser, the images were reconstructed as a function of *m/z* from a few hundreds of obtained spectra. In the obtained images, the existence of molecules was represented by pseudo color corresponding to the signal intensity. For the feasibility study, we picked up one peak, *m/z* 725, which was significantly present in the cancer region. MS/MS results indicated that *m/z* 725 was [sphingomyelin (SPM) 18:3]$^{+}$. Thus, we have successfully shown the feasibility of the MALDI-IMS as a tool for the analysis of pathological specimens to identify molecular markers. This method was considered to be applicable to variable tissues and cancers, including salivary gland and their tumors.

Video-B1
Clinical applications of gene transfer to salivary glands.

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Irreversible salivary hypofunction, such as occurs following radiation (IR) therapy or with Sjögren's syndrome (SS), causes considerable morbidity. Since there were no adequate conventional treatments available for either condition, in 1991 we began to explore the potential use of vivo gene transfer to salivary glands. We chose primarily to employ recombinant viral vectors, because viral-mediated gene transfer is most efficient. We deliver vectors via cannulation of the main excretory ducts of major salivary glands, similar to procedures commonly used clinically for contrast radiographs (sialograms). We have developed novel applications of gene transfer for both IR damage and SS, as well as conceived a strategy for using salivary glands as a target organ to correct systemic single protein deficiency disorders (SSPDDs). Our studies have achieved proofs of concept for all three-gene transfer applications in animal models, and this presentation will provide a brief summary of each. The gene transfer approach used to repair IR-damaged salivary glands involves transfer of the human aquaporin-1 cDNA to surviving ductal epithelial cells via a serotype-5 adenoviral vector (AdhAQP1). This approach has been successfully applied after IR to the salivary glands of rats [1] and minipigs [2], leading to transient increases in salivary flow (~80%). Very recently AdhAQP1 was approved for use in a phase 1/2 clinical study. The gene transfer approach employed for SS involves delivery of immunomodulatory transgenes via serotype-2 adeno-associated viral (AAV2) vectors. Two such vectors, encoding either interleukin-10 [3] or vasoactive intestinal peptide [4], have resulted in the preservation of salivary flow in the NOD mouse model of SS. As a model SSPDD, we have focused on the persistent anemia that accompanies chronic renal failure and used erythropoietin (EPO) as a model transgene. EPO is secreted physiologically via the constitutive pathway in the kidney, and as a transgene product from murine salivary glands most (>90%) EPO is found in serum versus saliva [5,6]. In order to control EPO expression and subsequent hematocrit levels, we have employed rapamycin regulation [7]. Using this system in a single AAV2 vector we have shown that after salivary gland gene transfer EPO secretion and hematocrits are tightly controlled in mice [8]. Overall, studies on gene transfer to salivary glands have made substantive progress, and we are optimistic about the future clinical utility of these approaches.

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Talk-B2

Morphogenesis and cleft formation of salivary gland epithelia: Exploration of new functional regulators.

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Salivary glands form multiple epithelial clefts during the process of branching morphogenesis in embryonic development. Extracellular matrix molecules regulate the dynamics of this complex process of organ formation. Fibronectin, laminin γ2, and tissue inhibitor of metalloproteinases-3 (TIMP-3) are differentially expressed in developing clefts or buds of submandibular glands. Integrin receptors for fibronectin and laminin cooperate with site-specific, developmentally regulated gene expression of fibronectin in this process. Fibronectin-mediated crosstalk between integrin cell-to-matrix and cadherin cell-to-cell adhesion systems promotes the formation of clefts. Experimental manipulation of the fibronectin/integrin systems can control branching morphogenesis. We have initiated studies to identify and characterize new molecules that may be important for cleft formation and fibronectin expression. Progress in understanding the mechanisms of salivary branching morphogenesis will provide novel approaches to future tissue engineering or regeneration of damaged salivary glands.

Poster-B1

A novel role of RbAp48 for tissue-specific apoptosis in the salivary glands depending on estrogen deficiency

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Although tissue-specific apoptosis in the salivary glands in estrogen-deficient mice may contribute to the development of autoimmune exocrinopathy such as Sjogren's syndrome, molecular mechanism responsible for tissue-specific apoptosis remains obscure. In this study, we have focused on the molecular mechanisms responsible for tissue-specific apoptosis caused by estrogen deficiency, and identified RbAp48 as a novel apoptosis-inducing gene exclusively in the salivary glands. In addition, we showed that RbAp48 overexpression induces p53-mediated apoptosis in the salivary glands caused by estrogen deficiency. RbAp48-inducible transfectant resulted in rapid apoptosis with p53 phosphorylation (Ser9), and a-fodrin cleavage. Reducing the expression of RbAp48 through small interference RNA (siRNA) inhibited the apoptosis. Furthermore prominent RbAp48 expression with apoptosis was observed in the salivary glands in ovariectomized (Ovx) C57BL/6 mice, not in Ovx estrogen receptor (ER) a $^{-/-}$, p53$^{-/-}$, and E2F-1$^{-/-}$ mice. Indeed, transgenic expression of RbAp48 gene induced apoptosis in the salivary glands, but not in other organs. These findings indicate that estrogen deficiency initiates p53-mediated apoptosis in the salivary gland cells through RbAp48 overexpression, and exert a possible gender-based risk of autoimmune exocrinopathy in postmenopausal women.

Poster-B2

Ionomycin inhibit the soluble protein transport between ER and Golgi.

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The cytoplasmic Ca^{2+} ion concentration is lower than that of extracellular space or intracellular Ca^{2+} storage such as endoplasmic reticulum (ER) or mitochondria. Ca^{2+} ion influx from the extracellular space or Ca^{2+} ion release from the intracellular storage result in the change of this balance and this change is quite important for variable cellular functions.

The initial step of protein exocytosis is the vesicular transport of protein from the ER to the Golgi apparatus. How can cytoplasmic Ca^{2+} ion concentration change influence on the protein transport between ER and Golgi? ER resident proteins such as immunoglobulin heavy chain binding protein (BiP) or immunoglobulin joining chain can be released from the retention mechanisms by the treatment of calcium ionophore, ionomycin, and can be transported to Golgi and ultimately secreted to the culture media.

In this study, we tried to check the influence of Ca^{2+} ion concentration change on the secretory protein transport between ER and Golgi. As cargo molecules, we chose polymeric immunoglobulin receptor (pIgR) or vesicular stomatitis virus glycoprotein (VSV-G). Transiently transfected cells were labeled with Tran-^{35}S-Label and chased for indicated times with or without ionomycin. The cargo molecules were immunoprecipitated and the ER-Golgi transport efficiency was estimated. 5 mM of ionomycin treatment inhibited pIgR and VSV-G transport between ER and Golgi, however, thapsigargin did not affect ER-Golgi transport. As these results indicated that the calcium influx from the outside of the cell may contribute to the inhibitory effect of ionomycin, we cultured the cells in the presence of EGTA and ionomycin. With this condition, however, ER-Golgi transport was completely shut down. Moreover, the cytoplasmic deletion mutant of VSV-G was used to check the contribution of the cytoplasmic domain on the inhibitory effect of ionomycin. Although overall transport efficiency was reduced compared to that of wild type VSV-G, ER-Golgi transport of the mutant protein was also inhibited. These results indicated that the extracellular domain might be indispensable for the ionomycin effect.

The molecular mechanisms underlying the ionomycin effect on ER-Golgi secretory protein transport will be discussed.

Poster-B3

Investigation on the influences of Chinese herbs on salivary secretion in rat submandibular gland.

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Objective: It has been found through long period of clinical traditional Chinese medicine researches that many Chinese herbs have curative effects on deficiency of saliva secretion. This study was conducted to investigate whether Chinese herbs have direct effects on salivary gland or not, and if direct effects exist, then to further investigate whether its efficacy was through inducing saliva secretion or increasing the rate of saliva secretion. To investigate the efficacy mechanism of Chinese herbs, sample prepared through rat vein perfusion of isolated salivary gland was adopted to detect the rate of saliva secretion with the interference of Chinese herbs.
Materials and methods: Submandibular gland of Wistar male rat (SPF grade, body weight 270-290g) was isolated and vascularly perfused. The excretory duct was cannulated and the secreted saliva was guided through a florine-tube to a cup placed on the balance. Saliva weight was continuously recorded in real-time and input into computer to perform differentiation analysis, thus the rate of secretion was calculated. Xuan Shen, Sheng Di, Sha Shen, Tian Huan Fen, Ge Gen etc totaled 10 kinds of Chinese herbs were chosen, whose final concentration in perfusion was converted according to clinical dosages to keep it basically equal with plasma concentration. Pre-preparation of Chinese herbs: extract granule bought from market was adopted, added distilled water and ultrasonic oscillated to dissolved, then centrifuged. Removing the sediment, supernatant was further filtrated through 0.45 μm micro-hole filtrated film and adjusted to a concentration of 1000 μg/ml for future use. 0.2 μM carbachol was adopted as control for saliva stimulation, which can stimulate the muscarinic receptors.
Results and discussion: As to control group, carbachol was given as stimulant 5 minutes before the start of experiment. It was observed that during the initial 30 seconds of induced saliva secretion, there was an ephemeral increasing peak, then the rate of secretion slowly increased and stepped into a sustained phase at 5 minutes. After washout of carbachol with control perfusion for 5 minutes the perfusate was shifted to the test solution with Chinese herb. By Chinese herb alone, no saliva secretion was induced in all kinds of Chinese herbs in the present experiments. However, after perfusion with single perfusion of Chinese herbs combined with carbachol for 5 minutes, the effect of saliva secretion promotion was induced in 5 kinds of Chinese herbs among 10 kinds of Chinese herbs. Phases in initial stage were not influenced but the secretion rate emerged to enhance the sustained phase. The enhancement was classified to three patterns: 1. Sustained phase in the secretion curve was overall slowly and continuously raised with a lifted baseline. 2. In sustained phase, the secretion curve rise continuously and reached its maximum value after 5~10 minutes, then sharply decreased to a lower baseline than control group with continuous secretion. 3. In sustained phase, the secretion continuously increased and reached its maximum value after 5~10 minutes, then decreased slowly. Above three types of secretion were in response to the categories of Chinese herbs (yin-nourishing agent, qi-enhancing agent and blood activating agent). Ten kinds of Chinese herbs, clinically observed with effects of enhancing saliva secretion, were chosen in this study, and only five of them emerged effects, which suggested that other five Chinese herbs effected in human body through nervous system and/or systemic hormonal changes.

Poster-B4

Possible Involvement of Clusterin in the Regeneration Process of Rat Submandibular Gland

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Clusterin, also designated as apolipoprotein J, SGP-2 and SP-40 is a heterodimerized glycoprotein, which can be observed in various organs. Although the discovery of the protein was nearly two decades ago, its biological roles remain largely unknown. Recently, a possible role of clusterin during pancreatic regeneration has been reported. However, the involvement of this protein during salivary gland regeneration has not been reported. The aim of this study was to investigate the gene expression and localization of clusterin during the regeneration process of rat submandibular gland. Male Wistar rats (nine weeks old) were anesthetized and the unilateral duct of submandibular gland was ligated. After ductal ligation, salivary gland immediately becomes atrophic. The gland regenerates after the ligation removal. The ligation was removed after one week and the gland was harvested at 12 hours, 36 hours, and 6 days after removal of ligation. The results from DNA microarray analyses showed immediate increase of clusterin expression after removal of ligation, which returned to almost basal level after 6 days. The gene and protein expression of clusterin was confirmed by RT-PCR and immunohistochemistry, respectively. In the normal gland, clusterin was equally distributed but limited in the cytosol of most of the parenchyma. After ductal ligation and removal, clusterin immediately accumulated intra-luminal space and strong expression was continuously observed along with the luminal surface of the atrophic acini until day 3. The expression was decreased after day 6 and the distribution returned to that of the normal gland. The results from this study demonstrated the dynamic change of this protein during regeneration process of rat submandibular gland, though the possible roles yet to be known.

Poster-B5

Effect of Dan Di Qiong Yu granule on salivary gland of Sjogren Syndrome mice.

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Objective: To investigate the curative efficacy and mechanism of Dan Di Qiong Yu Granule (DDQY) on Sjogren Syndrome (SS) mice.
Methods: SS model mice were administered with DDQY Granule at three different doses (low, middle and high) with normal saline (NS) as control to observe its influences on salivary flow, $CD4^+$ cells, $CD4^+/CD8^+$, and autologous antibodies in SS model mice.
Results: Model group have significant differences in salivary flow (11.00+0.67mg), $CD4^+$ T cells (32.19±2.47%), $CD4^+/CD8^+$ (2.10±0.23) compared with control group (9.03±0.87mg, 62.66±3.44% and 4.17±0.52), $P < 0.01$. Positive rate of autologous antibodies in control group is 0, and that of model group is 46.54%, showing significant differences through analysis using exact probabilities method ($P < 0.01$).

Except that salivary flow in low-dose group, other indexes in groups administrated with middle and high dose of DDQY Granule have significant difference before (9.31±0.83 and 8.86±0.69 mg in middle and high dose group) and after(10.50±0.93 and 10.58±0.49 ml in each group) the treatment ($P < 0.01$). But for normal saline group, all indexes have no significant difference before and after the treatment . Positive rate of autologous antibodies in normal saline group didn't change before and after the treatment with an identical value of 33.33%. For low and middle dose groups, the rates before and after treatment were respectively 50.00% and 30.00%. For high dose group, the rates before and after treatment were respectively 50.00% and 22.22%. Positive rates of autologous antibodies in DDQY Granule treated group have significant differences compared with that of normal saline group through exact probability method ($P < 0.01$).
Conclusion: DDQY Granule is effective in treating SS model mice and may function through constraining lymphocyte infiltration in salivary gland.

Talk-C1

Pilocarpine induces salivary secretion and thirst in rats.

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The numbers of patients complaining about xerostomia has increased recently. Pilocarpine, a muscarinic receptor agonist, is a typical sialagogue to treat hyposalivation. Although pilocarpine makes the oral cavity wet by increasing saliva, it is well-known in animal experiments that it also induces water intake. Mechanisms underlying the relationships between these events are unknown. In this symposium, we report the central as well as peripheral effects of pilocarpine by using behavioral, electrophysiological and histochemical studies. Intraperitonially injected pilocarpine increased both water intake and parotid salivary secretion in rats. Intracerebroventricularly injected pilocarpine also induced water intake but not salivary secretion. Intracerebroventricularly applied atropine, a muscarinic receptor antagonist, suppressed the increased water intake by the intraperitonially and intracerebroventricularly applied pilocarpine. Intraperitonially injected pilocarpine increased the number of c-Fos immunopositive cells in some nuclei of the circumventricular organs, hypothalamus and medula, which are related to thirst sensation. Intracerebroventricularly applied atropine suppressed the increased number of c-Fos immunopositive cells by the intraperitonially and intracerebroventricularly applied pilocarpine. Patch clamp recording shows that pilocarpine excites circumventricular organ neurons. We conclude that peripherally injected pilocarpine affects the parotid glands and the thirst center in the central nervous system.

Talk-C2

Electrophysiological analysis of the afferent activity from the submandibular salivary gland in the rat.

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Our recent histochemical study has shown that the sensory nerves travel in both the sympathetic and parasympathetic nerve routes supplying to the submandibular gland in the rat (Brain Research, 1060: 184-187, 2005). In the present study we analyze afferent neural activities in the peripheral cut ends of the sympathetic and parasympathetic nerve branches innervating the submandibular gland in urethane-anesthetized rats. The following results were obtained: 1) The afferent activity could be recorded from both sympathetic and parasympathetic routes; 2) both afferents had no spontaneous activities, but responded to mechanical pressure applied onto the gland; 3) when back pressure was applied from the main excretory duct by infusion of saline, both afferents showed tonic impulse discharges at pressure of higher than 100 mmHg; 4) the threshold pressure was little lower than the maximal secretory pressure which was induced by electrical stimulation of the parasympathetic secretory nerve (the chorda tympani); 5) there is no differences in the threshold pressures between afferents in the sympathetic and parasympathetic nerve routes; 6) on the other hand, the sensory nerve in the parasympathetic, but not sympathetic, nerve supply responded to mechanical pressure on the main excretory duct; 7) the sensory nerve in the sympathetic nerve supply discharged after ligation of the artery to the submandibular gland.

Previous histochemical studies show that substance P and CGRP-containing nerves considered as possible afferent fibers were identified frequently around small ducts and blood vessels. Our results suggest that the sensory nerve of the duct system is sensitive to excessive pressure of the fluid secretion, and may conduct pain due to salivary stone. Blood pressure and/or hypoxia may be monitored by afferents in the sympathetic nerve supply. Our results (6 and 7) suggest that the sensory nerves in the parasympathetic and sympathetic nerve supplies are more close to ducts and blood vessels, respectively.

Talk-C3

Effects of autonomic denervation and administration of SNI-2011 on the expression of AQPs in the rat submandibular gland

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[Purpose] The present study was aimed to explore the role of the autonomic nerves on the regulation of expression of water channels, aquaporins (AQPs). [Methods] Cervical sympathetic trunk denervation (CSTD) or chorda tympani denervation (CTD) was performed on 7-week-old male SD rats. From day 15th after operation, SNI-2011 (10mg/kg) or pilocarpin (0.3mg/kg) or chloroquine (50mg/kg) was administered orally everyday for 7 days. Protein and mRNA levels of AQP5 and AQP1 in the SMG were determined by Western and Northern blottings. [Results and Discussion] Expressions of AQP5 and AQP1 were not significantly affected by CSTD. However, both the SMG weight and the AQP5 protein expression were decreased by CTD, although the expression of AQP1 protein was not changed. On the contrary, the mRNA level of AQP5 was not affected by CTD. SNI-2011, a M3 receptor agonist, recovered AQP5 protein level which had been reduced by CTD, and increased the AQP1 protein expression above control. Pilocarpin injection showed no effect on AQP protein levels. The mRNA level of AQP5 was not affected by SNI-2011. The activity of cathepsin D/E was increased by CTD and this increase was inhibited by SNI-2011. The changes of cathepsin D/E activities by CTD and SNI-2011 were also observed in acinous cells isolated by Percoll. Administration of chloroquine, a denaturant of lysosome, recovered AQP5 protein level decreased by CTD. [Conclusion] The autonomic regulation of AQP5 levels in the SMG is suggested to be controlled by lysosomal system, not by transcriptional regulation.

Talk-C4

Neural- and Hormonal-induced Protein Synthesis and Mitotic Activity and the Dependence on NO-generation.

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In salivary glands, secretion of proteins and fluid is usually thought to be solely regulated by the autonomic nerves. In recent experiments on the rat parotid gland, however, some gastro-intestinal hormones caused protein secretion without any accompanying overt secretion of fluid. In rat parotid glands, the protein secretion may be partly dependent on nitric oxide (NO) generation. The present communication focuses on the effect of inhibition of NO generation on the expected increases in protein synthesis and mitotic activity in response to electrical stimulation of the autonomic innervation or to pentagastrin infusion in the anaesthetized rat. Incorporation of radiolabeled leucine and thymidine, respectively, into trichloroacetic acid-insoluble material of the parotid gland indicated protein synthesis and mitosis, respectively. Labeled leucine was injected I.V. 30 min after the end of the stimulation period, whereas labeled thymidine was injected I.P. 18 hr post-stimulation; the glands were removed 15 min and 1 hr, respectively, after the administration of the isotope.

NO is a likely parasympathetic non-adrenergic, non-cholinergic transmitter in the parotid gland, since the *parasympathetic nerves* of the gland contain NO-synthase. Parasympathetic stimulation (30 min) in the presence of atropine and adrenoceptor blockade increased the protein synthesis (compared to the gland of the unstimulated side), by 142% (10 Hz) and 200 % (40 Hz). Surprisingly, neither the neuronal type NO-synthase inhibitor N-PLA (30 mg/kg, I.V.), nor the unspecific inhibitor L-NAME (30 mg/kg, I.V.) reduced the response. Moreover, the parasympathetic non-adrenergic, non-cholinergic nerve (40 Hz for 30 min)-evoked increase in mitotic activity (65 %) was unaffected by the two types of NO-synthase inhibitors. *Sympathetic nerves* lack NO-synthase, yet inhibition of NO-generation influenced the β-adrenoceptor mediated response to sympathetic stimulation (50 Hz, 1 s every tenth second for 30 min). Whereas the protein synthesis increased by 192% in the presence of just α-adrenoceptor blockade, the response was more than halved in the presence of N-PLA (to 86%) or L-NAME (to 91%). Furthermore, the β-adrenoceptor mediated increase in mitotic activity (122%) to sympathetic stimulation (20 Hz, 4 min every fifth min for 30 min), under α-adrenoceptor blockade, was reduced to 49% and 47% in the presence of N-PLA and L-NAME, respectively. Despite being of the neuronal type, the NO-synthase generating NO in response to β-adrenoceptor stimulation was most likely of parenchymal origin. The α-adrenoceptor mediated increase (139%) in protein synthesis (under β-adrenoceptor blockade) was not affected by N-PLA or L-NAME. Just α-adrenoceptor activation caused no increase in mitotic activity. *Pentagastrin* (20 μg/kg, I.V. infused for one hour) caused the protein synthesis to increase by 17%. The increase engaged both cholecystokinin A and B receptors and most likely, was dependent on neuronal type NO-synthase of parenchymal origin, since the response was abolished by N-PLA. Interestingly, the cholecystokinin receptor blockers reduced the basal protein synthesis (by 20%), while N-PLA did not affect the basal protein synthesis. Thus, implying that strong rather than weak stimuli of the receptors activate neuronal type NO-synthase.

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Poster-C1

Relationship of chewing-stimulated whole saliva flow rate and salivary gland size in humans

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We have recently reported that unstimulated whole saliva flow rates (UWSFR) correlate positively with salivary gland sizes and body profiles of weight and body mass indices. In the present study, the correlations of chewing-stimulated whole saliva flow rates (CWSFR) with salivary gland sizes and the body profiles were investigated, and the results were compared with those of UWSFR. Saliva samples were collected from 24 healthy young males and 26 females by the spitting method while chewing paraffin and the CWSFRs were measured. The CWSFRs in all subjects and in males correlated positively with UWSFR, but not in females. The CWSFRs in all subjects correlated positively with parotid and/or submandibular gland sizes, weights and body mass indices, just as with UWSFR; however the correlation coefficients with salivary gland sizes were smaller than those of UWSFR. In contrast to the results of UWSFR, the correlation coefficients of the CWSFRs with parotid gland sizes in all subjects were larger than those with the sizes of submandibular gland. The CWSFRs in males correlated only with parotid gland sizes, and those in females did not correlate with any of the parameters. In conclusion, the results suggest that the larger the size of the salivary glands, the greater the CWSFR, at least, in males.

Poster-C2

Cardiac-related activity in superior salivatory nucleus neurons in anaesthetized rats.

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The superior salivatory nucleus (SSN) lies within the rostral part of parvicellular reticular formation (rRFp) in the brainstem. The preganglionic neurons in the SSN regulate vasodilation and secretion of glands in the orofacial region via submandibular and intra-lingual ganglia. In the nasal mucosa, oral mucosa, lacrimal gland and cerebral vasculature they regulate these functions via the pterygopalatine ganglion. Neuroanatomical study [1] has revealed a direct monosynaptic pathway from neurons in the cardiovascular nucleus tractus solitarii to pterygopalatine SSN neurons. We recently reported that neurons in the rRFp [4] including SSN exhibit a pulse-related activity as well as parasympathetic preganglionic cells in the ambiguus nucleus (2, 5) indicating that these neurons receive cardiac inputs. These morphological and physiological studies led hypothesis that preganglionic SSN neurons projecting submandibular and sublingual ganglia receive cardiac inputs. To test hypothesis, extracellular recordings were taken from SSN neurons projecting to submandibular and intra-lingual ganglia, and investigated whether these neurons exhibit a pulse-related activity in their excitatory amino acid induced activity in urethane-chloralose anaesthetized rats.

Single SSN neurons were identified by their antidromic spike responses following stimulation of the chorda-lingual nerve, chorda tympani branches or the lingual nerve [3]. In each identified SSN neurons, an ECG/ABP-triggered correlation histogram of the activity was constructed and cardiac coefficients were calculated. A neuron was classified as having a pulse-related activity if cardiac coefficient was more than 2.0 [4]. A total of 25 SSN neurons which induced to fire by ionophoretic application of the excitatory amino acid receptor agonist (AMPA, NMDA or DLH) were tested. The pulse modulations of the excitatory amino acid induced activity in the all tested neurons were not clear based on their firing manner. In 15 SSN neurons tested, the cardiac coefficient exceeded 2.0 (2.1- 13.0, 5.6 ± 0.7, n=15; mean ± SE, number), indicating exhibiting a pulse-related activity. Of the 15 SSN neurons that exhibit a pulse-related activity, 9 neurons had B fibre and 6 neurons had C fibre. These findings suggest that many of SSN neurons projecting to submandibular and intra-lingual ganglia receive cardiac inputs and are possibly involved in vasodilator function.

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Talk-C5

Electrophysiological study on the descending excitatory synaptic inputs to the superior salivatory nucleus in the rat.

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Introduction: . Copious salivary secretion is observed during feeding in various animals. During this behavior, the superior salivatory (SS) nucleus which is the primary parasympathetic center of submandibular and sublingual salivary secretion would be activated by the following inputs; oral sensory inputs via lower center and descending inputs from the higher center such as the feeding center. Recently, we have revealed that SS neurons receive glutamatergic excitatory, and GABAergic and glycinergic inhibitory synaptic inputs in rats (Mitoh *et al*, Brain Res 999: 62-72, 2004). To examine the descending excitatory synaptic inputs, we compared the excitatory responses to the SS neurons of the normal and decerebrate rats.
Materials and methods: We used neonatal Wistar rats (5-13 days old). The SS neurons innervating the salivary glands were labeled by retrograde axonal transport of a fluorescent dye. Additionally some rats were decerebrated in order to cut off the inputs from the higher center. Two days after operation, whole-cell patch-clamp recordings were performed in the labeled cell obtained from sagittal brainstem slices at a holding potential of - 60 mV. Excitatory currents were evoked by the application of glutamate in the presence of tetrodotoxin. The inhibitory inputs (GABAergic, glycinergic) were eliminated during the experiment.
Results and discussion: In many SS neurons (n=9/12) of the decerebrate rats, the amplitude of glutamate-induced currents was increased as compared with the normal rats. This suggests that glutamate receptors at the postsynaptic membrane were up-regulated due to decreased neurotransmitter release from the higher centers after the decerebration. Thus, such SS neurons receive descending excitatory inputs from the higher center. In some SS neurons (n=3/12) the amplitude of glutamate-induced currents was unchanged, suggesting they have hardly excitatory synaptic inputs from the higher centers.

Talk-C6

Functions, regulation and mechanisms of action of SUMOylation in salivary cells.

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Covalent attachment of ubiquitin (Ub) and small ubiquitin-like modifier (SUMO) to proteins plays a major role in regulating cellular functions. While ubiquitination generally promotes protein degradation, SUMOylation regulates a variety of cellular processes, including nuclear transport, genome integrity, signal transduction, and transcriptional regulation. Briefly, SUMO is activated in an ATP-dependent manner by an E1 activating enzyme, transferred to the E2 conjugating enzyme, and subsequently attached covalently to the lysine (K) acceptor site, within a particular sequence of ΨKxD/E (Ψ represents hydrophobic amino acid) of SUMO-target proteins by distinct E3 ligases. Similar to phosphorylation, SUMOylation is a reversible process catalyzed by deSUMOylation enzymes (SENPs). The list of known SUMO targets has grown substantially in recent years and remarkably, including RanGAP-1, HIF-1, p53, and many transcription factors, such as c-jun and c-fos. In many cases, SUMOylation leads to a modified protein-protein interaction of target protein(s), thus altering the biological consequences. Recently, a SUMO-binding motif (SBM) that interacts non-covalently with SUMOylated proteins has been described, and this SBM is found to exist in nearly all proteins known to be involved in SUMO-dependent processes. Collectively, SUMOylation appears to play a vital role in governing cellular adaptive responses to environmental stress and to modulate function of many important cellular proteins related to human diseases, such as neurodegenerative disorders.

Hypoxia is a (patho)physiological condition, which arises when cellular oxygen demand exceeds supply. Hypoxic stress results in a disrupted epithelial barrier function and ZO-1 assembly and activates genotoxic response. We investigate the relationship between the 1% O_2- or hypoxia-mimetic desferroxamine (DFO)-stimulated SUMOylation processes and the ability of cells to resist cell injury elicited by these treatments. By using salivary Pa-4 cells stably transduced with lenti-SUMO-1 and a cell permeant peptide harboring SUMO-binding motif to interfere with SUMO-dependent protein-protein interactions, we demonstrate that SUMOylation augments cell survival against DFO-treatment. This appears to be, at least in part, mediated through an attenuation of PKCδ activation and caspase-3 cleavage, hallmarks of pro-cell death signaling. We further show that constitutive SUMOylation facilitates 1% O_2- or DFO-induced NF-kB transactivation, possibly via activation of genotoxic signaling cascade. Intriguingly, DFO-induced DNA damage marker ATM S1981 phosphorylation precedes the activation of PKCδ and caspase-3. In addition, we report a transient preservation of transepithelial electrical resistance (TER) during the early stage of hypoxia (1% O_2) as well as enhanced TER recovery following prolonged hypoxia in SUMO-1-expressing cell monolayers. In conclusion, our results unveil a previously unrecognized mechanism by which SUMOylation and an intricate balance among activations of genotoxic response and NF-kB signaling pathways play critical roles in modulating salivary adaptive response to either 1% O_2 or DFO, governing salivary epithelial homeostasis.

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Talk-C7

Deciphering the Biological Roles of Salivary Glands: Comparative Cell Biology, Molecular Genetics, and Evolution

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Mammalian salivary glands have proven to be excellent models for physiological and developmental studies. They also have proven valuable as models for research on protein synthesis. But what is the biological role of this organ system in mammals? Surprisingly, a comprehensive answer to this question has remained elusive. Simple textbook answers, such as oral protection, lubrication, and digestion, are clearly inadequate. What we do know is that salivary glands evolve rapidly. Species differences in cell structure provide phenotypic evidence of evolution, whereas molecular genetic data provide evidence of diversity in secretory products. But how can we make sense of this diversity? The field of systematic biology is one source of help. Phylogenies based on algorithms that use nuclear and mitochondrial gene sequences provide a framework. Phenotypic, genetic, and physiological data from salivary glands can be mapped onto this framework and the resulting pattern interpreted in terms of species histories, relationships, and ecology. Through this process we can gain insight into the adaptive and biological roles of salivary glands. Another approach is to use secretory cell mRNAs as a starting point for interspecies comparisons of secretory proteins. Research along these lines has revealed the possibility that a protein secreted by rodent submandibular glands might influence mate selection and might be the most rapidly evolving protein yet observed in mammals. Although mode of action is unknown, the possibility exists that this salivary gland secretion is a “species generator,” which would be a remarkable and unexpected biological role of salivary glands.

Poster-C3

Electrophysiological study of the inhibitory inputs from the forebrain and brainstem to the superior salivatory nucleus in rats

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Objective; We have showed the excitatory (glutamate) and inhibitory (GABA and glycine) synaptic inputs to the superior salivatory (SS) neurons innervating the submandibular salivary glands and tongue in rats (Mitoh *et al*, Brain Res 999: 62-72, 2004). However, it has not been examined whether SS neurons receive inhibitory inputs from the higher and lower centers with respect to inhibitory salivary control. In the present study, we compared electrophysiological characteristics of the inhibitory inputs in brainstem slices obtained from normal and decerebrate rats.
Materials and methods; We used Wistar neonatal rats (9-14 days postnatal). SS neurons were labeled by retrograde axonal transport of a fluorescent dye. Whole-cell patch-clamp recordings were performed in the labeled neurons in sagittal brainstem slices obtained from normal and decerebrate rats. The inhibitory currents evoked by GABA and glycine application, and the inhibitory postsynaptic currents (IPSCs) evoked by electrical stimulation near the recording cell were recorded at holding potential of 0 mV.
Results and discussion; In all decerebrate SS neurons, GABA and glycine application produced larger amplitude of inhibitory currents as compared with normal SS neurons, suggesting that $GABA_A$ and glycine receptors at the postsynaptic membrane were up-regulated due to decreased neurotransmitter release from the higher centers after decerebration. All normal SS neurons invariably showed IPSCs. In decerebrate SS neurons, 17 % showed no IPSC and 83 % displayed enhanced IPSCs. These results suggest that most SS neurons receive descending inhibitory synaptic inputs from the higher centers, with 17 % receiving inhibitory inputs exclusively from the higher centers, and 83 % having inhibitory inputs from the higher and lower centers.

Poster-C4

Role of the feeding center for submandibular salivary secretion during feeding behavior in the rat.

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It is well known that salivary secretion is mainly induced during feeding behavior. In the present study we examined role of the feeding center on flow rate of saliva induced by feeding of various texture of food, using normal and feeding center-destroyed rats. For this examination animals were placed on a food deprivation schedule and allowed access to various food for 3 hours per day for one week. Water is available ad lib. The foods were food pellets, power diet, and 3 types of mashed diet (power diet : water = 1:1.5, 1:1, and 1:0.5). After the training schedule, the animals were anesthetized and the duct of left submandibular gland was cannulated with polyethylene tubing for recording salivary flow rates, and a pair of stainless electrodes was inserted into the masseter muscle for recording EMG activities. The feeding center (lateral hypothalamus) was destroyed by passing of DC current (0.2 mA, for 20 s) through a stainless wire (200 μm). After recovery from anesthesia, the recording experiment was performed. The following results were obtained: 1) In normal rats, the average salivary flow rates for 3 min were in the order power diet > hard mashed diet > medium mashed diet > food pellet > soft mashed diet; 2) Dry weights of food consumption were in the order soft mashed diet > medium mashed diet > hard mashed diet > power diet and food pellet; 3) Total EMG activities for 3 min were similar in various foods; 4) When the ipsilateral feeding center was destroyed, about 80 % decrease in the flow rates of saliva was observed during feeding of various foods; 5) Destruction of the contralateral feeding center yielded about 40 % decrease in salivary flow rates; 6) No changes were observed in total chewing cycles or total EMG activities for 3 min. These results suggest that the movement of the jaw, sensory inputs from the periodontal membrane, or the amount of food consumption is not correlated with the flow rate of saliva, and descending effects from the feeding center is essential for salivation during feeding.

Poster-C5

The Facial Nerve and Its Influence on the Parotid Gland

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Twigs of the facial nerve transverse the parotid gland in both humans and animals. The facial nerve is, however, not thought to exert any effects on the gland. Nevertheless, in a series of experiments we have found the facial nerve to exercise both short- and long-termed influences on the parotid gland of the Sprague-Dawley rat:

1) Electrical stimulation of the peripheral end of the cut facial nerve at the level of the stylomastoid foramen evoked a small flow of saliva, about 10% of that to stimulation of the auriculo-temporal nerve, from the duct-cannulated gland under pentobarbitone-anaesthesia, which was blockable by atropine.

2) Reflex secretion elicited by pouring citric acid on the tongue of the awake animal caused a profound secretion of parotid saliva. The flow rate was reduced by 88% after section of the auriculo-temporal nerve. A further reduction, to 95%, occurred by including facial nerve section (at the level of the stylomastoid level) in the surgical procedure.

3) The total acetylcholine synthesizing capacity of the parotid gland, as judged by the activity of choline acetyltransferase activity, was reduced by 15% seven days after facial nerve section (at the level of the stylomastoid level). Section of the auriculo-temporal nerve lowered the acetylcholine synthesizing capacity by 76%. The combined surgery induced an even greater fall, by 89%.

4) The total amount of calcitonin gene-related peptide in the parotid gland, a neuropeptide known to be confined to parasympathetic nerves and to sensory nerves, was reduced by 16% seven days after facial nerve section (at the level of the stylomastoid foramen).

5) The secretory cells of the parotid glands were sensitized to secretagogues seven days after section of the facial nerve (at the level of the stylomastoid level), as revealed by increased amounts of saliva secreted from the gland in response to the intravenous injection of the muscarinic agonist methacholine.

Most of the facial secretory nerve fibres innervating the parotid gland originated from the otic ganglion, since following otic ganglionectomy (and allowing time for nerve degeneration), the secretory response to facial nerve stimulation and the reflexly elicited secretion were almost abolished.

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Poster-C6

Melatonin-evoked Protein Secretion from the Rat Parotid Gland *In Vivo*

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Melatonin, a pineal hormone, is also produced in the gastro-intestinal tract. Its receptors are found in the stomach, intestine and pancreas. Though, the general view is that hormones do not exert acute effects on salivary glands, we have recently found pentagastrin and cholecystokinin to cause in vivo secretion of protein from the parotid gland of the rat. In analogy with some parasympathetic neuropeptide transmitters, the protein secretion was not accompanied by any overt fluid of secretion from the duct-cannulated gland.

The present work aimed at exploring the acute effect of melatonin on the secretory response of the parotid gland in the pentobarbitone-anaesthetized rat under adrenoceptor blockade. The intravenous infusion of melatonin (5 mg/kg or 25 mg/kg over 10 min) evoked no secretion of saliva. However, a dose-dependent secretion of protein occurred. This response was revealed by means of a subsequent wash-out flow of saliva evoked by a bolus dose of methacholine (5µg/kg, I.V.) given 10 min after the end of the melatonin-infusion period. The effect of melatonin was compared to that of a corresponding period of saline infusion. The amount of saliva secreted to the standard methacholine injection after the infusion of saline or melatonin was the same as before the infusion period. In the control rats, subjected to saline infusion (and a 10 min long pause), the concentration of protein and amylase activity was increased by 22% and 26% in the post-infusion methacholine-evoked saliva compared to that of the pre-infusion methacholine-evoked saliva. The corresponding percentage figures were after melatonin, at a dose of 5mg/kg, 53% for the concentration of protein and 53% for the concentration of amylase activity and, at a dose of 25 mg/kg, 102% for the concentration of protein and 73% for the concentration of amylase activity. The unselective melatonin receptor antagonist luzindole (2mg/kg, I.V.) reduced the melatonin-evoked responses. Further, the presence of melatonin receptors in the parotid gland was demonstrated by immunoblotting.

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Poster-C7

Induction of general anesthesia with propofol increases salivary flow.

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Aim: The use of ketamine resulted in a significant increase in salivary secretions [1)]. However, propofol-ketamine or midazolam-ketamine, sedation-analgesia, induced no significant increase in salivary flow [2)]. These results imply that administration of anesthetics affect salivary flow. In this paper, we investigated salivary flow after induction of general anesthesia with propofol or sevoflurane.
Methods: Subjects underwent oral surgery with general anesthesia. Patients who regularly used medicines and had salivary gland diseases were excluded. We divided the subjects into two groups (n=10 each); general anesthesia was induced by 1) propofol (2 mg•kg^{-1}, iv) or 2) sevoflurane (5%, inhal). Midazolam (3 mg, im) and 0.1 mg•kg^{-1} vecronium bromide were given to all patients, and then nasotracheal intubation was performed. To measure salivary flow, four dental cotton rolls were put on the floor of the mouth (sublingual carunclae) and oral vestibules (parotid papillae) for 1 min, and the weight of cotton rolls were measured at 30 sec and 10 min after intubation.
Results: In the propofol-treated group, salivary flow was significantly increase at 30 sec after intubation and then returned to the control level at 10 min after intubation. On the other hand, in the sevoflurane-treated group, salivary flow was no clearly changed at 30 sec and 10 min after intubation.
Conclusions: It is necessary to reduce salivation in induction of general anesthesia with propofol for preventing airway troubles, although propofol is a useful general anesthetic for rapid induction.
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Talk-D1

Regulation of epithelial tube formation in developing mouse submandibular gland.

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Epithelial tissues in most organs show tubular structures of polarized cells with defined apical or luminal, and basolateral plasma membrane domains. The tight junction (TJ) forms the structural barrier separating the two membrane domains and regulating solutes across cellular sheet, in which the claudin transmembrane proteins are directly involved. In vertebrate organs such as the submandibular salivary gland (SMG), epithelial tissues initially arise as solid cell masses with no lumens, which cells lack TJs. Subsequently, lumens form in the cell masses, with the de novo establishment of TJs and the apical and basolateral cell surfaces defined by the junctions. However, how TJ formation is coordined with membrane biogenesis in and contribution to tubulogenesis is largely unknown. Here, we investigated assembly of TJ proteins and roles of claudins in lumen formation of the developing mouse SMG. Immunofluorescence microscopy revealed that TJ proteins sequentially assemble in the solid cell masses, with claudins last recruited before the formation of definite lumens. To investigate the role of claudins in lumen formation, we employed a *Clostridium perfringens* enterotoxin fragment (C-CPE), which specifically binds to and remove several claudin species from TJs. Incubation of the developing SMG with C-CPE caused downregulation of most claudins without affecting the expression of ZO-1 and occludin and blocked lumen formation in epithelium by interfering with apical surface separation of apposing cells. In addition, apical but not basolateral membrane proteins were mislocalized in C-CPE-treated glands. Furthermore, we found that hedgehog signaling promoted epithelial lumen formation together with the formation of claudin-bearing TJs and apical membrane biogenesis. These findings suggest that claudin-dependent apical membrane biogenesis participates in lumen formation during organogenesis and is positively regulated by hedgehog signaling.

Talk-D2

FGF10 regulates branching morphogenesis during salivary gland development.

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Fibroblast growth factors (FGFs) are a family of heparan sulfate-binding growth factors that regulate branching morphogenesis of mouse submandibular glands. Mutations in human FGF10 are associated with salivary gland aplasia/ hypoplasia, and mice lacking FGF10 have no salivary glands while mice heterozygous for FGF10 have hypoplatic glands. FGF10 stimulates proliferation and morphogenesis of isolated salivary rudiments in organ culture in vitro. Addition of exogenous FGF10 to epithelial rudiments induces cell proliferation at the tips of the ducts resulting in branching and duct elongation. This FGF10 mediated morphology is modified by exogenous heparin fragments containing 6-O-sulfation, suggesting that mesenchymal 6-0-sulfates may present/localize FGF10 to the epithelium to mediate cell proliferation. Indeed, in situ analysis shows mesenchyme localization of 6-O-sulfotransferase increased expression adjacent to areas of proliferating epithelium. Heparitinase treatment also inhibits growth of FGF10 treated epithelial rudiments. These data suggest FGF10 induced proliferation in the epithelium is dependant on an epithelial heparan sulfate proteoglycan. PCR and in situ analysis show increased gene expression of heparan sulfate 3-O-sulfotransferases, and heparan sulfate proteoglycans in the periphery of proliferating epithelium. In addition, siRNA knockdown of the heparan sulfate 3-O-sulfotransferases reduces the amount of branching in FGF10-treated epithelium. Collectively, our data suggest that FGF10-mediated cell proliferation is regulated by HSPGs with 6-O-sulfation in the mesenchyme, and 3-O-sulfation in the epithelium. The differential localization of heparan sulfate proteoglycans with different patterns of sulfation may modulate the biological activity of FGF10 in different tissues during salivary gland development.

Talk-D3

Involvement of a subtilisin-like proprotein convertase, PACE4, in branching morphogenesis and AQP5 expression in the rat embryonic submandibular gland

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The submandibular gland (SMG) develops under the epithelial-mesenchymal interaction; *i.e.*, the oral epithelium downgrows into the mesenchyme, and continually develops by repeating dichotomous branching of the epithelial buds (branching morphogenesis; BM). This process is mediated by various growth/differentiation factors including activin and BMP, which are synthesized as inactive precursors and activated *via* the limited proteolysis at their R-X-(K/R)-R site by subtilisin-like proprotein convertases (SPC). In this study, we focused upon the BM and expression of the water channel AQP5 of the SMG as differentiation markers, and analyzed the effects of various protease inhibitors, antibodies, and siRNAs to clarify the molecular mechanism of the SMG development. In the organ culture system, Dec-RVKR-CMK, a potent inhibitor for SPC family, inhibited the BM of rat embryonic SMG and decreased AQP5 expression. However, other inhibitors for trypsin-like serine proteases such as leupeptin, soybean trypsin inhibitor, Dec-RVRK-CMK, and H-D-FPR-CMK, did not affect the BM and AQP5 expression of the SMG. Dec-RVKR-CMK also decreased the PACE4 expression, a member of SPC, but not furin, another member of the family, suggesting the participation of PACE4 in development of the SMG. Heparin, which is known to translocate PACE4 in ECM to medium in culture cells, and the specific antibody for the catalytic domain of PACE4 also affected the BM and AQP5 expression of the SMG. These inhibitory effects of Dec-RVKR-CMK were partially rescued by addition of recombinant BMP2 whose precursor is one of the candidate substrates for PACE4 *in vivo*. Further, the inhibition of PACE4 expression by siRNAs caused both the decreased expression of AQP5 and inhibition of the BM in the present organ culture system. These observations support the idea that SMG development is regulated by subtilisin-like proprotein convertase PACE4 *via* the activation of growth/differentiation factor(s).

Poster-D1

Freeze fracture studies of tight junctions in mouse salivary glands and cultured salivary cell lines.

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Introduction: Aquaporin 5 (AQP5) mediates transcellular water movement in salivary glands, whereas paracellular flow is regulated by tight junctions (TJs). The mechanisms that coordinate fluid secretion by these two pathways are unknown.
Objectives: This study was designed to examine the ultrastructure of TJs in the parotid (PG) and submandibular (SMG) glands of wild type and AQP5 knockout mice, and TJs of cultured rat SMG epithelial cell lines (SMG-C).
Methods: TJs were studied in freeze-fracture replicas of glutaraldehyde-fixed PG and SMG of wild type and AQP5 knockout mice (129SvJxBlackSwiss/Aqp5 +/+, -/-). Saliva secretion was stimulated in some animals by pilocarpine treatment. SMG-C cells were transfected with AQP5 or mock transfected and cultured in transwell dishes. Some cultures were treated with carbachol. Fixed cells were scraped off the transwell membrane and prepared for freeze-fracture. TJ structure was analyzed by counting the mean number of strands/TJ, the number of free ends and disruptions/mm of TJ length, and TJ complexity was determined by fractal geometry (Kniesel et al., Tissue & Cell 26:901, 1994). Statistical significance of the results was assessed by ANOVA.
Results: Acinar TJs usually were short with few strands whereas those of duct cells were longer and had more strands. The number of strands/acinar TJ was slightly increased ($p=0.0506$) in the parotid glands of AQP5 -/- compared to +/+ mice, and after pilocarpine treatment of both +/+ and -/- mice ($p<0.05$). There were no differences among groups in the number of free ends and disruptions, or in TJ complexity. In the SMG, the number of strands/granular duct cell TJ was significantly greater than for acinar TJs ($p<0.001$). However, no differences were observed among the four groups (+/+, -/-, unstimulated, pilocarpine stimulated) in the number of strands/acinar TJ. TJs of SMG-C cells were well developed with numerous strands. After carbachol stimulation, the mean number of strands/TJ in the mock-transfected cells was significantly increased ($p<0.001$). In the AQP5 transfected cells, the mean number of strands/TJ decreased after carbachol stimulation, although not significantly ($p=0.069$).
Conclusions: In many tissues, TJ strand number is correlated with junctional "tightness". The increased number of TJ strands after pilocarpine stimulation and in AQP5 -/- mice suggests decreased TJ permeability. In +/+ mice, AQP5 and TJ components may communicate to control fluid secretion pathways. In SMG-C cells, transfected AQP5 also may interact with TJ components; the initial data suggest similarities with the *in vivo* situation.
Supported by The Nippon Dental University (Tokyo, Japan), NIH grant DE13823 and UCHC.

Poster-D2

Shared or Non-overlapping Intracellular Signaling Pathways Activated by EGFR or FGFR Differentially Regulate Branching Morphogenesis in Fetal Mouse Submandibular Glands.

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Although growth factor signaling is required for the embryonic development of many organs, the individual signaling mechanisms regulating these specific organogenetic processes are just beginning to be defined. Branching morphogenesis in the fetal mouse submandibular gland (SMG) is controlled by several families of growth factors, including epidermal growth factor (EGF) and fibroblast growth factor (FGF) ligands and their receptors. We have previously reported that EGF is expressed in the fetal mouse SMG, and stimulates development and branching morphogenesis of the SMG by activation of Erk-1/2, PI3K, PLCg1, and PKCs.

In this study, we compared signaling activated by three growth factors, EGF, FGF7, or FGF10, and correlated it with the specific morphogenetic processes elicited by the growth factors. Western blotting analysis showed that EGF strongly and clearly stimulated the phosphorylation of Erk-1/2 and weakly stimulated phosphorylation of PLCg1 and PI3K in cultured E14 SMG. However, FGF7 stimulated the phosphorylation of both PLCg1 and PI3K, but elicited only minimal phosphorylation of Erk-1/2. FGF10 also stimulated the phosphorylation of both PLCg1 and PI3K, but did not stimulated Erk-1/2 phosphorylation. None of these growth factors led to phosphorylation of JUN-kinase or p38MAPK, the two other principal substrates of MEK phosphorylation.

Morphological study of mesenchyme-free epithelium of E13 SMG cultured in Matrigel revealed that EGF induced cleft formation of end buds, and that FGF7 stimulated both cleft formation and stalk elongation, but that FGF10 specifically induced only stalk elongation. In mesenchyme-free SMG epithelium in Matrigel simultaneously cultured with EGF, FGF7 and FGF10, the specific inhibitor for Erk-1/2 activation, PD98059, completely blocked cleft formation, whereas U73122, a PLCg1 inhibitor, suppressed stalk elongation.

These finding suggest that EGF stimulates the process of cleft formation and drives branch formation via Erk-1/2 signaling, and that FGF7 stimulates both cleft formation and stalk elongation via PLCg1 and partly via Erk-1/2, but that FGF10 stimulates stalk elongation of fetal SMG epithelium via PLCg1, but not Erk-1/2, signaling pathways.

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Poster-D3
Cellular Aspect of Salivary Gland Branching Morphogenesis.

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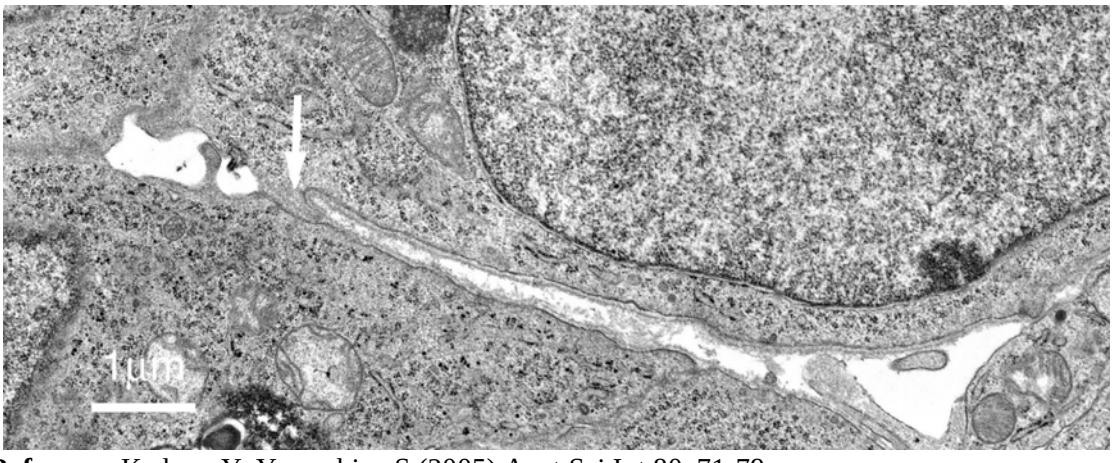
Epithelial branching morphogenesis is a fundamental process that generates complex tissue architecture of various exocrine organs. The overall process of the branching has been shown to depend on growth factors, extracellular matrices, and cytoskeletal elements. Although epithelial branching starts with a formation of cleft at the basal surface of epithelium, direct mechanism involved in this early step remains to be described. One possible reason for this is the fact that only some of the new clefts become stable, while others disappear shortly.

We have recently found that one laminin peptide, LVLFLNHGH (a functional peptide sequence located in the connecting loop between strands *E* and *F* of LG4 module of laminin-a5), perturbed *in vitro* branching morphogenesis of rudimental submandibular gland (SMG) and resulted in irregularities in the contours of its epithelium, with formation of many clefts. To reveal the structural aspect of epithelial cleft formation, here we studied the ultrastructure and the localization of cytoskeletal elements of the clefts in the SMG rudiments treated with the peptide.

Embryonic day-13 SMG rudiments of mice were cultured for 3 days in serum-free DMEM/F12 medium supplemented with LVLFLNHGH peptide (250mg/ml). The rudiments were either fixed with glutaraldehyde and processed for TEM or fixed and stained with phalloidin-rhodamine for whole mount staining.

By TEM, typical clefts were 0.3mm width and deepened between adjacent cells. The length of the cleft occasionally reached more than 10 mm. The contour of epithelial cell facing bottom and cleft was covered with continuous basement membrane. At the deepest end of the cleft, short cytoplasmic projection, hereafter “hook”(see figure, arrow), was noted in more than 2/3 cases. Phalloidin-rhodamine staining showed that the expression of actin filament was seen along the cell surfaces facing neighboring cells but less extensively along those facing basement membranes.

We suggest that the epithelial cleft formation is an active cell shape change driven by physical force that is generated by epithelial cells. “Hook” may play role, but further studies are needed to elucidate an entire process of the cleft formation.



Reference Kadoya Y, Yamashina S (2005) Anat Sci Int 80, 71-79.

Poster-D4

The expression of cellular markers of duct/acini and side population dynamics in the duct-ligated mouse submandibular gland.

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[Purpose] Tissue injury induces regenerative responses including cell proliferation/ differentiation. Ligation of the main excretory duct of the submandibular gland (SMG) induces apoptosis of acinar (Ac) cells and proliferation of duct cells. Intercallated duct (ID) cells in the developing gland are considered to serve as the stem cells for striated ducts (SD) / granular convoluted tubules (GCT) and Ac cells, but this idea is not established yet. [Methods] The main excretory duct of unilateral gland of C57BL/6 mice was ligated carefully so that the accompanying nerves and blood vessels are not injured. The contralateral gland was left intact and used as a control. Mice were killed at 0, 1, 3, and 6 days after ligation and the expressions of AQP5, kallikrein mK22, Na^+,K^+-ATPase, and Sca-1 were examined by Western blotting and immunohistochemistry. Side population (SP) cells were identified and analyzed on the basis of the feature that these cells efflux Hoechst 33342. [Results] AQP5, kallikrein mK22, Na^+,K^+-ATPase as markers for Ac, GCT, SD cells, respectively, were decreased at 1, 3 and 6 days after ligation. However, at 1 day after ligation, the expression of Sca-1, a stem cell marker, was significantly increased. Since Sca-1 is known to be expressed on some of SP cells, we hypothesized that ligation induces generation of SP cells. We found changes of numbers of SP cell were well concurred with the changes of Sca-1 positive cell. By immunohistochemistry, Sca-1 was expressed only in the ID cells, ED and myoepithelial cell on 0 day. On the 1st day after ligation, Sca-1 was induced in many species of ducts including of ID, SD, GCT and ED. [Discussion] In this study we showed that Sca-1 was expressed in the SMG and its expression was not restricted in the ID cell. By ligation, Sca-1 became to express in SD, GCT and ED, besides ID. These results implied that ligation have induced proliferation in all cells in the duct systems. Acinar cells would not enter into the proliferation phase probably because Sca-1 was not expressed. This idea is not contradictory to the known fact that ligation induces apoptosis of Ac.

Poster-D5

Role of PDGF in salivary gland morphogenesis.

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Objectives: Branching morphogenesis of submandibular gland (SMG) is regulated by multiple growth factor. For example, fibroblast growth factor and its receptor is highly expressed in SMG. Recent studies suugest a primary role of FGF10/FGFR2b signaling in the initiation of the gland, an essential role of FGF8 at later stages. Platelet-derived growth factor (PDGF) receptor was also expressed in mouse SMG. However, the role of PDGF and their receptor has never been clearly understood. The purpose of this study is to analyze the effect of PDGF in organ cultured SMG.

Methods: 1) SMG was dissected from embryonic day 13 ICR mice, placed on polyethylene terephthalate filters, and cultured in D-MEM/F12 medium supplemented with 10 ng/ml PDGF-AA or 1 ng/ml PDGF-BB. SMGs were photographed, counted the number of end buds, and analyzed the epithelial cell proliferation at every 24 hours. E13 SMGs were incubated in 1.6 U/ml of Dispase in Hanks' balanced salt solution at 37℃ for 20 minutes. Epithelium were separated from the mesenchyme with fine forceps. 2) Total RNA was extracted from E13-E16 SMGs using TRIzol reagent. Expression of PDGFs and their receptors were analyzed by RT-PCR.

Results: SMGs branching increased 1.5-fold by PDGF-AA and 1.8 fold by PDGF-BB, respectively, compared with control after 72hr incubation. PDGF-A gradually increased and peaked at E15 SMG. PDGFαR expression was highest at E13. PDGF-B and PDGFβR expression were highest at E15 during embryonic stage. At E13 SMG, PDGF-A was mainly expressed in SMG epithelium, however, PDGF-B was only expressed in mesenchyme, PDGFαR and βR were mainly expressed in mesenchyme. Further, the expression of FGFs, especially FGF-7 and FGF-10 were induced by the stimulation of PDGF-BB. These results suggest that PDGF-AA and PDGF-BB may affect the expression of FGFs in mesenchymal cells, and then enhaced the branching morphogenesis of SMG.

Poster-D6

The change of the subcellular localization of CD38 in the rat sublingual gland during saliva secretion

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CD38 has been known to produce the intracellular Ca^{2+}-mobilizing molecules “cyclic ADP-ribose” and “NAADP” from NAD^+ and NADP, respectively. However, since CD38 is an ectoenzyme, the topological paradox has been argued; both cyclic ADP-ribose and NAADP functions in the cytosolic area, but these molecules are produced into extracellular or/and intravesicular spaces. Between the three major salivary glands in the rat, the most strong expression of CD38 has been found in the sublingual gland. In this study, we investigated the change of the subcellular localization of CD38 during saliva secretion in this gland. With no stimulation, the sucrose density gradient centrifugation (SDG) profile of CD38 showed two major peaks; one is the heavy density peak (d=1.15) and the other is the light density peak (d=1.05). Since the heavy density peak showed well coincidence with the SDG profile of Na^+/K^+ ATPase, the plasma membrane localization of CD38 was suggested. MG1, mucous secretory protein, was fractionated into the light density peak. When salivary glands were stimulated with pilocarpine, an agonist of parasympathetic nervous system, the SDG separation profile of CD38 changed; the heavy density peak area increased and the light density peak disappeared, but a new peak (d=1.11) was appeared, which also contained MG1. Morphologically, under parasympathetic stimulation, the sublingual acinar cells shrank and secretory granules completely vanished, which indicated the discharge of secretory materials. The pre-treatment of rats with atropine, an antagonist of parasympathetic nervous system, before pilocarpine administration, completely inhibited not only the parasympathetic saliva secretion but also the change of the SDG separation profile of CD38. On the other hand, when salivary glands were stimulated with isoproterenol, an agonist of sympathetic nervous system, the SDG separation profile of CD38 did not changed. Under sympathetic stimulation, the sublingual acinar cells showed almost no morphological changes. These results suggested that CD38 located in the both plasma and secretory granule membrane in the rat sublingual mucous acini. Parasympathetic stimli induced salivary secretion from this gland and CD38 located in the secretory granules would fuse to the plasma membrane.

Talk-D4

Submandibular and sublingual glands in Nkx2-3 mutant mice.

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Introduction: The differentiation and development of the salivary glands is dependent upon the appropriate expression of numerous growth factors, their receptors and associated signaling molecules, and the activity of cell and organ specific transcription factors. The NK genes encode homeodomain transcription factors involved in specification of positional information, cell type determination and morphogenesis. Nkx2-3 is expressed in acinar and ductal cells of the developing and adult mouse sublingual gland (SLG), lingual mucous glands, and weakly in the submandibular gland (SMG) (Biben et al., *Int J Dev Biol* 46:415, 2002). Histological examination of the SLGs of Nkx2-3 homozygous mutant mice revealed delayed mucous cell development, disruption of cellular architecture and amplification of a "non-acinar" cell type.
Objective: The goal of this study was to determine the effects of targeted mutations of Nkx2-3 on mouse salivary gland differentiation, development and cell specific secretory protein expression.
Methods: SLG and SMG were dissected from Nkx2-3 mutant ($129SvJxC57BL6J^{Nkx2\text{-}3\text{-}/\text{-}}$) (Wang et al., *Dev Biol* 224:152, 2000) and wild type (wt) (C57BL6J, Jackson Laboratories) mice on the day of birth and at various ages up to 4.5 months. The glands were fixed in 4% paraformaldehyde in Na cacodylate buffer and prepared for low temperature embedding in LR Gold resin. One micrometer sections were stained with methylene blue-Azure II for morphologic evaluation, or labeled with antibodies to salivary secretory proteins using an immunogold silver staining procedure.
Results: The wt SLG was well differentiated at birth, with large, pale-staining mucous acinar cells and small serous demilune cells. The main histological change noted during postnatal development was differentiation of occasional granular duct cells in some of the intralobular ducts of sexually mature males. In neonatal Nkx2-$3^{-/-}$ mice, the SLG serous cell component was more prominent than in wt, whereas mucous acinar cells were small and their granules occasionally exhibited dense cores similar to those seen during mucous cell differentiation in wt glands (Wolff et al., *Anat Rec* 266:30, 2002). By 20 days of age, the mucous cells had increased in size, but remained smaller than those of wt mice. Adult Nkx2-$3^{-/-}$ SLG mucous cells expressed low levels of Smgc, a splice variant of sublingual mucin (Muc19); Smgc normally is restricted to the neonatal SMG. Serous demilune cells were more numerous in adult Nkx2-$3^{-/-}$ glands, and expressed higher levels of Dcpp and Psp than wt. The duct system of the adult Nkx2-$3^{-/-}$ SLG was markedly altered, with an increase in the number and size of intralobular ducts. In males, the duct cells appeared similar to SMG granular duct cells, with numerous secretory granules containing high levels of EGF. Similar but less pronounced changes were seen in the ducts of female glands. The morphology of the SMG of both male and female Nkx2-$3^{-/-}$ mice was generally similar to that of wt mice at all ages examined.
Conclusions: These results suggest that Nkx2-3 has cell specific effects in the SLG. It apparently enhances mucous cell differentiation and may regulate serous cell proliferation and function. In the SLG Nkx2-3 also appears to repress development of the SMG granular duct phenotype.

Supported by NIH grant DE09428, UCHC and The Nippon Dental University.

Talk-D5

A novel mouse protein differentially regulated by androgens in the submandibular and lacrimal glands

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We characterized a cDNA clone derived from female mouse submandibular gland (SMG). The transcripts of this cDNA were approximately 1.2 kb in size and predicted to code a 165 amino-acid protein with a putative signal peptide for secretory pathway. This protein, named SMARP (submandibular androgen-repressed protein), had amino acid homology at the N-terminal regions with members of the glutamine/glutamic acid-rich protein (GRP) family of rats. Northern blot analysis revealed that SMARP mRNA is expressed, out of the major mouse organs, only in the SMG and exorbital lacrimal gland (LG), with much more abundance in the former. For SMG, the level of SMARP mRNA was 36 times higher in females than in males, whereas for LG it was 28 times higher in males than in females. Furthermore, the level of SMARP mRNA was increased in SMG but reduced in LG with castration of males, whereas it was reduced in SMG but increased in LG after administration of testosterone to females or castrated males. In situ hybridization detected the signal for SMARP mRNA in female SMG, and immunohistochemistry detected the signal for SMARP protein in female SMG and male LG. In female SMG, SMARP mRNA and protein were localized intensively in a subpopulation of acinar cells, whereas in male LG SMARP protein was distributed diffusely in all acinar cells. These results suggested that SMARP is a secretory protein whose expression is regulated by androgens negatively in SMG and positively in LG.

Talk-D6

Identification and therapeutic potential of salivary gland side population cells

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Purpose Stem cell based-therapy is a promising treatment for patients suffering from severe dry mouth. We identified side population cells (SP cells), which are highly enriched for stem cell activity, in salivary glands to apply them for the cell therapy of hypofunction in the damaged glands.

Materials and methods Submandibular glands obtained from C57BL/6J (6-wk-old, male) mice were digested with collagenase and hyaluronidase. After the digestion, salivary organoids (ductal and lobuloalveolar structures) were isolated and disaggregated in trypsin-EDTA, followed by filtration through a 40μm mesh. Isolated epithelial cells were incubated in Hoechst 33342 and resuspended in HBSS containing propidium iodide. Cells from the fractions of SP cells and main population cells (MP cells) were sorted on a flow cytometry into tubes. By RT-PCR analysis, expressions of Bcrp1 and several stem cell markers were examined in SP and MP cells. We also analyzed differential gene expressions between SP and MP cells by cDNA microarray. In addition, to examine whether SP cell transplantation can recover the function of the glands, we isolated SP cells from submandibular glands of transgenic mice expressing green fluorescent protein (GFP) and transplanted them into mice with hypofunction in the glands induced by irradiation.

Results Isolated SP cells expressed Bcrp1 and Sca1, but not Oct4, c-Kit, and CD34. By cDNA microarray, we could identify a SP cell-specific gene in salivary glands. Furthermore, SP cell transplantation enabled recovery from the hypofunction in the irradiated glands, although the transplanted cells were only sparsely distributed in the glands of the recipient mice, suggesting that soluble factors secreted from SP cells are possibly involved in the rescue of secretory function.

Conclusion Our data suggests that SP cell transplantation is available for the recovery of secretory function in damaged glands.

Poster-D7

Heat Shock Protein 27kDa Regulates Differentiation and Regeneration of Acinar Cells of the Rat Submandibular Gland

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Heat shock proteins are induced by heat shock and other physiological stresses, and protect cells from cell death through their roles as "molecular chaperone". The 27kDa heat shock protein (Hsp27) has been suggested to work at the crucial cellular checkpoints for proliferation, apoptosis and differentiation. In the present study, we have examined the chronological immunolocalization of Hsp27 in the submandibular gland of postnatal developing rats as well as in the acinar regeneration after unligation of the submandibular duct of adult rats.

Postnatal development

At 3w, Hsp27 immunoreactivity disappeared in acini and ducts, but was intensely accumulated in the cells forming a small group at the center of acini. These cells were identified as the terminal tubular (TT) cell. At 4w, Hsp27-immunopositive cells were still observed in the acinar center, whereas the TT cells were hardly found in this area. Electron microscopically, Hsp27-immunoreactivity was localized in the "immature" acinar cells. After 5w, Hsp27 immunoreactivity was no longer detected in acini and ducts except for some intercalated duct (ID) cells. Submandibular glands at 3w consecutively injected with isoproterenol (IPR) to induce the acinar differentiation showed smaller number of Hsp27-immunopositive cells than the control glands.

Duct ligation experiment

In the control and normal submandibular glands, Hsp27-immunoreactivitv was observed in the nerves and blood vessels but not in the duct-like structures. Three days after duct unligation, small number of Hsp27-immunopositive cells was found in the distal ends of regenerating acinar portion. Hsp27-immunoreactivity was intensely localized in the cytoplasm of small and round-shaped epithelial cells. Hsp27-positive cells were increased in number until 7 days after unligation, but no positive cells were recognized in the acini after 14 days after unligation. Histologically, the acinar regeneration had completed after 14 days. Double immunohistochemistry of Hsp27 plus proliferation marker (PCNA) or acinar differentiation marker (GRP-a) demonstrated that Hsp27 was transitionally expressed at a boundary phase between proliferational and differentiational periods during acinar regeneration.

Conclusion

These results suggested that TT cell is the pleiotropic precursor of ID cells as well of acinar cell, and that, a part of TT cells die by apoptosis, and that Hsp27 plays important roles in the downregulation of proliferation and upregulation of differentiation of acinar cells during the development and regeneration of salivary glands. In addition, Hsp27 was suggested to be an useful marker to detect the acinar progenitor cells of the rat submandibular gland.

Acknowledgements

The authors wish to thank Prof. S. Iseki and Prof. A.R. Hand for their kind support.

Poster-D8

Temporary accumulation of glycogen in the epithelial cells during developmental differentiation of the mouse submandibular gland revealed by the high-pressure freezing/freeze substitution-TEM.

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Glycogen storage during late stages of embryonic development is known in various organs. However, glycogen accumulation in developing submandibular glands (SMG) has not been reported. A combination method of high pressure freezing (HPF) which provides a deep vitreous freezing of biological samples in milliseconds and subsequent freeze substitution (FS) has been expected to minimize the loss and redistribution of a variety of cellular and tissue elements, even water-soluble materials without limiting membrane, such as glycogen. In the present study, we report temporary accumulation of glycogen in the mouse submandibular epithelial cells detected by light microscopic histochemistry and transmission electron microscopy (TEM) using a HPF/FS method. For light microscopy, in order to avoid the loss of glycogen, tissues were fixed with ethanol and embedded in JB-4 resin which does not require dehydration and substitution processes. Glycogen was detected by PAS staining, periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) reaction, and digestion test with α-amylase. At embryonic day 14 (E14), glycogen accumulation initiated in proximal portions of the developing epithelial cords, and then extended to the distal portions as the cord branching and elongation continued. The appearance of glycogen deposition in the duct cells paralleled the duct lumen development and glycogen particles were found at the basal portion of the cells. Glycogen storage in the duct cells was retained by around postnatal day 12 (P12d). At E17, an abrupt appearance of glycogen accumulation occurred in the secretory cells of the terminal bulb portions. TEM observations showed that a large mass of flocculent substance which was not bordered by limiting membrane, varying in shape and size, was conspicuous in the cytoplasm of the differentiating secretory cells of E17. PA-TSC-SP reaction stained the substance as homogeneous dense particles, suggesting that this flocculent substance is glycogen. In the secretory cells of neonatal SMGs, each glycogen accumulation mass reduced in size associated with the abundant cytoplasmic organella and localized in Golgi area adjacent developing secretory granules or lysosomes. In the postnatal SMG, glycogen accumulation in the secretory cells of the terminal units became gradually inconspicuous in parallel with an increase of secretory granules, and finally completely disappeared by P3d. It was noted that, throughout the SMG organogenesis, the epithelial cells in the neck portions between the developing terminal bulbs and elongating cords remained free of glycogen storage. Furthermore, BrdU-labeling analysis showed that appearance of glycogen accumulation in duct cells and acinar cells is an event subsequent to the cessation of cell proliferation. These results show that the HPF/FS is useful for the preservation of unstable substance ultrastructurally, such as water-soluble glycogen accumulation. It is also revealed that during the organogenesis of SMG, glycogen accumulation occurs at the defined stages, immediately after the proliferation and before the special differentiation, such as luminal formation and secretory granule formation, confirming a significant involvement of the carbohydrate metabolism.

Poster-D9

Effects of actin-related drugs on exocytosis in parotid acinar cells.

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Objective: In parotid acinar cells, activation of b-adrenergic receptors provokes exocytotic amylase release. Since apical actin network undergoes reorganiztion during the regulated exocytotis, regulation of actin cytoskeletal structure is considered to be involved in the exocytosis. Actin-related drugs have been used to investigate the relation between amylase release and reorganiztion of actin cytoskeleton. In this paper, we investigated the effects of actin-related drugs on amylase release and actin depolimerization in rat parotid acinar cells.

Methods: Parotid glands were removed from 6-weeks old Sprague-Dawley rats, and the dispersed acinar cells were prepared using trypsin and collagenase A. For determination of amylase release, acinar cells were stimulated by the b-receptor agonist isoproterenol in the presence of actin-related drugs, and amylase activity released into medium was assayed by the method of Bernfeld. For the visualization of F-actin, cells were labeled with Alexa-488 phalloidin and the fluorescence was measured with a laser scanning confocal microscope.

Results: In the presence of the actin polymerization inhibitor cytochalacin D, the actin stabilization reagent jasplakinolide and actin-related protein inhibitor Y-27632, isoproterenol-induced amylase release was partially reduced. In confocal microscopy, F-actin was observed in cortical layer in the unstimulated cell, and adhesion of Ω-shaped F-actin to apical membrane was induced in the isoproterenol-stimlated cells. Cytochalacin D and jasplakinolide inhibited isoproterenol-induced adhesion of Ω-shaped F-actin, reduced fluorescence intensity and induced aggregation of cortical actin. Furthermore, jasplakinolide induced apoptosis. Y-27632 had no significant effect on microscopic observation in the isoproterenol-stimulated cells.

Conclusion: These results suggest that amylase release inhibition by actin-related drugs is caused by not only detriment of actin regulation but also other factors.

Poster-D10

Control of paracellular transport and its morphological evidence in perfused rat submandibular gland

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Introduction: The para-cellular route for fluid secretion, in particular, is still a long-standing question. The aim of this study is to visualize the alterations on TJ structure caused by isoproterenol (IPR) on carbachol (CCh) stimulation in the isolated, perfused rat submandibular gland (SMG), using freeze-fracture (FF) replicas on rapidly frozen tissues.
Materials & Methods: Isolated perfused rat SMG were non-stimulated as controls, and stimulated with CCh/IPR. For three-dimensional analysis by the quick freezing deep etching FF replica method, fresh submandibular tissues obtained from the perfused rat submandibular gland system were cut, immediately transferred, and rapidly frozen with liquid helium by metal contact without chemical fixation (MF-7000/Meiwa). After cutting and deep etching at -110 to -100 °C, freeze-fracture replicas were obtained from the fractured surfaces of both specimens by rotary shadowing with platinum and carbon coating at –100 °C using the freeze fracture equipment system (BAF 400/BARZERS). These replicas were cleaned with a solution of sodium hypochlorite and distilled water and examined by transmission electron microscopy (H-7100 and H-7650/ Hitachi).
Results and Discussions: In present observation, the strand particles of TJs formed 2-5 lines along most of the luminal side at rest, and microfilaments and also 10nm intermediate filament located below the TJ strands. Furthermore the bridging structure was recognized as directly connecting between TJ membrane particles and submembranous filament. In the vertical fracture surface, submembranous cytoskeletal filament arranged as thick layer. TJ strand on the lateral membrane, and secretory granules were connected with thick cytoskeletal filament. After CCh/IPR stimulation, the canaliculi became dilated, and the tight junction structure in the intercellular canaliculi became more contracted, meandering, and intermittent. The strand particles of TJs rearranged with free ends and terminal loops. Notable structural change occurred on the luminal membrane; the microvilli completely disappeared except just cell adjacent area, and numerous secretory granules fused to luminal membrane. These structural changes might be results of rearrangement of actin filaments networks under the luminal plasmamembrane. Direct interactions between the TJ membrane particles and submembranous actin cytoskeleton may modulate a paracellular permeability mechanically. Accordingly, contraction of submembranous actin cytoskeleton during the exocytosis by CCh/IPR stimulation, may cause rearrangement of TJ strands particles and thus increase of paracellular permeability. Rearrangement and movements of TJ membrane particles such as occludin and claudins may involve reconstruction of the subluminal membranous actin filament network through the intermediary of interstitial molecules such as ZOs.

Conclusion: After CCh/IPR stimulation, TJ strand are arranged roughly and become straggled and interrupted, and these changes on TJ may be related to a novel arrangement of the subcellular structures especially of the actin filament network and the TJ associated protein. The structure of tight junction can be changed by secretory stimulation to allow paracellular transport.

Poster-D11

Localization of G proteins in the main excretory duct of the rat submandibular gland with special reference to the MED tuft cell and the taste bud type II cell.

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The epithelium of the main excretory duct (MED) of the rat submandibular gland is composed of heterogeneous cell types, including type I and II light cells, dark cells, tuft cells and basal cells. Tuft cells resemble taste bud type II cells with regard to the distribution of their cell organella (1). The taste bud type II cells express Gα-gustducin, the heteromeric GTP-binding protein α-subunit, implicating a role in taste signal transduction (2). In addition, phospholipase C β 2 (PLC β 2) is also an immuno-cytochemical marker of type II cells. Gα-o is the most abundant G-protein in the brain, and is known to regulate ion channels (3). In this study, we examined the expression of several G proteins in the main excretory duct of the rat submandibular gland to elucidate its function by comparison with the corresponding expression in taste bud type II cells.

MEDs and circumvallate papillae were obtained from 9-week-old male Wistar rats. Unfixed tissues were embedded in Tissue Tek, frozen in liquid nitrogen, and 5-μm-thick serial sections were cut using a cryostat. After mounting the sections on glass slides, immunohistochemical staining was performed using the indirect immunofluorescence method with antibodies against Gα-gustducin, PLC β 2 and Gα-o.

Gα-gustducin: In the MED, tuft cells and type II light cells with a bulging apex were strongly stained. In the taste bud, type II cells were strongly stained. PLC β 2: In the MED, tuft cells and type II light cells with a bulging apex were strongly stained. In the taste bud, type II cells were stained. Gα-o: In the MED, tuft cells were strongly stained. Type II light cells with a bulging apex and the other cells were weakly stained. In the taste bud, type II cells were stained.

Judging from their expression of Gα-gustducin, tuft cells in the MED seem to have a chemoreceptive function. However, MED tuft cells and taste bud type II cells appear to be attached by efferent-type nerve endings, as there is an absence of synaptic vesicles in the area of nerve contact. Thus, there is a possibility that tuft cells, including the other cell types, are involved in ion metabolism.

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Talk-E1

Ultrastructure of the Ovine parotid gland.

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The parotid gland of adult sheep was examined by electron microscopy. The endpieces are in the form of relatively long tubules. Based on secretory granule structure, the endpieces consist of two distinct types of serous cells and scattered seromucous cells. Irrespective of secretory cell type, the basal surface of each cell is thrown into a series of slender folds into which membranous cytoplasmic organelles cannot fit. The folds are associated with an elaborate system of microvillus-lined intercellular canaliculi, a relationship geared to intercellular passage of solutes and water. Proceeding distally, the basal folds disappear, followed soon thereafter by the disappearance of the intercellular canaliculi. Intercalated ducts consist of unremarkable simple low cuboidal epithelium. The succeeding duct, viz., the striated duct, has two morphologically distinct segments. The proximal portion of the duct lacks basal striations, but has slender, organelle-free, horizontally-oriented basal folds. The unique feature of the proximal duct cells is the presence of microvillus-lined luminal diverticula that may function in a manner analogous to the intercellular canaliculi in the endpieces. The distal portion of the striated duct is morphologically identical to prototypical striated ducts in other salivary glands and species. The interlobular duct is, for a short distance, identical to the distal striated duct, but quickly assumes the lineaments of typical excretory ducts. Coupled with the paucity of organic secretory products in the final saliva, the morphology of the sheep parotid gland suggests that this organ is geared more to fluid production than to secretion.

Talk-E2

A morphometric study by HRSEM of the secretory responses of human salivary glands stimulated in vitro by various secretagogues.

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In order to investigate the dynamics of human salivary secretion at the cellular level, we have set up an in vitro incubation method of samples of salivary glands obtained at surgery. With our modification of the osmium maceration technique which, removing all cytoplasmic organelles, exposes the internal surfaces of the plasmalemma, we observed, by HRSEM, the changes of the portions of membrane involved into secretion after stimulation with secretagogue drugs. In some experiments, specific adrenergic and muscarinic inhibitors were added. Besides carbachol and isoproterenol, respectively muscarinic and β-adrenergic agonists, we used clozapine, a dibenzodiazepine derivative used in psychotic patients that provokes hypersalivation, a fastidious side effect of therapy. The effects of the drug, used alone or in combination with carbachol, have been compared with those observed after treatment with drugs acting on specific receptors. To quantify the response to stimulation, we have calculated (with statistical methods) the number of microvilli and microbuds (corresponding to pits seen in images obtained by transmission electron microscopy) per square micrometre of the cytoplasmic surface of the intercellular canaliculi luminal membrane in images obtained by high-resolution scanning electron microscopy. Clozapine, when directly acting on human submandibular specimens, induces a small secretory response in serous cells; this is partially decreased by muscarinic and adrenergic antagonists and by combined incubation with carbachol, thus confirming its behaviour as a partial agonist to muscarinic receptors. We also suggests that the drug acts on the nerve terminals contained within the glandular specimens. Results of other experiments that dealed, both in submandibular and parotid glands, with the action of specific inhibitors were compared and discussed.

Talk-E3

Expression and localization of aquaporin-6 in parotid acinar cells.

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Objective: Aquaporins (AQPs) are integral membrane proteins that function as channels in the transfer of water and small solutes across the membrane. In mammalian cells, 13 isoforms (AQP0-12) have been identified so far, which differentially expressed in various types of cells and tissues. AQP6 has been identified in kidney and considered to function as an anion channel. We investigated the expression and localization of AQP6 in the rat parotid gland.

Methods: Expression of mRNA and protein of AQP6 was detected by RT-PCR and western blotting analysis using polyclonal anti-AQP6 antibody, respectively. In morphological study, localization of AQP6 was examined by confocal microscopy and immunolabelled ultrathin cryosection. Secretory granules and plasma membrane fractions were isolated using Percoll gradient.

Results & Disucssion: In RT-PCR, 262 bp band as AQP6 mRNA was detected in rat parotid acinar cells. In western blotting analysis using anti-AQP6 antibody, 33 kDa band was detected in the plasma membrane and secretory granule membrane of the rat parotid gland, whereas 33 and 60 kDa bands were detected in the rat kidney membrane. In confocal microscopy, positive reactions on anti-AQP6 antibody were confirmed in tight junction area nearby the tight junction protein ZO-1 and cytosolic area of parotid acinar cells. In immunolabelled ultrathin cryosection, positive reactions on anti-AQP6 antibody were indicated nearby tight junction and around granule membrane. Positive reactions on anti-AQP6 antibody on purified parotid granules were confirmed by immunoelectoron microscopy. As AQP6, 33 kDa band was also detected in isolated granule membrane by western blotting. When parotid glands were stimulated by the b-agonist isoproterenol, anti-AQP6 antibody positive reactions accumulated in luminal side of acinar cells. These results suggest that AQP6 is involved in secretory functions in parotid acinar cells.

Talk-E4

Analysis of secretory dynamics in mouse parotid acinar cells reveals multiple pathways for secretory granule fusion.

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Work from our laboratory using electrophysiological and optical assays of exocytosis in mouse parotid acinar cells revealed that there are multiple Ca^{2+} dependent vesicle pools. For example, photolytic Ca^{2+} elevation induced two phases of exocytosis: an initial phase that could not be resolved as individual fusion events by either membrane capacitance measurements or time-differential imaging analysis, and a second phase comprised of resolvable events that reflected both sequential compound and, in contrast to pancreatic cells, multivesicular fusion of granules. These observations are consistent with minor and major regulated secretory pathways (1). Fusion of secretory granules produced persistent post-fusion structures that were often found to coincide at similar subcellular sites or "hot-spots" (2). The delay between Ca^{2+} elevation and the first exocytotic event, as well as the delay between sequential events at identified hot spots was significantly shorter than that reported for pancreatic cells (3). When exocytotic events were mapped onto images of selected acini, they primarily aligned with the apical or lateral aspects of the cells. Application of muscarinic or adrenergic agonists induced secretory activity of similar magnitude, but with different kinetic properties. Additionally, a significant component of non-cholinergic, non-adrenergic amylase release has previously been demonstrated (4). The signaling that underlies this residual component of amylase release is not fully understood, but likely includes purinergic receptor activation because extracellular ATP can increase amylase release from rat parotid acini (5) and mouse parotid gland (6). We now demonstrate that ATP-mediated Ca^{2+} influx via ionotropic P2X4 receptors is an important signal for amylase release. A rapid and significant increase in the numbers of events was found to correlate with the ATP-evoked rise in cytosolic Ca^{2+}. Combined treatment with the P2X4R-selective sensitizing compound ivermectin induced an increase in both the secretory rate and total number of fusions. Moreover, fused secretory granules could be visualized using the fixable fluid phase marker Texas Red-dextran (7). These data demonstrate that activation of specific Ca^{2+} signaling routes can selectively evoke multiple secretory pathways.

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Poster-E1

Relation of proteoglycan to sorting amylase into secretory granules/vesicles.

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It has been known that sulfated proteoglycan is involved in the secretory granule formation in some exocrine cells, whereas the relation of sulfated proteoglycan to the vesicular secretion is unknown. We investigated the process of unstimulated secretion from rat acinar cells. The unstimulated secretory pathways in parotid acinar cells have been reported; the constitutive-like pathway which is mediated by immature secretory granules, and unstimulated granule exocytosis[1]. The constitutive secretion, which has been reported in various cells, may also occur in parotid acinar cells, but there is little investigation. Here, we indicated the presence of constitutive secretory pathway in parotid acinar cells, and the relation of sulfated proteoglycan to the vesicular secretion.

Biosynthetically labeling of the acinar cells was performed with ^{35}S-[Met and Cys] by 5 min-pulse and 5 min-chase to label the newly synthesized amylase. Then, the homogenate from the acinar cells was fractionated by centrifugation on a Redi Grad-sucrose density gradient to obtain granule and vesicle fractions. The newly synthesized amylase was contained in the vesicle fractions but not in the granule fraction including mature and immature granules. Chondroitin sulfate was detected in the fluid in the secretory granules at 40 ~60 kDa, whereas it was not detected in the fluid in the secretory vesicles.

To investigate the secretion, acinar cells were incubated with or without isoproterenol (IPR, 10^{-6}M) for 20 min. The medium under the unstimulated conditions contained the newly synthesized amylase but not chondroitin sulfate. On the other hand, the medium from the IPR-stimulation contained chondroitin sulfate and a large amount of unlabeled amylase, but little of the newly synthesized amylase. These indicated that the newly synthesized amylase secreted under the unstimulated conditions may be through the constitutive secretory pathway neither through the constitutive-like pathway nor by the unstimulated granule exocytosis. The unstimulated secretion of the newly synthesized amylase was strongly inhibited by PNP-bxyloside, an inhibitor of proteoglycan synthesis. This indicated that amylase transport into the constitutive secretory vesicles involved proteoglycan-relating processes, although the secretory vesicles did not contain chondroitin sulfate.

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Poster-E2

Activation of cAMP-dependent protein kinase by cGMP in the rat parotid acinar cells

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Amylase release from the rat parotid gland is mainly mediated in a cAMP-dependent protein kinase (PKA)-dependent manner. In the previous studies, we reported that salivary secretion by muscarinic-acetylcholinergic stimulation enhanced cGMP/cGMP- dependent protein kinase (PKG) signaling to evoke amylase release, although this secretion was a low level compared to amylase release evoked by β-agonist.

In the present study, we investigated whether cGMP-mediated amylase release might be due to cGMP/PKA pathways, as well as cGMP/PKG pathway. Activation of PKA by cGMP was required 100-1000 times higher concentration than that by cAMP in the parotid cytosol fraction. However, in the presence of 0.1 μM cAMP, low concentration of cGMP activated PKA, and the synergistic activation of PKA was found by the combination of both cyclic nucleotide.
Permeabilized derivatives, diBu-cAMP and 8-pCPT-cGMP released amylase, and the combination of both released amylase synergistically form parotid acinar cells. cGMP dose-dependently stimulated amylase release from saponin- permeabilized parotid acinar cells. Phosphorylation by cGMP produced phosphorylated proteins of the same size as those produced by cAMP. Phosphorylation by cGMP was inhibited by the addition of PKA inhibitor, H-89. These results indicated that cGMP activates not only PKG but also PKA. Thus, it appears that both cGMP/PKG and cGMP/PKA pathways mediate amylase release from rat parotid acinar cells.

Poster-E3

Roles of Rab27 and its effectors in isoproterenol-induced amylase release from rat parotid acinar cells

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Small GTPase Rab is a large family of putative membrane trafficking proteins. We have reported that Rab27 and its effectors, Slac2-c/MyRIP and Slp4-a/granuphilin-a, regulate isoproterenol (IPR)-stimulated amylase release from rat parotid acinar cells [1, 2]. In the present study, we investigated intracellular localization and translocation of Rab27 effectors in rat parotid acinar cells. We further examined whether Noc2, one of the effectors, is involved in IPR-stimulated amylase release from the acinar cells.

Acinar cells and subcellular fractions were prepared from parotid glands of male Wistar rats (approximately 10 weeks old). Since a presence of Noc2 in the parotid acinar cells wasn't known yet, mRNA expression of Noc2 was investigated by RT-PCR at first. Next, subcellular distribution of Rabs and Rab effectors was investigated by Western blotting and immunohistochemistry. Rab3D, Rab8, Rab26, Rab27A, Rab27B, Slac2-c, Slp4-a and Noc2 were detected in the secretory granule membrane fraction. Rab27, Slac2-c and Munc13-4 were translocated to the apical plasma membrane after IPR-stimulation during 5 min, and decreased from the membrane after 30 min. Although Noc2 was expressed in SGM bound to Rab27, Noc2 was not translocated to APM and the Noc2/Rab27 complex was disrupted after stimulation with isoproterenol (IPR) for short time. In addition, the anti-Noc2-Rab-binding-domain antibody inhibited IPR-induced amylase release from SLO-permeabilized parotid acinar cells. The results suggest that the Noc2/Rab27 complex is an important constituent of the early stages of IPR-stimulated amylase release.

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Poster-E4

Subtype-specific regulation of inositol (1,4,5)-trisphosphate receptors by protein kinase A

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Prior work from our laboratory has established that cAMP and calcium signaling pathways can intersect in exocrine acinar cells of the pancreas and the parotid salivary gland. Specifically, increasing cellular cAMP attenuates calcium signals initiated by activation of muscarinic receptors in the pancreas while the same treatment enhances calcium signals in parotid acinar cells (1, 2). One possible explanation for the observed differences is the subtype-specific regulation of calcium release from inositol 1,4,5-trisphosphate receptors mediated by protein kinase A phosphorylation. The type 2 and type 3 $InsP_3Rs$ are the predominant isoforms expressed in exocrine acinar cells and both isoforms are physiological substrates for PKA. However, acinar cells express all three subtypes of $InsP_3Rs$ and the receptors may form heterotetramers, making it difficult to analyze subtype-specific regulation in these cells. The purpose of this study was to compare the functional effects of phosphorylation on type 2 and type 3 $InsP_3Rs$ in isolation. In order to determine the effects of phosphorylating individual sites in each isoform, expression constructs harboring various phosphorylation-site mutants were generated. The specific regions of the type 2 receptor that are substrates of PKA were identified using epitope-tagged subclones of the receptor. The effects of mutating the proposed phosphorylation sites of the type 3 receptor were also examined. The functional effects of mutating individual phosphorylation sites were tested using calcium imaging techniques in DT40-3KO cells expressing individual receptor isoforms, while transfected COS7 cells were used to directly monitor phosphorylation events. Results presented here indicate that while PKA-mediated phosphorylation can alter the calcium release activities of both isoforms, the functional effects of phosphorylation are elicited through different molecular mechanisms.

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Poster-E5

Exocytosis and fluid secretion in exocrine glands studied by two-photon microscopy

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A molecule can transit with an extremely low probability from the ground state to the first electronically-excited one by simultaneous absorption of multiple photons. Such process is called as multi-photon excitation process. A new optical microscopy based on such multi-photon excitation process ("two-photon microscopy") has been used widely in biological and medical sciences, because two-photon microscopy can visualize living cells in near intact states for a long time within deeper layers of organs. Such extremely low probability of two-photon excitation causes a property that pigments are excited and fluorescence is generated only at the focal point of the objective lens. This is because it is at the focal point where the laser photon density is maximized. By another attractive property of two-photon microscopy, multicolor excitation capability, we have established a less-invasive imaging method for quantifying intracellular Ca^{2+}, and simultaneously visualizing fluid transports and a single episode of fusion pore opening during exocytosis. In pancreatic acinar cells, we have found a sequential compound exocyotsis for the first time. Sequential compound exocyotsis has been found in many kinds of secretory glands including salivary glands. Further exploration revealed dynamics and physiological roles of actin cytoskeleton, fusion-pores, and SNARE proteins. Furthermore, we have succeeded in visualizing calcium-dependent water and electrolyte transport in nasal mucosal epithelial and salivary glands, and these may be used as an assay system for allergic rhinitis. As discussed above, this extremely rare quantum mechanical process results in extremely localized excitation ($< 10^{-15}$ *l*). By applying such localized two-photon excitation for photolysis of caged Ca^{2+}compund, we have achieved functional mapping of ion channels involved in water and electrolyte transport in pancreatic acinar cells. Because fluid transport is a general function of the exocrine gland, this technique may be applicable to various physiological and pathological studies. In addition, we have recently succeeded in observing intact neurons deeper than 0.9 mm from the surface of the brain cortex in an anesthetized mouse. In the same preparation, we can determine morphologically dendritic spines and axon terminals, suggesting that their long-term changes can be chased in a living mouse. Two-photon microscopy will therefore advance the researches on mechanisms of exocytosis and fluid secretion in secretory cells in intact glands *in vivo*.

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Poster-E6

Multi-photon imaging of cellular heterogeneity in the sensitivity of Ca^{2+} responses in rat parotid ducts.

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The salivary ductal cells modify the primary saliva and change the ion component of saliva. It has been considered that rat salivary ducts consist of morphologically and functionally distinctive cell types. We examined the heterogeneity of ductal cells with regard to their sensitivity to several Ca^{2+}-mobilizing agonists by visualizing Ca^{2+} responses with two-photon microscopy. Our data demonstrate that low concentrations (0.1 μM) of epinephrine (Epi) induced Ca^{2+} responses in ~ 30% of ductal cells. These Epi-sensitive cells were scattered throughout the duct and varied markedly in their Ca^{2+} responses to 0.1 μM Epi with regard to the time of response onset. Approximately 80% of these Epi-sensitive cells responded immediately to the subsequent stimulation with 1 μM Epi. A similar heterogeneity in Ca^{2+} responses were observed when the ducts were stimulated with low concentrations of phenylephrine (PhL), carbachol (CCh) and ATP. Analysis of the ductal cells that were sequentially stimulated with low concentrations of PhL, CCh, and ATP revealed that the majority (69%) could only respond to one of the three agonists; only a small minority (9%) were capable of responding to all three agonists. Thus, salivary ductal cells exhibit significant heterogeneity in their sensitivity to Ca^{2+}-mobilizing agonists. These data also show that low concentrations of PhL, CCh, and ATP activate different sub-populations of ductal cells.

Poster-E7

MORPHOLOGICAL CHANGES INDUCED BY HISTATINS IN *Candida albicans*: A MICROSCOPIC AND SUBMICROSCOPIC COMPARISON

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In spite of the numerous studies performed in an attempt to clarify the issue, the mechanism of action of salivary histatins remains unclear. The aim of the present study was to investigate on and, possibly, correlate histatin-induced morphological changes on *Candida albicans* by fluorescence microscopy (FM), transmission electron microscopy (TEM), and high resolution scanning electron microscopy (HRSEM).

Each of the fluorescent dyes used by FM (i.e. TMRM for mitochondrial potential, Lysotracker for lysosome acidic compartment, and DAPI for DNA) exhibited a specific staining in control cells. Following histatin treatment, we observed a recurring staining pattern, corresponding to fluorescence concentration along the cell periphery, suggesting a loss of dying specificity. Ultrastructural analysis was subsequently carried out to assess histatin-induced cytoplasmic modifications. After treatments with histatins, TEM revealed characteristic intracellular modifications including: vacuole overgrowth, nuclear disappearance, loss of organelle identity, as well as the appearance of electron-dense membranes, likely of mitochondrial origin. Additionally, structures resembling autophagosomes were occasionally observed. By HRSEM, mitochondrial swelling was invariably the first sign of a histatin-induced effect. Other modifications included: intracellular membrane disarrangement, organelles confusion, and a large central cavity drifting deformed bodies to the cell periphery, similarly to what was detected by TEM. In summary, our study illustrates the occurrence of ultrastructural modifications following administration of histatins. Observations made with FM, TEM and HRSEM provided different views of the same signs, demonstrating a definite action of histatins on *C. albicans* morphology. The possible functional meanings of these morphological results has been discussed in the light of the most recent biochemical data on histatin fungicidal activity.

Poster-E8

Sequential appearance of Golgi proteins during *de novo* formation of the Golgi apparatus in parotid acinar cell.

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Our immunohistochemical and electron microscopic study indicated that an anti-microtubular agent, nocodazole induced *de novo* formation of Golgi apparatus in rat parotid acinar cell. Immediately after incubation of parotid tissue pieces in the culture medium containing 10μg/ml nocodazole at 37 °C, numerous GRASP55-positive spot-like structures appeared in the basal region of cytoplasm which was ordinarily occupied by the rough endoplasmic reticulum (RER). As GRASP55 is a Golgi-stacking protein, these small spots were thought to be an element of the Golgi apparatus. Electron microscopy indicated such spots were smooth-surfaced vesicular structures seemed to be generated from the RER. At 30 min after the incubation, the vesicular structures aggregated to form small membranous clusters composed of tubules and vesicles, and became positive in one another Golgi-stacking protein, GM 130. Immunogold electron microscopy revealed positive reaction for anti-GM130 on such smooth-surfaced membranous clusters. Whereas, no remarkable change could be observed on pre-existent Golgi apparatus in the supranuclear region of the cell.

During further incubation, such membranous clusters were increased in size and developed into small Golgi stack in the basal cytoplasm, and the immunoreactivity for various Golgi proteins, e.g. mannosidase II, β-COP, GBF1 and TGN38, became positive followed by the formation of secretory granules. Such process of Golgi formation was partially inhibited by the incubation with nocodazole at low temperature. At 25 °C, only GRASP55-positive spots were appeared, but the addition of the other Golgi-resident proteins and the development of the membranous clusters were completely inhibited followed by marked accumulation of GRASP55-positive small vesicular structures throughout the cytoplasm.

These results suggested that the primary form of the Golgi apparatus was GRASP55-associated vesicles bud from the RER. Addition of GM130 led to form membranous cluster by aggregation of the vesicles. Various Golgi-resident proteins were sequentially appeared on the developing Golgi clusters. Finally, Golgi stack with secretory function was established. Intracellular transport at normal temperature is requisite for this process of the Golgi development.

Poster-E9

Comparison of cluster formation of GFP-IP_3 receptors in HSY, a human salivary cell line, and COS-7 cells.

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The inositol 1, 4, 5-trisphosphate (IP_3) receptor is an IP_3-gated intracellular Ca^{2+} channel responsible for mobilizing stored Ca^{2+}. It has been reported that IP_3-generating agonists evoke clustering of green fluorescent protein (GFP)-tagged IP_3 receptor type 1 (GFP-IP_3R1) in COS-7 cells. Previously, we constructed a plasmid vector containing full-length rat IP_3R type 3 linked to GFP (GFP-IP_3R3) and visualized the distribution of GFP-IP_3R3 expressed in various cultured cells including HSY cells, a human parotid duct cell line [Morita et al., Cell Calcium 31, 59-64, 2002; Morita et al., Biochem. J. 382, 793-801, 2004]. In the present study, we examined whether stimulation with ATP or IP_3 induced the cluster formation of GFP-IP_3R3. When COS-7 cells were stimulated with 100 μM ATP, clustering was observed in ~80% of the cells expressing GFP-IP_3R3. Thapsigargin and ionomycin did not induce cluster formation of GFP-IP_3R3, and the ATP-induced clustering was observed even after depletion of the Ca^{2+} store by pretreatment with thapsigargin. These results suggest that Ca^{2+} is not a direct trigger for the clustering of GFP-IP_3R3. On the other hand, when HSY cells were stimulated with ATP, clustering of GFP-IP_3R3 was detected in only <5% of the cells examined. In order to show the involvement of IP_3 in the cluster formation, we examined the effects of IP_3 using saponin- or b-escin-permeabilized cells. Application of 10 μM IP_3 evoked rapid cluster formation of GFP-IP_3R3 in both of permeabilized HSY and COS-7 cells, and the IP_3-induced clustering was inhibited by heparin, a competitive antagonist of IP_3R. This result provides evidence that the clustering of GFP-IP_3R3 is induced by IP_3 binding to the receptors. Monitoring of IP_3 using the IP_3 biosensor LIBRA suggested that ATP-induced production of IP_3 in HSY cells was very low as compared with that in COS-7 cells. ATP-induced clustering of GFP-IP_3R3 may be dependent on the extent of IP_3 production.

Poster-E10

Possible Involvement of Myosin-ATPase in the spatio-temporal regulation of exocytosis in adrenal chromaffin cells.

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We have suggested previously that myosin light chain kinase (MLCK) which regulates actin-myosin interaction has an essential role in ATP-dependent priming of exocytosis in adrenal chromaffin cells (1). Further we suggested that reorganization of actin cytoskeleton and actin-myosin interaction have indeed an essential role for the vesicle recruitment from the reserve pool (2). The role of myosin II in vesicle transport was also suggested recently by Gutiérrez and his colleagues (3). In order to clarify the involvement of myosin ATPase in the role of myosin as a molecular motor, we examined the effects of 2,3- butanedione monoxime (BDM), an inhibitor of myosin ATPase, on the secretory events and on the vesicle dynamics.

The secretory events in cultured bovine adrenal chromaffin cells were detected by amperometry using a single carbon fiber electrode (2). The vesicle dynamics was studied by an imaging of chromaffin vesicles labeled with Lyso Tracker Green DND-26 as was described (4).

Secretory response to both depolarizing stimulation and inonomysin were decreased by BDM. Examination of high K^+-evoked events with the amperometry indicated that BDM decreased the frequency of exocytotic events in the sustained response, but not in the initial burst. BDM dramatically changed the vesicle movement during repolarizing phase; the increase of the vesicle population with the faster movements was abolished. Exocytotic events caused by sucrose, which indicates the exocytosis from the releasable pool, was abolished by BDM. All of these results suggested that myosin ATPase is required for the recruitment of the vesicles from the reserve pool to the releasable pool in adrenal chromaffin cells.

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Talk-F1

THE ION AND FLUID SECRETION MECHANISM.

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Transepithelial chloride movement is thought to be the driving force for the secretion of fluid and electrolytes by salivary gland acinar cells. The currently accepted salivary gland secretion model predicts that transepithelial chloride movement and the subsequent excretion of fluid and electrolytes by acinar cells requires the coordinated regulation of multiple water and ion transporter and channel proteins. Saliva is produced when most, if not all, of these transporters and channels are activated of by an agonist-induced increase in the intracellular free [Ca^{2+}]. This presentation will review the transport mechanisms thought to be involved in this process as well as recent developments in confirming the molecular identities of these proteins. The cloning of these transporter and channel proteins has improved our understanding of this process at the cellular level. The physiological roles of individual transporter and channel proteins have been revealed through the characterization of mice with null mutations in many of these genes.

Talk-F2

Structural and functional significance of the dimerization of the secretory Na-K-2Cl cotransporter (NKCC1)

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The diuretic-sensitive cation-chloride-cotransporters (CCCs) are members of a small gene family consisting (in vertebrates) of 7 electroneutral chloride transporters and 2 membrane proteins of unknown function. Classical physiological studies and more recent observations with knockout mice have demonstrated that the CCCs play important roles in many biological processes including exocrine fluid secretion, renal salt and water homeostasis, hearing, olfaction, pain perception, spermatogenesis, and neurotransmission. The CCCs exist as homodimers in the plasma membrane but the molecular interactions that underlie this dimerization as well as its functional significance are still unclear. In this regard, it has been suggested that modulation of CCC behavior via heterodimerization may play a significant role in their biological properties. We have used the secretory Na-K-2Cl cotransporter (NKCC1) as a model CCC and employed NKCC1/CCC chimeras, chemical cross-linking and a novel co-immunoprecipitation assay to explore these problems. Our results demonstrate that the residues responsible for NKCC1 dimerization are contained entirely within its 50 kDa intracellular C-terminus. When we replaced the C-terminus of NKCC1 with that of other CCCs we found that these chimeras interacted very weakly, if at all, with wild-type NKCC1, indicating that the formation of CCC heterodimers is unlikely to be of biological significance. By systematically swapping out regions of the C-terminus of NKCC1 with those of NKCC2, a close homologue with which NKCC1 does not dimerize, we have been able to localize the amino acids involved in dimerization. In HEK-293 cells, over-expression of a non-functional NKCC1 mutant that dimerizes with the endogenous NKCC1 has little effect on fluxes via the endogenous protein. Thus the wild-type unit of a wild-type/inactive dimer-pair is still active, indicating that each subunit of a dimer pair functions largely independently of the other.

Talk-F3

Localization and Function of CLCA in rat submandibular glands

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A molecular entity for Ca^{2+}-dependent Cl^- transport has not been well characterized in rat salivary glands even though it is known to be important for saliva production. We identified a new member of the CLCA family, rCLCA, which we isolated from rat ileum by a PCR-based strategy, and we found that it is strongly expressed in the submandibular gland (SMG). The full length of this isoform is 3.3 kb, and the predicted ORF encodes a 903-a.a. protein containing an N-terminal region located extracellularly. The amino acid sequence of rCLCA protein has 83% homology to murine CLCA1 and 2. Transient transfection of HEK293 cells with rCLCA cDNA resulted in expression of 120- and 86-kDa proteins in the membrane fraction. The latter appeared to be a proteolytic product of the former. Streptavidin recognized the 86-kDa protein immunoprecipitated from surface-biotinylated cells using the anti-rCLCA antibody, suggesting that the proteolytic product locates on the plasma membrane. The transfection with rCLCA revealed a marked Cl^- conductance evoked by ionomycin with an outward rectification in the I-V relationship. The current was dependent on Ca^{2+}, and it was sensitive to niflumic acid and DIDS. RT-PCR revealed expression of rCLCA in the stomach, lung and liver as well as the SMG. Intense immunostaining was detected in the striated ducts of SMG, but not in the acinar cells. Immunoblot of the membrane fraction yielded 137- and 90-kDa bands. N-glycosidase F reduced the size of the immunoreactive bands in the native and transfected cells to 100 and 75 kDa, suggesting that both species are linked to N-glycan. To address the question as to whether rCLCA is involved in Cl^- reabsorption in rat SMG during muscarinic stimulation, we evaluated the electrolyte content of saliva from the glands pretreated with rCLCA siRNA. Retrograde injection into a given submandibular duct with the siRNA significantly increased Cl^- concentration in saliva collected after administration of pilocarpine. These results represent a physiological significance of rCLCA in transepithelial Ca^{2+}-dependent Cl^- transport in the ductal system of rat SMG.

Poster-F1
Bond graph expression on an epithelial transport system

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Oster et al. (ref. 1) introduced bond graphs in network thermodynamics. The bond graph is very useful for a representation of thermodynamic systems in which Eulerian (stationary) co-ordinate frame is used. But it is inadequate for a representation of mechanical systems in which Lagrangian (moving) co-ordinate frame is used. We cannot use the Eulerian concept together with the Lagrangian concept in a method. This paper is limited to use the bond graph in thermodynamic systems like as membrane transport systems. The bond graph is composed with bonds, thermodynamic elements and connection elements. A bond, a half-arrow, can express a power with its direction and its causality. Each power is expressed by a product of through variable and conjugated across variable. The causality is expressed by appending to a bond, a vertical bar, called a causal stroke, with following meaning: an element adjacent to the vertical bar is governed by an across variable. The through variable is measured at one point in a circuit, and is additive at a parallel connection and called KCL variable. The across variable is measured by attaching the instrument to two connection points on the component, and is additive at a series connection and called KVL variable. In a thermodynamic system, through variables are a current, a volume flow, a mass flow, a reaction rate and etc., and the across variables are a voltage, a pressure, a chemical potential, a chemical affinity and etc. In a mechanical system, the through variables are a force, and the across variables are a velocity. This apparent reversal makes a confusion to use the bond for mechanics.

Elements in bond graphs are a parallel junction, a series junction, a capacitor C, a disspative conductor L and a generalized transducer TD. The capacitor has the independent variable of the through variable. A driving force across a dissipater (conductor) is a difference of two across variables. The dissipation disappears when the driving force is zero, that is, in equilibrium state. Two across variables can determine a through variable, but a through variable cannot determine two across variables. Therefore we can conclude that the dissipative conductor has the independent variable of the across variables. The transducer is a two-port element, which has an input port and an output port. An independent variable of the input port a driving force, and determines the output driving force. The independent variable of the output port is a through variable, and it determines the input through variable. I noticed an error on the coupling representation modeled by Oster et al. (ref. 2). The coupling is also a dissipative process. We realize the coupling between processes A & B by the fact, that a part of power A transfers to a power B process, or a part of power B transfers to a power A process, reversibly. The coupling disappears when the driving force X_A and the driving forces X_B have a certain ratio. Therefore we can conclude that the coupling module CPL has the independent variables of both driving forces. A coupling module CPL can be expressed by a series circuit of two transducers having transfer ratio of γ_A & γ_B and a dissipater L_C in series. In a two-powers system of A & B, the dissipative subsystem is modeled with two conductors L_A & L_B and a CPL. A simultaneous equation derived from the bond graph of the dissipative subsystem as follow;

$J_C = L_C (\gamma_A X_A - \gamma_B X_B)$

$J_A = L_A X_A + \gamma_A J_C = L_A X_A + \gamma_A L_C (\gamma_A X_A - \gamma_B X_B) = (L_A + \gamma_A{}^2 L_C) X_A - \gamma_A \gamma_B L_C X_B$

$J_B = - \gamma_B J_C + L_B X_B = - \gamma_B L_C (\gamma_A X_A - \gamma_B X_B) + L_B X_B = - \gamma_A \gamma_B L_C X_A + (L_B + \gamma_B{}^2 L_C) X_B.$

Thus the simultaneous equation is same form of the phenomenological equation that has a reciprocal (coupling) coefficient of $- \gamma_A \gamma_B L_C$.

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Poster-F2

A modular approach to computational modelling of epithelial electrolyte transport.

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Systems biology is an exciting and rapidly emerging field which attempts to combine genome, transcriptome and proteome data in order to synthesize a 'complete' description of a biochemical or physiological process at a cellular, tissue or organismal level. A crucial element of this approach is the development and subsequent refinement of a computational model of the process based on mathematical descriptions of the individual components and their interrelationships.

Although the systems biology concept is applied mainly to metabolic and signalling pathways at present, a very similar approach has been used sporadically to characterize epithelial transport processes for many years. Computational models, based on knowledge of the individual channels and transporters that are active in a particular epithelium, have successfully simulated many experimental results that have been obtained in the laboratory. Where differences have been observed between model predictions and experimental observations this has often led to improvements in the model which progressively result in a better match between prediction and observation.

What is lacking at present is a user-friendly software framework to enable transport physiologists, who may not have the time or training to write simulation programs from scratch, to assemble single-cell and epithelial transport models and test the behaviour of these models in simulated experiments. It should be possible, for example, to build a library of modules that describe the properties and behaviour of known channels and transporters, many of which are expressed in numerous different epithelia. A module representing an NHE1 Na^+/H^+ exchanger, for example, could then be 'plugged' into the basolateral membrane of an epithelial cell model in such a way that the Na^+ and H^+ fluxes that it generates realistically respond to and influence the electrochemical gradients that drive it. As more is learned about the kinetics and regulation of the individual channels and transporters, modules such as this could be gradually improved and updated.

This presentation illustrates our attempt to modularise an existing, highly successful computational model of electrolyte transport by pancreatic duct epithelium (Sohma et al. 2000). The aim is to create firstly a library of modules representing specific channels and transporters, and secondly a three-compartment framework that can be used as a basis for modelling other epithelia. Our initial work has made use of Simulink, the graphical interface of the MATLAB software environment.

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Poster-F3

Bicarbonate secretion by cultured salivary gland cells.

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Objectives: We investigated whether Par-C10 cells, established by immortalization of rat parotid acinar cells, are capable of transcellular bicarbonate secretion when cultured on permeable support.
Methods: The expression of potential key transporters in Par-C10 cells was studied by RT-PCR. Monolayers of cells were grown on Transwell-COL PTFE membranes. Transepithelial resistance (TER) was measured by EVOM volt-ohm-meter. Transepithelial ion transport was evaluated by short-circuit current (I_{sc}) measurements. To monitor regulation of intracellular pH (pH_i) and transcellular bicarbonate movements, microfluorometry using BCECF, a pH-sensitive fluoroprobe was applied. Bicarbonate secretion was estimated from the initial rate of decrease in pH_i following inhibition of basolateral bicarbonate uptake by EIPA or/and H_2DIDS.
Results: As expected, we found the expression of NHE1, NHE2, NHE3 and NKCC as well as low level of pNBC1 transporters and CFTR channel in Par-C10 cells. During culturing on Traswell membranes ParC-10 cells developed very high TER (2530±153 Ωcm^2 at day 5 vs 130±12 Ωcm^2 control values). I_{sc} was increased in response to either ATP or forskolin stimulation. Following an acid load by ammonium pulse, the pH_i increase was sodium-dependent and EIPA-sensitive on the basolateral but not on the apical side suggesting the role of NHEs. H_2DIDS independence, however, suggests a minor role, if any, for NBCs in pH_i regulation. The presence of Cl^-/HCO_3^- exchangers were revealed at both sides of the polarized Par-C10 cells when the removal of Cl^- from the HCO_3^-/CO_2 perfusion solution either on the basolateral or on the apical side resulted in an instant increase of intracellular pH in a reversible manner. Changes in pH_i following the blockade of basolateral bicarbonate accumulation confirmed that the cells achieve vectorial bicarbonate secretion. Simultaneous inhibition of NBC and NHE by basolateral application of EIPA and H_2DIDS induced significant pH_i decrease (-0,0103±0,0067 ΔpH/min) compared to control (0,0010±0,0019 ΔpH/min). Forskolin (-0,0522±0,0052 ΔpH/min) or ATP application together with NBC and NHE blockade significantly accelerated the pH_i decrease when compared to control. EIPA alone had similar effects as the two inhibitors together.
Conclusions: ParC-10 cells form polarized epithelium and develop high TER on Transwell membrane. These cells achieve highly efficient pH_i regulation. They also accomplish vectorial bicarbonate transport, in which basolateral NHEs play a crucial role. This transport can be stimulated by both forskolin and ATP suggesting the presence of multiple inducible intracellular pathways in these cultured cells.
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Poster-F4

Regulation of Cl^- secretion by muscarinic cholinergic and adrenergic stimulation in acinar cells of rat salivary glands.

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Muscarinic and α-adrenergic agonists induce fluid secretion from salivary glands via an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of acinar cells. β-Adrenergic stimulation itself does not evoke fluid secretion but modulates $[Ca^{2+}]_i$ increase-induced fluid secretion via an increase in the cytosolic cAMP level ($[cAMP]_i$). However, cellular regulation of fluid secretion from salivary gland by these agonists is still unclear. Since Cl^- secretion from acinar cells is believed to drive fluid secretion from salivary glands, we investigated the properties of $[Ca^{2+}]_i$ increase-induced Cl^- secretion from acinar cells and $[cAMP]_i$ increase-induced modulation of the Cl^- secretion to clarify the cellular regulation of the fluid secretion in parotid and submandibular glands.

The gramicidin-perforated patch recording techniques were adopted on rat parotid and submandibular acinar cells and anion current which performs intact Cl^- secretion from the cells was measured at the holding potential −80 mV. Carbachol (CCh) induced a bumetanide-sensitive oscillatory anion current in parotid acinar cells as was previously reported in submandibular acinar cells (1). The cAMP-increasing agents, forskolin + 3-isobutyl-methylxantine (IBMX), did not induce any oscillatory current but reduced the CCh-induced oscillatory anion current both in parotid and in submandibular acinar cells. Since the ionic current measured in the gramicidin-perforated patch configuration reflects on the transporter activity and the ion channel activity, these results suggest that cAMP suppresses one or both of the activities in the configuration. In the whole cell recording, in which the ionic current reflects not on the Cl^- transporter activity but on the ion channel activity, cAMP-increasing agents potentiate the Ca^{2+}-activated Cl^- channel activity of parotid acinar cells (2), and suppress the Cl^- channel activity of submandibular acinar cells (3). These suggest the difference between the mechanism of cAMP-induced suppression of the current in parotid acinar cells and that in submandibular acinar cells in the gramicidin-perforated patch configuration. In conclusion, muscarinic cholinergic stimulation induces oscillatory Cl^- secretion via $[Ca^{2+}]_i$ increase and β-adrenergic stimulation suppresses the secretion via $[cAMP]_i$ increase, both in parotid and submandibular acinar cells.

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Poster-F5

Voltage-dependent transient activity of Na^+/H^+ exchanger.

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Using the ion-selective micro-electrode technique, we investigated the H^+ transport mechanism in the bullfrog proximal tubule *in situ*. A peritubular acid load induced depolarization of the luminal membrane and cellular acidification simultaneously. We observed that the peritubular acid load induced a temporary inhibition of luminal fluid acidification despite the cellular acidification (paradoxical alkalinization). Moreover, removing the peritubular acid load, which repolarized the luminal membrane as well as recovered the cellular acidification, induced the further acidification of the luminal fluid while the intracellular pH was increased (overshoot acidification). Both paradoxical alkalinization and the over-shoot acidification were mainly mediated by H^+ transport via Na^+/H^+ exchanger (NHE). After simulating these phenomena with a mathematical model of the proximal tubule, we concluded that NHE activity was i) directly regulated by membrane potential, ii) membrane potential "change" dependent, and iii) membrane potential-independent in the steady state. Using a non-steady-state NHE kinetic model, we also examined a possible molecular mechanism of the transient voltage-dependent NHE activity, in which the conformational change of NHE molecule for the ion exchange action proceeded very low ($< \sim 0.3$ trun$\cdot s^{-1}$) with coupling an intrinsic electric charge movement ($z\delta = \sim -2.5$).

Talk-F4

A feedback control model of fluid transport in salivary gland.

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A new model is presented for the control of secretion rate and osmolarity in a fluid transporting epithelium. The system has three basic elements : a cell system actively transporting solute (ions), an aquaporin acting as an osmotic sensor at one membrane [1] and a paracellular system transporting a hypotonic fluid. The cellular movement of water is osmotic but the paracellular movement is an active convection [2]. With these elements, described in the steady-state by three equations, all the main properties of isotonic fluid transport can be predicted [3].

This model has been applied to the *in vitro* rat SMG system in which hypertonic perfusions with sucrose were applied [4]. The system responds by producing a hypertonic secretion but the decrease in flow rate is much greater than that expected for osmotic adjustment. Retro-perfusion of the apical membrane with Hg^{+} ions results in reduced secretion rates but the system still behaves in a non-osmotic way to hypertonic sucrose challenges. Experiments with rats containing genetically-lowered amounts of AQP5 (present at both membranes) show that when there is virtually no AQP5 at the basal membrane, sensor control seems to be lost and the system behaves osmotically; intermediate AQP5 levels have no apparent effect.

The model predicts all these effects and is consistent with a feedback system centred on AQP5 at the basal membrane. AQP5 at the apical membrane controls the size of the osmotic signal presented to the basal sensor. In spite of the presence of these AQP systems, very little water is passing the cell route [5] due to zero osmotic clamping of the epithelium by the feedback system.

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Talk-F5

Evidence for interaction between transcellular and paracellular water transport pathways: signaling between Aquaporin-5 and the tight junction complex in mouse salivary glands.

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To investigate potential physiological interactions between the transcellular and paracellular pathways of water transport, we asked whether targeted deletion of Aquaporin 5 (AQP5), the major transcellular water transporter in salivary acinar cells, affected paracellular transport of 4 kD FITC labeled dextran (FITC-D), which is transported through the paracellular but not the transcellular route. Following intravenous injection of FITC-D into either AQP5 wild-type or AQP5 -/- mice and pilocarpine induced saliva collection for fixed time intervals, we show that the amount of FITC-D in the saliva of -/- mice is half that in matched +/+ mice, indicating a 2 fold decrease in permeability of the paracellular barrier in mice lacking AQP5. We also found a significant difference in the proportion of transcellular vs. paracellular transport, between male and female mice. This was reflected in differences in the number of tight junction strands detected by electron micrographs in male and female glands. Average acinar cell volume was increased in glands from AQP5 -/- mice, suggesting an alteration in the volume sensing machinery of the cell. Western blots revealed that expression of Claudin-7, Claudin-3 and Occludin, critical proteins that regulate the permeability of the tight junction barrier, were significantly decreased in -/- compared to +/+ salivary glands, and also showed different patterns of change in male and female salivary glands. These findings reveal the existence of a gender influenced molecular mechanism involving AQP5 that allow transcellular and paracellular routes of water transport to act in conjunction with one another.

Talk-F6

Change of Claudin Expression in Primary Cultured Parotid Acinar Cells.

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Tight junction is an essential structure for epithelial cells to keep their polarity. We have previously established a system for primary culture of parotid acinar cells that retained abilities to generate new secretory granules and to secrete proteins signal-dependently (1). Because cell polarity and cell-cell adhesion are prerequisites for epithelial tissues, we investigated structures of tight junctions in the culture. We found that the cells formed two kinds of structure: monolayers and semispherical clumps. Tight junctions were observed at the apical part of lateral membrane between cells in the monolayers and cells at the surface of the clumps. The cells inside the clumps surrounding lumens also had tight junctions. The cells inside the clumps kept secretory granules better than the other two types of cell, suggesting that they retained the original character as acinar cells. Although claudin-4 was not detected in the cells just after the isolation from the glands, it began to be expressed and its expression level increased during culture. Immunofluorescence microscopy showed that claudin-4 was expressed in the monolayers and at the surface of the clumps, but not inside the clumps. Only claudin-3, which is expressed in the original acinar cells just after the isolation and in the intact gland, was detected inside the clumps. These results suggest that the difference in claudin expression is related to the three-dimensional structures of cell cultures and the functions of acinar cells. Inhibition of cellular signaling to induce claudin-4 expression may lead to prolongation of lifespan of the culture.

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Poster-F6

Impaired aquaporin-5 distribution in salivary glands from a Sjögren's syndrome mouse model.

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The non-obese diabetic (NOD) mouse, an appropriate model to study autoimmune exocrinopathy prevalent in Sjögren's syndrome patient, was used to study the expression and localization of AQP5 in salivary glands.

After hematoxylin-eosin staining, the salivary glands from NOD mice of 8 weeks of age and Balb/c of 8 and 24 weeks of age had normal histological characteristics, while those of NOD mice of 24 weeks of age revealed numerous focal inflammatory infiltrates and important damage of the tissue. Computer-assisted microscopy was performed to quantitatively evaluate AQP5 distribution in the immunopositive acini of both Balb/c and NOD mice of 8 and 24 weeks of age. Salivary gland acini from NOD mice of 24 weeks old exhibited higher labeling index (LI; percentage of acinus area stained with anti-AQP5) at the basal membrane, but lower LI at the apical membrane when compared to acini from Balb/c mice. Real time RT-PCR quantification of the relative AQP5 mRNA expression revealed significant higher AQP5 mRNA expression in submandibular glands, but not in parotids, of mice aged of 8 and 24 weeks. AQP5 expression in salivary gland membranes, detected by Western Blot, was similar in NOD and Balb/c mice of 8 weeks of age, while it was 2 to 3-fold higher in NOD mice of 24 weeks of age compared to Balb/c mice. Salivary flow, measured in response to pilocarpine stimulation, was not different in NOD and Balb/c mice of 8 weeks of age, while it was decreased by ± 29 % in NOD mice of 24 weeks of age compared to Balb/c.

Taken together, these data suggest that impaired AQP5 trafficking contributes to the observed lower saliva flow in NOD mice of 24 weeks of age. The unexpected increase in AQP5 expression observed in NOD mice of 24 weeks of age could reflect a compensatory mechanism of the infiltrated and damaged gland.

Poster-F7

Molecular and cellular analyses of mutant AQP5 which occurred naturally in Spraque-Dawley rats

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A naturally occurring point mutation in the AQP5 water channel was found in SD rats (308G-308A; 103Gly-103Asp), which resulted in 8-times lower secretion of saliva in this animal. The mechanism was studied how such malfunction of saliva secretion is caused by this mutation. This mutation was found to be present in the 3rd transmembrane domain, which are located at the remote site from the aqueous pore. The fact implies that mutation may not affect the AQP function. In fact, measurement of osmotic water permeability by *Xenopus oocyte* expressing normal and mutant AQP5 confirmed that mutant AQP5 is functional. Immunohistochemistry of the frozen section of the SMG from normal and mutant rats showed an extreme reduction of membrane expression of the mutant molecule. MDCKII cells have been used as a model system to study distribution and trafficking of AQP5 molecule. The wild-type GFP-AQP5 chimeras transiently expressed in MDCKII cells showed positive labeling in intracellular vesicles, with strong fluorescence in apical membrane. On the other hand, apical expression of the chimeric protein was minimum in those cells transfected with mutant GFP-AQP5. Using AQP5-gene-transfected MDCKII cells, the trafficking of AQP5 to the plasma membrane was examined by stimulation with thapsigargin, an inhibitor of ER Ca-ATPase. The existence of mutation significantly reduced the AQP5-membrane trafficking in MDCKII cells. The AQP5 mutant has a significantly lower salivary secretion probably because of abnormal membrane trafficking and therefore the diminished membrane expression.

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Poster-F8

Regulation of intercellular junctions in polarized salivary cells.

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Objective: Intercellular junctional sturucture including tight junction is important for epithelial cell function. Intercellular junctions regulate paracelular permeability across epithelial cell sheets and also function as a barrier to intramembrane diffusion of components between apical and basolateral membrane domains. SMIE cells are derived from rat submandibular gland, and the cell monolayer keeps junctional structure and the function. In this study, we examined the effect of neurokinin A (NKA), a neuropeotide, on transepithlial resistasnce (TER) and dextran permeability in SMIE cells.

Methods: SMIE cells were kindly provided from Dr. Bruce Baum (NIDR). Confluent SMIE cells were plated on collagen-coated transwell culture chambers (24-mm-diameter). After 3 or 4 days, the monolayers of the SMIE cells were used for experiments. TER was measured using a Millicell ERS epithelial voltohmmeter. For the measurement of dextran permeability, FITC-dextran (4 kDa) was added to the medium on the basolateral side of the cells, and fluorescent level in the solution on the apical side was determined by a fluorimeter.

Results and Discussion: When NKA was added to the medium on the basolateral side of SMIE cell monolayer, TER was clearly increased in a time- and dose-dependent manner. The Ca^{2+}-ATPase inhibitor thapsigargin and the Ca^{2+} ionophore A23187 also increased TER. However, NKA had no effect on permeability of FITC-dextran. These observations suggest that 1) NKA regulates intercellular junctions and decreases permeability of smaller molecules than 4 kDa, and 2) the effect of NKA is coupled to the increase in intracellular Ca^{2+} levels in SMIE cells.

Poster-F9

The use of gene disruptions and isolated, perfused glands to examine mouse submandibular function.

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The isolated, perfused gland technique has been extensively used to elucidate submandibular fluid secretion, morphological changes and tight junction permeability in relatively large animals: rat, rabbit and guinea pig. However, the production of mice with gene disruptions of individual ion transport proteins has lead to a renewed interest in applying this technique to the mouse. We have recently established the mouse submandibular gland perfusion technique and applied it to knockout mice.
Aqp5$^{-/-}$ (water channel) and *Nkcc1*$^{-/-}$ ($Na^+/K^+/2Cl^-$ cotransporter) mice showed 60-70% decreased saliva flow when glands were stimulated with 0.5μM carbachol at room temperature (22°C). Both *Ik1* (intermediate conductance K channel) and *Slo1* (large maxi-K channel) knockout mice secreted normally, but the double *Ik1*$^{-/-}$/*slo*$^{-/-}$ mouse secreted ~70% less salivation. The amount of K^+ excreted in saliva almost doubled in *Aqp5*$^{-/-}$ mice (>100mM vs. ~55mM in wild-type), but was not significantly changed in *Nkcc1*$^{-/-}$ mice. K^+ excretion was slightly higher in *Ik1*$^{-/-}$ mice, but was much less in *Slo1*$^{-/-}$ (~15mM) and *Ik1*$^{-/-}$/*Slo1*$^{-/-}$(~15mM) mice. Moreover, the Na^+ concentration in these latter animals (*Slo1*$^{-/-}$ and *Ik1*$^{-/-}$/*Slo1*$^{-/-}$) was significantly increased (>80mM vs. ~45mM in wild-type mice).
The dramatically elevated levels of K^+ secretion from *aqp5*$^{-/-}$ were further investigated. When perfused with a hypertonic solution (sucrose added), glands from wild-type mice produced less fluid (~70% decrease) and high K^+ in their saliva (>100mM). This response was nearly identical to *Aqp5*$^{-/-}$ glands perfused with a physiological solution. When *Slo*$^{-/-}$ and *Ik1*$^{-/-}$/*Slo1*$^{-/-}$ glands were perfused with a hypertonic solution, saliva flow decreased as well; however, the K^+ concentration increased only modestly (<20mM).
The above results suggest that the isolated, perfused gland technique is useful for analyzing fluid secretion from knockout mice, where pharmacological studies have encountered limits due to non-specific effects. The maxi-K channel, which is located on the basolateral membrane of acinar cells, appears to also play a central role in regulating the K^+ concentration in the saliva secreted by the mouse submandibular gland. The maxi-K channel seems to also regulate Na^+ absorption, possibly mediated by a functional relationship with epithelial Na^+ channels and/or Na^+/H^+ exchangers in the duct cells.

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Poster-F10

Water permeability as measured by NMR in salivary gland cells.

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1. The diffusive water permeability of mouse parotid acinar cells was measured by ^1H nuclear magnetic resonance relaxation method using an extracellular relaxation reagent, gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) (1).

2. Parotid glands were minced, separated by collagenase and trypsin, then acinar cells were suspended in KRB solution with 0.5% BSA. The shape of acinar cells are almost sphere, and it radius is 8.1 ± 0.4 μm (mean ± SEM). The final cytocrit is ca. 10 % (2).

3. The rate constant for water efflux from the cells was estimated to be ca. 5 s^{-1} at 29°C which would be consistent with diffusive water permeability (P_d) of ca. 1.3 x 10^{-3} cm s^{-1}. This value is the same range of those obtained in perfused rat submandibular gland (3 x 10^{-3} cm s^{-1}), *Necturus* gallbladder (1.6 x 10^{-3} cm s^{-1}) and human red blood cells (2.4 to 4.7 x 10^{-3} cm s^{-1}) (1, 5 and their references).

4. Activation energy of water transport through the cell membrane (E_a) was estimated from temperature dependence (5–30°C) of the rate constant for water efflux. From the slope of Arrenius plot, E_a value is estimated to be ca. 3 kcal/mol. This activation energy is much smaller than that obtained in the lipid bilayer vesicles (12- 14 kcal/mol) (3,4), and is the same range of red blood cells with AQP1 (E_a = 5 kcal/mol)(5). Thus, this indicates that water transport of acinar cells should be done by a channel-mediated pathway, and suggests contribution of AQP5.

5. A potential inhibitor of AQP5 water channel, $HgCl_2$, was applied to the acinar cells. The acinar cells are so sensitive to the presence of $HgCl_2$. After counting trypan-blue uptake of cells with various concentration of $HgCl_2$, we choose 50 μM of $HgCl_2$ since more than 50% of cells could survive in this concentration. The rate constant for water efflux from the cells did not change significantly, but only a tendency to decrease. Since 50 μM of $HgCl_2$ is a half of 100 μM used for perfused rat submandibular gland(6), it is possible that Hg^{2+} did not reach the effective concentration to inhibit AQP5. We are now under preparation to measure E_a value of acinar cells with $HgCl_2$.

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Talk-G1

Calcium signaling mechanisms in salivary gland cells and other epithelial cells.

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In salivary gland cells and other epithelial cells, activation of phospholipase C by G-protein-coupled receptors results in release of intracellular Ca^{2+} stores and activation of Ca^{2+} entry across the plasma membrane. Early work on mechanisms of calcium entry in the parotid gland revealed that Ca^{2+} entry involves signaling from depleted intracellular stores to plasma membrane Ca^{2+} channels, a process referred to as *capacitative calcium entry* or *store-operated calcium (SOC) entry*. The best characterized electrophysiological current associated with capacitative calcium entry is the calcium-release-activated calcium current, or I_{crac}. The mechanism of activation of SOC or CRAC channels involves an endoplasmic reticulum Ca^{2+} sensor, Stim1. Recently, an integral plasma membrane protein, Orai1, has been described that is essential for I_{crac} in lymphocytes. We have found that in HEK293 cells, RNAi knockdown of either Stim1 or Orai1 significantly reduces SOC entry. Yet, overexpression of Stim1 was without effect, and overexpression of Orai1 inhibited entry. However, when we co-expressed these two proteins together, huge store-operated Ca^{2+} entry and store-operated Ca^{2+} currents were observed. These currents resembled I_{crac} in a number of ways, including a positive reversal potential, strong inward rectification, fast inactivation, complete inhibition by 1 μM Gd^{3+}, activation by 1 μM 2APB and complete inhibition by 30 μM 2APB. Thus these two proteins appear to completely recapitulate both the activation mechanism and permeation mechanisms for I_{crac}. In addition to Orai1, two similar proteins, Orai2 and Orai3 also support store-operated Ca^{2+} entry. Noise analysis of single channel conductances indicate that overexpression of Orai1 and Orai2 result in currents with distinct single channel conductances. This indicates that Orai is likely a pore-forming subunit of the SOC channel. Stim1 appears to act by redistributing within a small component of the endoplasmic reticulum, approaching the plasma membrane, but does not appear to translocate into the plasma membrane.

Talk-G2

Monitoring IP_3 and Ca^{2+} dynamics in salivary and other cell lines.

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Monitoring temporal changes in intracellular concentrations of inositol 1,4,5-trisphosphate ($[IP_3]_i$) is of vital importance to the ultimate understanding of a variety of spatiotemporally complex intracellular Ca^{2+} signals. We have described a ratiometric fluorescent biosensor consisting of the IP_3-binding domain of the rat type3 IP_3 receptor fused between the FRET pair CFP and YFP, and preceded by a membrane-targeting signal. This novel biosensor, which we refer to as LIBRA, allows for quantitative monitoring of $[IP_3]_i$ in single living cells.

LIBRA localized to the plasma membrane and Golgi area when expressed in HSY human salivary cell line or other cell line such as COS-7, MDCK, SH-SY5Y, DT40, and most of their fluorescence was retained by the cells after permeabilization with saponin. When permeabilized cells were exposed to 10uM IP_3, LIBRA showed an increase in 480 nm signal (~4.5%) and a parallel decrease in the 535 nm signal (~3.6%). Changes in the LIBRA 480nm/535nm emission ratio had a monophasic dependence on $[IP_3]$ with apparent dissociation constant (K_d) of 404nM. We estimate that inositol 1,3,4,5-tetrakisphosphate, inositol 4,5-bisphosphate, and inositol 1,3,4-trisphosphate bind to LIBRA with K_ds of ~15uM, ~40uM and >>10uM, respectively. Neither Ca^{2+} (0-1000 nM) nor ATP (0-3 mM) had any effect on the emission ratio.

We found that changes in pH affected the emission ratio as a result of the well-known effects of pH on YFP fluorescence. Therefore, we replaced the FRET accepter EYFP to the more bright and pH-stable mutant Venus (LIBRAv). We next engineered IP_3-binding domain of LIBRAv. Mutation of a critical amino acid of IP_3-binding domain (K508A) attenuated its response to IP_3, whereas the mutation R440Q gave 2-fold increase in the affinity for IP_3. We also constructed another high affinity LIBRA using IP_3-binding domain of type 2 IP_3R with a R441Q mutation (LIBRAv-2S), which exhibits 10-fold increase in the affinity for IP_3 and 2-fold increase in Rmax.

Using of LIBRAv and LIBRAv-2S we monitor the dynamics of $[IP_3]_i$ after ATP stimulation of intact HSY and COS-7 cells. Stimulations with 3-100uM ATP increased the emission ratio of LIBRAv-expressing COS-7 cells. Similar effects of ATP in HSY cells were observed with LIBRAv-2S but not with LIBRAv, suggesting that the IP_3-response in HSY cells are smaller than that in COS-7 cells. We also monitored IP_3 and Ca^{2+} signals simultaneously using LIBRA variants and fura-2, and found a monophasic increase in IP_3 level during oscillatory changes in Ca^{2+} concentrations with in HSY and COS7 cells. We also summarize the spatial and temporal characters of Ca^{2+} responses in salivary grand cells, including acinar and ductal cells, and discuss the future application of LIBRA variants.

Talk-G3

INTRACELLULAR CALCIUM SIGNALING: MECHANISTIC INSIGHT FROM ANALYSIS OF DISTINCT SIGNALS IN PAROTID AND PANCREAS.

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Exocrine cells from the parotid and pancreas are morphologically and functionally related. We have compared the spatial and temporal characteristics of intracellular Ca^{2+} signals in these two cell types to gain mechanistic insight into how the specific characteristics of these Ca^{2+} signals are generated and subsequently impart regulatory control over fluid and protein secretion. Our central hypothesis is that the localization, abundance and regulation of the Ca^{2+} signaling machinery defines the specific characteristics of the Ca^{2+} signals in these cells. We have investigated the contribution of inositol 1,4,5-triphosphate (IP_3)-induced Ca^{2+} release by imaging Ca^{2+} signals following flash photolysis of caged-IP_3. Global, uniform flash photolysis resulted in Ca^{2+} signals which were initiated in the apical pole in both pancreas and parotid. In parotid acinar cells, the Ca^{2+} signal invariably propagated throughout the cell, independently of stimulus strength. In contrast, Ca^{2+} signals remained localized to the apical third of pancreatic acinar cells following photolysis of low [IP_3]. Focal uncaging (spot size~1 mm^3) of IP_3 in either the apical or basal region of acinar cells initiated an apical to basal Ca^{2+} wave. The Ca^{2+} signals in parotid acinar cells exhibited considerably faster kinetics than in pancreatic acinar cells. These characteristics are consistent with the localization and relative abundance of IP_3R and Ca^{2+} clearance machinery in these cell types. Total Internal Reflection Microscopy (TIRFM) allows imaging with unsurpassed resolution in a limited zone at the interface of the plasma membrane and cover-slip. Using TIRFM we have monitored Ca^{2+} influx following store depletion in both cell types. These measurements revealed substantially greater Ca^{2+} influx in parotid when compared to pancreatic acinar cells. In summary, while both pancreas and parotid acinar cells use similar mechanisms to increase Ca^{2+}, the spatial and temporal characteristics of the signals are distinct. The characteristics of Ca^{2+} signals in parotid acinar cells, in particular the spatial characteristics and rapid global kinetics appear tuned to activate spatially separated ion channels required for fluid secretion. In contrast, the more graded responses in pancreatic acinar cells are ideally suited primarily to sustain exocytosis without the potentially deleterious effects of sustained global Ca^{2+} signals.

Talk-G4

Regulation of the Plasma Membrane Ca^{2+}-ATPase in Parotid Acinar Cells

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Effective fluid secretion in parotid acinar cells is critically dependent on the exquisite spatio-temporal control of intracellular Ca^{2+} ($[Ca^{2+}]_i$) signals by the concomitant activation of cAMP pathways. Such signalling cross-talk is believed to be important for the precise control of apically located Ca^{2+}-dependent Cl^- channels and basolaterally located Ca^{2+}-dependent K^+ channels which maintain maximum ion and thus water movement. My previous studies in acutely isolated parotid acinar cells have demonstrated that the key molecular mechanisms for this signalling cross-talk are the PKA-mediated modulation of inositol 1,4,5-trisphosphate receptors ($InsP_3R$), that control Ca^{2+} release, and plasma membrane Ca^{2+}-ATPase (PMCA), that control Ca^{2+} clearance [1,2]. Specifically, PKA potentiates and phosphorylates the PMCA but only in the presence of $[Ca^{2+}]_i$-raising agents (2). More recently, using a convenient model for assessing PMCA activity in intact cells, activation of PKA (using 10 μM forskolin) differentially potentiated $[Ca^{2+}]_i$ clearance at the apical region compared to the basolateral region. This was further investigated using the immortalised parotid acinar cell line, Par-C10 cells, grown on permeable transwell supports that allowed the separate perfusion of apical versus basolateral surfaces of the epithelial cell layer. It was therefore possible to selectively inhibit Ca^{2+} efflux using La^{3+} applied to either the apical or basolateral side. These experiments revealed that the apical PMCA was the major route for Ca^{2+} efflux in Par-C10 cells and also confirmed that PKA activation differentially potentiates the apical PMCA.

Finally, Western blotting revealed that PMCA1, 2 and 4 are expressed in acutely isolated parotid acinar cells and Par-C10 cells. Immunofluorescence revealed that PMCA1 was distributed throughout all regions of the plasma membrane, whereas PMCA4 was localized to the apical membrane. Likewise, the PDZ-containing accessory proteins, ezrin and EBP50 also exhibited an apical distribution. *In situ* phosphorylation assays demonstrated that PMCA1 and PMCA2 were phosphorylated by the combined treatment with forskolin and CCh.

Collectively these data suggest that PMCA is phosphorylated by PKA in a Ca^{2+}-dependent manner that differentially regulates Ca^{2+} clearance in the apical region of parotid acinar cells. This likely involves a Ca^{2+}-mediated assembly of a signalling complex that brings PKA closer to the PMCA allowing targeted regulation specifically at the apical plasma membrane. Such tight spatial regulation of Ca^{2+} efflux may represent an important mechanism for the fine-tuning of Ca^{2+}-dependent effectors at the apical membrane important for the regulation of fluid secretion and exocytosis.

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Poater-G1

Muscarinic receptor mobilization of plasma membrane Ca^{2+}-ATPase in epithelial cells: Role of the NHERF2 PDZ scaffold.

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The Na-H exchanger regulatory factors (NHERF1 and NHERF2) are PDZ scaffolds with an increasing number of roles in diverse cell types. NHERF-2 has been shown to play a role in agonist mediated Ca^{2+} signalling in epithelial cells by interacting with phospholipase C beta isoforms and is also reported to interact with the b splice variant of the neuronal isoform of PMCA2 interacts. The current study was undertaken to determine if PMCA interacted with NHERF-2 in HT-29 epithelial cells and whether this interaction was relevant during muscarinic receptor (M3) evoked Ca^{2+} signalling. HT-29 cells express only the M3 isoform of the muscarinic receptor. RT-PCR and Western blotting showed that HT-29 cells expressed the PMCA1 and PMCA4 isoforms. HT-29 cell lysates were probed with GST-fusions of NHERF1 and NHERF2 and we found that that NHERF2 but not NHERF1 interacted with PMCA via the PDZ2 module of NHERF2. Co-immunoprecipitations confirmed that PMCA and NHERF2 associated in HT-29 cells. In response to stimulation with carbachol (CCh) there was a significant increase in the cell surface level of PMCA as determined by surface biotinylation methods. The levels of PMCA increased to 154.0± 11.6% ($P<0.01$; $n=4$) of control levels within 60s of agonist exposure. An increase in cell surface associated NHERF2 was also observed and importantly, this recruitment of NHERF2 to the membrane preceded that of PMCA. The association of NHERF2 with the plasma membrane fraction increased to 145.3±4.9% of control ($P<0.01$; $n=4$) within 30s. Confocal immunofluorescence demonstrated that PMCA and NHERF2 colocalised at the membrane following exposure to CCh. Silencing RNA was used to knockdown endogenous NHERF2 in HT29 cells. This resulted in a reduction of PMCA at the membrane under resting conditions to 54.0±5.0% ($P<0.01$; $n=3$) of control and the CCh mediated translocation of PMCA was totally abolished. Fura-2 measurements of intracellular Ca^{2+} revealed that silencing of NHERF2 caused a decrease in the rate of recovery of Ca^{2+} to resting levels due to reduced Ca^{2+} efflux. Our findings demonstrate that muscarinic stimulation in a native cell model results in a rapid translocation of PMCA to the plasma membrane. The recruitment and maintenance of PMCA to the membrane appears to require the PDZ scaffold NHERF2 which may serve to nucleate a Ca^{2+} efflux complex.

Poster-G2

Regulation of KCNQ2/3 by the ubiquitin ligase Nedd4-2.

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The muscarine-sensitive K^+ current (M-current) stablizes the resting membrane potential in neurons, thereby limiting excitability. Modulation of the M-current can have drastic effects on neuronal excitability, as observed in disease states such as epilepsy and chronic pain. The M-current is mediated by a heteromeric channel consisting of KCNQ2 and KCNQ3 subunits, and thus far, little is known about how cell surface levels of KCNQ2/3 channels are regulated. One mechanism by which the membrane density of ion channels can be modulated is by ubiquitination by the ubiquitin protein ligases Nedd4 and Nedd4-2, which typically results in internalisation and destruction of the channels. We have previously shown that Nedd4 and/or Nedd4-2 can regulate the renal Cl^- channel ClC-5 (1) and neuronal voltage-gated Na^+ channels (2). The C-terminal tail of KCNQ3 exhibits a proline-rich motif (PPXPPY), a potential target for the ubiquitin-protein ligase Nedd4/4-2 (1).

This study was undertaken to determine whether KCNQ2/3 was regulated by Nedd4/4-2. The current amplitude of KCNQ2/3 was not altered by co-expression with Nedd4 in *Xenopus* oocytes. In contrast, in the presence of Nedd4-2 the K^+ current amplitude was down-regulated to 53.7 ± 5.2 % ($n = 70$, $p<0.01$) of control levels, whereas a ligase deficient Nedd4-2 mutant was without effect. Mutation of the PPXPPY motif in KCNQ3 (Y698A) had no effect on the down-regulation of the heteromeric channel by Nedd4-2. To determine whether Nedd4-2 physically interacted with KCNQ2/3, we performed GST-fusion pulldown andco-immunoprecipitation experiments. These results showed that Nedd4-2 interacted with both KCNQ2, KCNQ3 and KCNQ3(Y698A). Ubiquitination experiments using the KCNQ2 and KCNQ3 subunits were then performed in HEK293 cells. We found that KCNQ2, KCNQ3 and KCNQ3(Y698A) were all ubiquitinated in the presence of Nedd4-2. Taken together, the data from this study show that Nedd4-2 functionally interacts with the KCNQ2/3 heteromer, but not via the PY motif. It is possible that Nedd4-2 binds to a different intracellular motif in KCNQ2/3, or alternatively interacts with the channel via an accessory protein.

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Poster-G3

Secretagogues stimulate phosphorylation of MARCKS in parotid acinar cells.

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Objective: Myristoylated alanine-rich C kinase substrate (MARCKS) is a major cellular substrate for protein kinase C (PKC). MARCKS has been demonstrated to be implicated in cell motility, phagocytosis, membrane trafficking and mitogenesis. Phosphorylation of MARCKS has been reported to be involved in secretory functions, such as catecholamine release in bovine adrenal chromaffin cells, noradrenaline release in SH-SY5Y human neuroblastoma cells, mucin exocytosis in human airway epithelial cells and glucose-induced insulin secretion in rat pancreatic islets. In rat parotid acinar cells, the activation of b-adrenergic and muscarinic cholinergic receptors provokes exocytotic amylase release and water and ion secretion. We investigated the effects of secretagogues on phosphorylation of MARCKS in rat parotid acinar cells.

Methods: Rat parotid acinar cells were isolated using trypsin and collagenase. Membrane and cytosolic fractions were isolated by ultracentrifugation. Secretory granules were purified using Percoll gradient. Protein expression was determined by western blotting analysis using anti-MARCKS and anti-phosphorylated MARCKS (p-MARCKS) antibodies.

Results and Discussion: In western blotting analysis, MARCKS and p-MARCKS were detected in membrane and cytosolic fractions and secretory granule membrane. The PKC activator phorbol-12-myristate-13-acetate, the b-adrenergic agonist isoproterenol and the muscarinic agonist carbachol time-dependently stimulated phosphorylation of MARCKS in rat parotid acinar cells. These agonists had no effect on total amount of MARCKS. These results suggest that phosphorylation of MARCKS is involved in regulation of secretory function in rat parotid acinar cells.

Talk-G5

Regulation of TRPC channels by STIM1

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TRPC channels are activated by receptor stimulation to mediate Ca^{2+} influx. TRPC channels are activated by multiple mechanisms that include activation by depletion of ER Ca^{2+}, activation by lipid mediators and activation by translocation to the plasma membrane. Regulation of TRPC channels by store depletion has been controversial and the potential ER Ca^{2+} sensor that can mediates regulation by store depletion remains unknown for many years. A breakthrough was made with the identification of STIM1, which has been suggested to regulate SOC and I_{crac} by functioning as the ER Ca^{2+} sensor. How STIM1 activates the Ca^{2+} influx channels and whether STIM1 affect the activity of TRPC channels remains unknown. We found that STIM1 binds TRPC1, TRPC4 and TRPC5 but not TRPC3, TRPC6 and TRPC7. The binding was mediated by the ERM domain of STIM1. As expected, functional assays showed that STIM1 activates TRPC1 and TRPC5. TRPC5 can be activated by receptor stimulation and by La^{3+}. Activation of TRPC5 by receptor stimulation was completely dependent on STIM1, whereas activation by La^{3+} was not, indicating that a) regulation of TRPC5 by agonists and La^{3+} occurs by different mechanisms and b) regulation by STIM1 does not involve translocation of the channels to the plasma membrane. Remarkably, the cytosolic C-terminus of STIM1 was sufficient for activation of SOC, I_{crac} and TRPC channels, even when native STIM1 is depleted by siRNA. This indicates that STIM1 is not a sudunit of the channel and that the regulatory domains of STIM1 resize in its C terminus. Activity of STIM1 requires an ERM domain and a cationic lysine-rich region, which is essential for gating of TRPC1. However, the lysine-rich region was not involved in binding of STIM1 to TRPC channels, suggesting that it regulates channel activation or gating. Deletion of the ERM or lysine-rich regions in the constitutively active STIM1(D76A) yields dominant negative mutants that inhibited the nativity of the SOC and TRPC channels. Finally, deletion of STIM1 in cultured intralobular parotid ducts inhibited Ca^{2+} influx activated by store depletion. These findings implicate STIM1 as a key regulator of activity rather than a channel component, and reveal similar regulation of SOC, I_{crac} and TRPC channel activation by STIM1.

Talk-G6

Shank2 as a key regulator of epithelial transport in the apical membrane.

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Aberrant ion transport caused by either hypo- or hyper-functioning of epithelial transporters is associated with a number of diseases from cystic fibrosis to Sjögren's syndrome. Accumulating evidence suggests that protein-protein interactions play a critical role in the regulation of fluid and ion transport of secretory epithelia including salivary glands[1]. PDZ (PSD-95/discs large/ZO-1)-based adaptors have emerged as a large group of proteins that sequester functionally-related groups of transporters, receptors, and other effector proteins into integrated molecular complexes[2].

Recently, we found that Shank2, a PDZ domain-containing protein, is localized at the apical region of many secretory epithelia including colon, pancreas, and salivary glands, and associates with two key epithelial transporters, the cystic fibrosis transmembrane conductance regulator (CFTR)[3] and the Na^+/H^+ exchanger 3 (NHE3)[4]. Interestingly, Shank2 affects the membrane expression of CFTR and NHE3 and the cAMP-dependent regulation of these transporters. For example, Shank2 decreased the cAMP/PKA-dependent phosphorylation and activation of CFTR in heterologously expressed cells and in T84 epithelial cells. In the case of NHE3, the cAMP/PKA-dependent inhibition of NHE3 activity was attenuated by Shank2. Moreover, it was found that Shank2 and EBP50/NHERF1, a PDZ-based adaptor which recruits PKA, compete with each other in associating with CFTR, and the CFTR Cl^- channel activity was dynamically regulated by the competition of Shank2 and EBP50 binding. These results indicate that Shank2 is one of the key organizers at the apical membrane of secretory epithelia, and controls the membrane expression and activity of CFTR and NHE3.

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Talk-G7

The regulation of epithelial Na^+ channels by UTP.

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Epithelial Na^+ channels are expressed in the apical membranes of the epithelia of the colon, the respiratory tract, the renal collecting duct and the excretory ducts of the exocrine glands. Their activity is regulated by hormones such as aldosterone and insulin. It is also regulated by autocrine factors, such as ATP, which are released from the epithelium in response to mechanical stimuli and contact with pathogens such as *Pseudomonas aeruginosa* and parainfluenza virus. ATP acts via metabotropic receptors in both the basolateral and the apical membranes of epithelial cells to inhibit the activity of epithelial Na^+ channels. Despite its physiological importance, and the potential role of purinergic agonists as therapies for cystic fibrosis, the mechanism(s) by which ATP inhibits epithelial Na^+ channels remain unclear.

We have been using Fisher Rat Thyroid (FRT) cell monolayers transfected with the a-, b- and g-subunits of ENaC and with siRNA directed against candidate signalling mediators to investigate the intracellular mechanisms by which the purinergic agonist, UTP, inhibits epithelial Na^+ channels. Our data indicate that the inhibitory effects on Na^+ channels of both apical and basolateral UTP are mediated by increased intracellular Ca^{2+} consequent on the activation of P2Y2 receptors and pertussis toxin-sensitive G proteins. They further show that the effect of basolateral UTP and a component of the effect of apical UTP are mediated by phospholipase b4. The mediator of the non-phospholipase Cb4-mediated component of the inhibition produced by apical UTP is unclear, although it does not appear to be one of the other 3 isoforms of phospholipase Cb. Finally, we found that the inhibitory action of intracellular Ca^{2+} on the Na^+ channels is dependent on changes in intracellular Cl^- concentration.

Talk-G8

Signaling pathways regulating Na^+ transport in salivary ducts

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The plasma-like primary saliva from the salivary endpieces undergoes modification during its passage along the salivary duct. Here Na^+ is absorbed through the epithelial sodium channels (ENaC) that are expressed at the apical membrane of the duct cells. ENaC is a heteromultimeric protein that is expressed in several Na^+ absorptive epithelia including the kidney, the colon, the lung and ducts of the sweat and salivary glands. It plays a critical role in the regulation of blood pressure, extracellular fluid volume and the thickness of the fluid layer coating the respiratory passages as well as in the production of hypotonic saliva. Given the physiological importance of its functions, ENaC activity is tightly regulated. Regulators of ENaC include the hormones aldosterone and insulin, which upregulate the channels, and purinergic agonists as well as intracellular Na^+ and Cl^- concentrations which downregulate the channels.

Recent studies have indicated that many regulators of ENaC exert their effects via protein kinases. For example, it is believed that insulin upregulates ENaC activity via the serum- and glucocorticoid-dependent protein kinase, Sgk. Sgk upregulates ENaC by phosphorylating Nedd4-2. This phosphorylation, in turn, inactivates Nedd4-2 and renders it unable to ubigutinate and inactivate ENaC. In addition to Sgk, ENaC is upregulated by G protein-coupled receptor kinase, Grk2. Unlike Sgk, Grk2 does not phosphorylate Nedd4-2 but rather directly phoshorylates the b subunit of ENaC. This phosphorylation of ENaC prevents the channel from interacting with Nedd4-2. Hence, as is the case for Sgk, Grk2 inhibits internalization of ENaC.

In addition to being regulated by kinases, ENaC is regulated by membrane lipids. It has been suggested that insulin may exert its effects on ENaC via phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PIP3). The detailed mechanism of the cellular pathway that phospholipids use to control ENaC activity is still not known. This raises questions of the extent to which the effects of insulin on ENaC are mediated by kinases and the extent to which they are mediated directly by phospholipids, and whether the effect of phospholipids on ENaC is also mediated via Nedd4-2.

Poster-G4

Ca^{2+} influx induced by ionomycin under a high $[K^+]_o$ in rat submandibular acinar cells.

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An increases in extracellular K^+ concentration ($[K^+]_o$) inhibits Ca^{2+} entry in rat submandibular acinar cells. The high $[K^+]_o$ induced inhibition of TG-stimulated Ca^{2+} entry is thought to be induced by decreases in the driving force for Ca^{2+} entry. In the present study, we examined the effects of increasing $[K^+]_o$ on Ca^{2+} entry in TG-stimulated and ionomycin (IM)-stimulated rat submandibular acinar cells.

Submandibular acinar cells were obtained by a collagenase treatment. The control solution (solution I) contained (mM): NaCl 121, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 1.5, $NaHCO_3$ 25, NaHEPES 5, HHEPES 5 and glucose 5 (pH 7.4). The high K+ solution contained (mM): KCl 125.5, $MgCl_2$ 1, $CaCl_2$ 1.5, $KHCO_3$ 25, HHEPES 10 and glucose 5 (pH 7.4). Various K^+ concentrations of test solution were prepared by mixing appropriate amounts of the control and the high K^+ solution. All solutions were aerated with 95 % O_2 and 5 % CO_2 at 37 °C. $[Ca^{2+}]_o$ was measured by Fura 2-fluorescence.

Cells were first perfused with Ca^{2+}-free control solution and were stimulated with TG (2 μM) for 10 min. Then suddenly Ca^{2+} (1.5 mM) was added (reintroduction of Ca^{2+}). The introduction of Ca^{2+} increased $[Ca^{2+}]_i$. Increases in $[Ca^{2+}]_i$ induced by the reintroduction of Ca^{2+} were examined in TG-stimulated submandibular acinar cells perfused with various $[K^+]_o$. As increment of $[K^+]_o$, $[Ca^{2+}]_i$ increases induced by the reintroduction of Ca^{2+} were suppressed.

Similar experiments were performed using IM (5 μM). In IM-treated cells, the introduction of Ca^{2+} also increased $[Ca^{2+}]_i$. However, as increment of $[K^+]_o$, $[Ca^{2+}]_i$ increases induced by the reintroduction of Ca^{2+} were not suppressed. IM-induced $[Ca^{2+}]_i$ increases were also inhibited by Gd^{3+} (1 μM). The final $[Ca^{2+}]_i$s were similar in various $[K^+]_o$, although the rate of $[Ca^{2+}]_i$ increase in the high K^+ solution was slow compared with that in the control solution.

The present study demonstrated that the driving force for Ca^{2+} entry is sufficient in the high K^+ solution in rat submandibular acinar cells. A high K^+ solution, which depolarizes membrane potential appears to modulate Ca^{2+} entry pathways in rat submandibular acinar cells.

Poster-G5

Regulation of epithelial sodium channel by caveolin.

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The epithelial Na^+ channel (ENaC) expressed in the kidney and the distal colon plays an important role in sodium/fluid homeostasis and in maintaining normal blood pressure. In the ducts of salivary glands, Na^+ reabsorption via ENaC determines hypotonicity of the final saliva. Activity of ENaC in these tissues is regulated by several humoral and homocellular mechanisms including by feedback inhibition of the channel by cytosolic Na^+. In salivary duct cells, cytosolic Na^+ feedback downregulates ENaC via a pathway that involves the ubiquitin protein-ligase, Nedd4-2. At high cytosolic Na^+ concentrations, Nedd4-2 interacts with the C-termini of the β– and γ–subunits of ENaC. The interaction between Nedd4-2 and ENaC induces ubiquitin-dependent internalisation of the channel. The detailed mechanism of the internalisation pathway of ubiquitinated ENaC mediated by Nedd4-2 is currently not known.

It is well established that internalisation of plasma membrane proteins is mediated via clathrin-dependent and clathrin-independent pathways. The internalisation of membrane proteins via the clathrin-independent pathway depends on the activity of an integral membrane protein caveolin (Cav). We have been undertaking short-circuit current studies to investigate regulation of ENaC expressed in Fisher Rat Thyroid (FRT) cells and have found that ENaC is downregulated by caveolin-1 (Cav-1). Co-expression of Cav-1 and ENaC significantly decreased both activity and surface expression of the channel. Furthermore, our data indicate that internalisation of ENaC in FRT cells by the caveolin-dependent mechanism is Nedd4-2-sensitive. These findings suggest that Nedd4-2-dependent downregulation of ENaC is, at least in part, mediated via the clathrin-independent pathway. The mechanism by which Cav-1 downregulates ENaC, in particular the role of Cav-1 in Nedd4-2 mediated internalisation of the channel is the focus of our current investigations.

Poster-G6

The role of TRP channels in the salivary gland fluid secretion.

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The fluid secretion in the salivary glands is achieved by a coordinated sequence of intracellular signaling events, including the activation of membrane receptors, generation of the intracellular second messenger, inositol 1,4,5, trisphosphate, internal Ca^{2+} release, and Ca^{2+} influx. It is well-established that prolonged fluid secretion is regulated via a sustained elevation in $[Ca^{2+}]_i$ that is primarily achieved by the influx of Ca^{2+} into the cell from the external medium. Ca^{2+} influx channels are activated shortly after agonist stimulation of cells. This Ca^{2+} influx determines the sustained elevation of $[Ca^{2+}]_i$ in stimulated cells, which regulates several key ion channels that drive fluid secretion. The TRPC family of proteins has been proposed as molecular components of these Ca^{2+} channels. Studies from our laboratory have identified TRPC1 as component of the store-operated Ca^{2+} channels in salivary gland cells. In recent studies we have further established the role of TRPC1 in submandibular gland acinar cell fluid secretion by using TRPC1 null mice. Pilocarpine stimulated fluid secretion was significantly reduced (>50%) in the mice compared to control wild-type. Further, thapsigargin- as well as carbachol-stimulated Ca^{2+} influx as well as Ca^{2+}-currents were comparably reduced in dispersed acinar cells from these glands. Ca^{2+}-dependent K^+ currents were also diminished. In aggregate, these data provide strong evidence that TRPC1 is a critical regulator of salivary gland fluid secretion.

Salivary gland fluid secretion is also determined by the ability of acinar cells to regulate their water permeability and volume. Changes in osmolarity of the extracellular medium induce water fluxes that result in swelling or shrinkage of cells. Regulation of cell volume in response to such changes in osmolarity is critical for cell function and survival. AQP5 has been previously shown to be involved in this mechanism. Recently we provided evidence that TRPV4 is activated during cell swelling and that Ca^{2+} entry via this channel is required for regulatory volume decrease. Further, we reported a novel association between TRPV4 and AQP5 that is involved in regulation of cellular response to hypo-osmotic stress. Our data suggest that TRPV4 and AQP5 are assembled in a signaling complex that responds to anisosmotic conditions and coordinates cellular volume regulation.

Thus we show that members of transient receptor potential family, TRPC1 and TRPV4, contribute to salivary gland function.

Poster-C8

Salivary Gland Dysfunction and Abnormal Eating Behavior in Mice Lacking Muscarinic Acetylcholine Receptors.

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We disrupted *Chrm3* (muscarinic acetylcholine receptor, subtype 3) gene in mice (M3KO mice). The most obvious phenotype was their small body size. They retarded in growth around 3-5 weeks after birth, and some of them even died. Because antagonism of muscarinic acetylcholine receptors is known to inhibit salivary secretion, we hypothesized that they could not eat dry pellet around weaning. When wet paste was fed daily, their growth improved significantly, and no pups died of malnutrition. The M3KO mice did not secrete detectable amount of saliva, even after a pilocarpine injection (1 mg/kg, i.p.). We published these observations in a Journal (Matsui et al. Proc Natl Acad Sci USA. 2000 Aug; 97: 9579-84). Surprisingly, Wess et al. published a paper in Nature later, stating that their M3KO mice secrete enough saliva but suffer from central anorexia, thus developing growth retardation phenotype (Nature. 2001 Mar; 410: 207-12). I felt uncomfortable at their statement and decided to study the salivary function of M3KO mice more in detail. I believed strongly that it was important to examine their salivation under physiological condition. In order to address this issue, I observed eating behavior carefully and noticed that the M3KO mice access water nozzle more frequently than wild type during eating dry pellets. This characteristic behavior was not observed when fed wed paste. Thus, I concluded that the M3KO mice do encounter significant difficulty in eating dry pellet, which is caused by insufficient salivation. In addition, I and Dr. Nakamura (deceased this summer) examined calcium signaling of dispersed submandibular gland cells in response to various concentrations of carbachol. The response was significantly decreased in M3KO mice, and completely lost in M1M3KO mice. This indicates that M3 and M1 receptors contribute to ACh-induced calcium signaling in major and minor manners, respectively. We published these observations in a Journal (J. Physiol. 2004 Jul; 558: 561-75). Soon after, Wess et al. published a paper stating that their M3KO mice also suffer from insufficient salivation (Mol Pharmacol. 2004 Aug; 66: 260-7). Accordingly, the M3KO mice are now proved to be a good model of dry mouth, and I hope this model is used by many researchers for salivary gland studies.

Authors Index

SOME TRADITIONS REGARDING THE OLD ANATOMY THEATRE OF PADUA UNIVERSITY

MAURIZIO RIPPA BONATI

The old Anatomy Theatre of the University of Padua has, justifiably, aroused the interest and curiosity even of outsiders, such that many aspects of its origins, function and use have been "explained" in terms that often have little to do with reality.

Just over four hundred years ago, on the 16th of January 1594, the Anatomy Theatre of the University of Padua was inaugurated. Still today, it arouses both curiosity and admiration among the numerous visitors to the Palazzo Bo. It is easy to understand this reaction both now and in the past: the chronicler of the *Natio Germanica* (one of the "nations", or groups, students were organised into), who was usually not given to effusioness, proudly wrote that "almost everyone in the city" had hastened to take part in the celebrations[1]. We do know though, that the impressive wooden structure can only accommodate about two hundred people, thus, can reasonably assume that in reality, on that auspicious day, very few "ordinary" Paduans had the rare chance of entering the hallowed, exclusive, academic precincts of this unique Anatomy Theatre itself. Probably, however, few people outside the academic world were really able to understand the aims and the significance of the various phases of this disquieting, perhaps threatening, "operation".

Portal, when discussing the more obscure and controversial aspects of anatomical research, observed that "the public exaggerates everything and it considers anything it doesn't understand to be a marvel"[2]. If this is true then it is not difficult to suppose that any event which excited interest will have fed popular myths rumours and credences, many of which still survive. Indeed, today there are many traditions and tales told, regarding the construction and use of the Anatomy Theatre[3], whose origins are difficult to trace, even though they are much less exaggerated than they might have been seeing that the Anatomy Theatre was in use for almost three centuries[4], and that the dissection of human bodies, autopsy, is always surrounded by an air of understandable secrecy and superstition. The rumours and traditions, the "metropolitan legends", described here offer a balanced mixture of reality and imagination, a cocktail, that explains people's continuing interest in the vicissitudes of the Anatomy Theatre which today, after long and careful restoration work, is once again open to visitors[5].

Tradition, says: the Anatomy Theatre of Padua University is the oldest in the world. It was built, and paid for, by Girolamus Fabricius ab Acquapendente who, at the time, was Professor of Anatomy and Surgery. The structure was designed by fra' Paolo Sarpi. Dissection was carried out in secret as it contravened both the civil and religious Laws of the period.

The first commonly accepted "fact", that the Anatomy Theatre "is the oldest in the world", is neither right nor wrong, but should be qualified: it is the oldest permanent Anatomy Theatre, still in existence today. "Permanent" and "still in existence" must be specified. On the one hand because we know that temporary anatomy theatres were in use in Padua from the mid-15th century on and, on the other, because Padua is not the only University that claims to have been the first to construct a permanent classroom-laboratory. In the first case, however, the "Theatre" was a simple, frame structure which was only assembled when a practical lesson was given and then dismantled immediately after use. These temporary structures are known today thanks to both Alessandro Benedetti (c. 1455 - 1525)[6], who, in his work *De Anatomia*, published in the early 16th century, describes the uses and usefulness of such a theatre for autopsies and to Andrea Vesalio (1514 - 1564), who chose a frontispiece for his work *De humani corporis fabrica* which offers a lively representation of just such a structure[7]. This image , which has been elaborated so as to eliminate the one hundred-odd onlookers, is, currently, the only existing pictorial evidence of early anatomy theatres.

The two authors above both mention that tiers became necessary when more people wished to attend the autopsy than could comfortably be accomodated around the dissecting table. This problem first arose in Padua in the 1470s, when Paduan academic life was, both quantitatively and qualitatively, flourishing[8], It arose again in the 1550s when, after the Cambrai crisis, the student population in Padua grew to a remarkable size for those days 1,300 students[9] and, it seems, these students all attended Professor Vesalio's anatomy lessons[10].

The passage from a temporary to a permanent structure is less clear, particularly if one remembers that teaching autopsies could only be carried out in the colder months of the year and, furthermore, only lasted for two or three weeks. However, even though it is hard to know whether the descriptions we have are simply accounts of projects which were never carried out or are accounts of structures that really existed, from the mid-16th century on there are increasingly detailed and frequent references to permanent anatomy theatres but, there is no concrete proof. For example, both the Frenchman, Charles Estienne (Carolus Stephanus)[11] and the Tuscan, Guido Guidi (Vidus Vidius)[12], wrote of theatres but they offer descriptions that are as full of details as they are lacking in any documentary confirmation. Currently, the earliest documented structures are the "House of Anatomy" in Zaragoza which was built in 1586[13] and a theatre in Ferrara, even though, in this latter case, the theatre is not directly described but merely mentioned in passing in documents concerning the Palazzo del Paradiso inside which, in 1588, there was such a structure[14].

However, in Padua, in January 1584, there was already an anatomy theatre functioning "certainly not in a provisional manner, but a permanent structure"[15] and this is the earliest of all the structures we have reliable evidence about at present[16].

The statement that the Anatomy Theatre was paid for by Girolamus Fabricius ab Acquapendente (1533- 1619) is probably the most widely accepted popular "truth": it is also the most easily disproved. Both the first and the second Anatomy Theatres, like any other public building of the day, were paid for by public or "state" funds. The belief that it was Aquapendente who paid for the structure probably gained ground because the name of the famous anatomist is inscribed above the door of the

Theatre[17] or, maybe, because his coat of arms is partially preserved in the room next door, today, the Hall of Medicine[18]. Furthermore, the connection between Girolamus Fabricius ab Acquapendente and the Anatomy Theatre is logical: he was the only Professor of Anatomy and Surgery for almost 30 years and he was famous everywhere: he was considered to be the best anatomist of his day. Indeed, it is no mere accident that when Pietro Damiani painted his famous work showing the miracles of St. Anthony of Padua, he used Girolamus Fabricius ab Acquapendente as the "model" for "the surgeon in the Miracle of the Miser's heart"[19].

But, even though it is not true that Acquapendente personally paid for the Anatomy Theatre out of his own pocket, it is possible that a rich and famous Professor, aware of his professional image, should have, in some way or another, participated in such a major project. For example, in the same period as the Anatomy Theatre was being planned and built, Acquapendente paid for a series of anatomical drawings, in colour, by gifted artists. At the same time his ex-pupil and subsequent rival, Giulio Casseri (c. 1552 - 1616), who had an anatomy theatre in his own house in Via del Pozzo dipinto and later, in 1614, was to construct another, "built largely at his own expense in a room in the Palazzo del Capitaniato"[20] also commissioned similar scientific drawings.

Even more interesting, but harder to explain, is the fact that the design has been attributed to fra' Paolo Sarpi (1552 - 1623). Unfortunately the origins of this popular credence are obsure but, by the 18th century, this attribution had already been widely accepted even among the teaching staff: Domenico Cotugno for example in his *Iter Italicum*[21],attributes the design to Sarpi and he probably received the information, in 1765, from Leopoldo Marc'Antonio Caldani or, perhaps, from the great Giambattista Morgagni in person.

In reality, notwithstanding long and patient research, nothing has been found that has made it possible to identify, with certainty, the person who designed the Padua Anatomy Theatre. The same is true for that other great 16th century scientific project in Padua: the Botanical Garden. However, the fact that neither of these works has a definite author could suggest that they were co-operatively designed, using the "internal" skills available in and around the University at the time. It is fairly certain that Andrea Moroni had a hand in the design for the Botanical Garden. At that time, 1545, he was overseer of St Justina, of the Palazzo Podestarile and had probably already been selected to oversee the restoration of the Palazzo Bo buildings[22]. The "technician" involved in the design of the Anatomy Theatre could have been the architect and painter Dario Varotari (1542 - 1596)[23], summoned, perhaps informally, by Aquapendente himself. Varotari had already designed Aquapendente's country house and had executed some of the anatomical paintings mentioned earlier[24]. The same, however, could be true of Paolo Sarpi (1552 - 1623). He was a friend, a patient, and, although less well known, a scientist who worked with Aquapendente and worked so well that the anatomist, who was usually very sparing with his praise, congratulated Sarpi in public for his contribution to the understanding of pupillary contraction[25]. But this is guesswork, and Paolo Sarpi's role, if he had one, in designing the theatre would remain pure conjecture but for two other coincidences. Firstly, we know that Aquapendente began to study the anatomy of the eye again[26] in 1592, precisely when the first permanent theatre "erat destructum"[27] and had to be replaced and, secondly, a drawing of an ox's eyeball inserted into his work on sense organs, is very like a bird's eye view of the theatre itself.

This coincidence makes us bold enough to suggest that the design for the anatomy theatre could have been developed during the period when the two friends were working on the anatomy of the eye. There may even be a hint of irony in the design, given the old Professor's character[28]; But the problem the theatre design had to solve, and did solve, was how to create a structure that would allow the maximum number of people to have the best possible conditions when participating in an unrepeatable experiment: the dissection of a corpse.

So much for the origins of the Anatomy Theatre, now let us turn to the "proof" that autopsies, even if not explicitly forbidden, were still something that was better done in secret. There are many rumours to investigate here: that there is a canal under the Palazzo Bo which ensured that the bodies could at least enter and leave the Anatomy Theatre discreetly, that the windows of the Theatre were bricked up so that there could be no casual onlookers and, lastly, that the dissecting table could be turned over so that the human body would disappear fast and an animal appear in its place. But before dismissing these popular myths out of hand it hould be remembered that human dissection was never explicitly forbidden even though the Religious authorities, at different times and in different places, interpreted the instructions issued by Boniface VIII in 1300 in very different ways. However, the Papal Bull *De sepulturis*, was really more concerned with certain "ferocious" practises during funerals, which damaged the corpse, than with postmortem operations to establish the cause of death or to add to knowledge about human anatomy.

As regards Padua, proof that human dissections were not illegal lies in the fact that part of the money destined for such dissections was used to celebrate funerals in the Church of San Martino[29], which was near the Palazzo Bo, and there is also a rare commemorative document[30], published in relation to the Church of Santa Maria dei Servi regarding such funerals. The public nature of these religious ceremonies serves to discredit the idea of a secret underground canal, which, in fact, has never existed. The tradition of the secret canal may have arisen because of student pranks that went too far: for example, one night in 1582, students did not only steal the corpse but "indignis modis dilaniatum, in perfluenten Brentam proiecerunt"[31] (briefly: they threw it in the nearby River Brenta!). But the real reason for the canal myth is probably the fact that the corpses were, indeed, brought to the Bo by canal, the main Naviglio Interno (Inner Canal), which ran through the city and passed quite close to the University buildings before going underground.

The question of the bricked up windows may have a practical explanation too, in that all the windows were bricked up, but only because the wooden theatre structure functioned best when lit from above since the focus of interest, the dissecting table, lies at the bottom of the inverted cone.

Lastly, as regards the use of a table designed to be turned over quickly, hiding the corpse and revealing an animal, this can be explained because of the former custom of carrying out both human and animal dissections during the same lesson so as to offer a comparative anatomy

lesson. Indeed, in 1584, when the first permanent theatre came into use, it was soon established that animal dissections could be carried out in any, and not only the usual, period. This custom of animal dissection was a very old one and, to cite the case of 16th century Padua, one only has to remember the text and illustrations in *De humani corporis fabrica* by Vesalio and the activities of Gabriele Falloppio (1523 - 1562) who, in February 1553, won the applause of his exacting student spectators by dissecting a monkey instead of the usual dog or pig and again, in December of the same year, dissected a "caput phocae", probably a Dolphin, to see that animal dissections were a part of normal University practise.
In 1556, animals were, once again, the subject of a request Falloppio sent to the Riformatori in which he splendidly sums up the didactic and, indirectly, the social importance of dissection lessons. To summarise his request: since the times that "invite anatomy" were getting mear, the magistrates were encouraged to provide a "soggetto" as quickly as possible because, if there were no such subject available soon, there was a risk that many students would leave Padua and go to Bologna or Ferrara to study. In the meantime he proposed to fill the gap and "far con gl'orsi e la simia una bellissima Anatomia" (carry out beautiful Anatomy with bears and monkeys), lessons he invited the Riformatori themselves to attend[32].
To conclude with a doubly curious observation that, once again, demonstrates the strange logic of myth, rumour and tradition. The one thing that is never mentioned, indeed seems to have been entirely forgotten, is that in the 16th and 17th centuries music was often performed in the Anatomy Theatre. Once again we have to thank the record keeper of the *Natio Germanica*[33], for this information. He tells that this music was used both to keep the students calm while they waited for the teacher and to create a relaxed atmosphere in breaks during a practical lesson. It seems very strange that in the city, which was certainly much queiter than it is today, this music did not attract any attention at all. It seems equally strange that speculation about the music did not fuel yet another intriguing, metropolitan legend about the Anatomy Theatre of Padua.
Recently, Maurizio Rippa Bonati has promoted the recording of a CD of Paduan lute music of the XVI – XVII centuries played in the Anatomy Theatre by Terrel Stone. Such disk is entitled *De auditu* (*about the ear*) to pay homage to Fabricius who, during his lifetime, published a book with the same heading.

[1] A. Favaro (a cura), *Atti della Nazione Germanica Artista nello Studio di Padova*, "Monumenti storici pubblicati dalla R. Deputazione Veneta di Storia Patria", XX, vol. 2, Venice 1911-12; see vol. II, p. 58.
[2] A. Portal, *Histoire de l'Anatomie*, Paris 1770, vol. I, p. 272, quoted by D. Giordano, *L'anatomia dei vivi*, in *Scritti e discorsi pertinenti alla storia della medicina ...*, Milan 1930, p. 22.
[3] M. Rippa Bonati, *Le tradizioni relative al teatro anatomico dell'Università di Padova con particolare riguardo al progetto attribuito a Fra' Paolo Sarpi*, "Acta Medicae Historiae Patavina", vol. 25-26, 1988-89 and 1989-90, pp. 145-168.
[4] The Anatomy Theatre in the Bo was in use until 1872 when teaching activities were transferred to the Institute in Via San Mattia.
[5] C. Semenzato (ed.), *Il Teatro anatomico. Storia e restauri*, Padova 1994; see especially V. dal Piaz, *Architettura, trasformazioni, restauri: da laboratorio scientifico a monumento della scienza*, pp. 83-113; A. M. Spiazzi, *Criteri generali sull'intervento di restauro e annotazioni in margine ai manufatti lignei in Padova nel Secolo XVI*, pp. 135-140 and the *Schede tecniche*, pp. 141-175.
[6] A. Benedetti, *Anatomice, sive de Hystoria corporis humani libri quinque*, Argentorati 1528; bk. I, chap. I, "De utilitate anatomices, et de cadavere eligendo deque temporario theatro constituendo"; although many authors mention an edition that was purportedly printed in Venice in 1493, we have found no trace of this edition nor any evidence that it ever really existed.
[7] A. Vesalio, *De humani corporis fabrica libri septem*, Basle 1543.
[8] F. Dupuigrenet Desroussilles, *L'Università di Padova dal 1405 al Concilio di Trento*, in *Storia della Cultura Veneta*, vol. 3/II: *Dal primo Quattrocento al Concilio di Trento*, Vicenza 1981, pp. 607-647; see especially the section "L'età d'oro (c. 1475-1509)", pp. 619-623.
[9] Archivio di Stato di Venezia, *Capi del Consiglio dei Dieci, Lettere di Rettori*, busta 82, c. 2 (21 March 1542).
[10] Archivio Antico dell'Università di Padova, *Atti dell'Università Artista*, Raccolta Minato vol. 28, c. 113r.
[11] C. Stephanus, *De dissectione partium corporis humani libri tres*, Parigi 1545, bk. III, chap. XL, "Anatomici theatri apparatus", pp. 346-348; the semicircular wooden structure, able to accomodate up to 500 people, would have been set up in the open air and, possibly covered by a *velarium*.
[12] V. Vidius, *De anatome corporis humani*, Venezia 1611, bk. I, chap. VIII, "De Lumine, Loco, ac Scamno idoneo ad Anatomen", pp. 12-13; an octagonal wooden structure, constructed inside a square area is described in this work which, although it was not printed until the early 17th century, had in fact been written some fifty years before.
[13] A. San Vicente, *Monumentos diplomaticos sobre los edificios fundacionales de la Universidad de Zaragoza*, Zaragoza 1981, pp. 7-9 and 167-168.
[14] G. Muratori e G. Guidorizzi, *Documento unpublished, 1588, concerns the construction of the permanent Anatomy Theatre in the University of Ferrara*, in *Acts of the III Convegno della Marca per la Storia della Medicina*, Fermo 1960, pp. 267-268.
[15] Archivio Antico dell'Università di Padova, *Cronaca delli professori riformatori*, t. II, n. 20, *Del modo con cui trattavasi anticamente l'Anotomia, e del Sistema presente*. G. Cervetto, *Di alcuni illustri anatomici italiani del Decimoquinto Secolo - Indagini per servire alla storia della scienza*, Verona 1842; see art. V, par. I, "Cenni storici sul teatro anatomico Padovano", pp. 133-146.
[16] A. Gamba, Il primo teatro anatomico stabile di Padova non fu quello di Fabrici d'Acquapendente, "Atti e Memorie dell'Accademia Patavina di Scienze Lettere ed Arti", XCIX (III), 1986-87, pp. 157-161; thanks to the information contained in the *Atti* della *Natio germanica* and in the University archives it has been possible to reconstruct the early history of this theatre in even greater detail than that of its, still existing, successor which was built in 1594-95.
[17] HIERONYMO FABRICIO AB AQUAPENDENTE / XXX IAM ANNOS ANATOM[IAE] PROFESSORE; this inscription, which was clearly executed in 1595 can easily be associated with the epigraph above which commemorates the Civic Rectors and Riformatori in office in 1594:

THEATRUM ANATOMICUM / IUSTINIANOIUSTINIANO PRAETORI / NICOLAO GUSONI PRAEFECTO / IOANNI SUPERANTIO EQUITE / MARINO GRIMANO EQ[UITE] ET DI[VI] M[ARCI] PROC[URATORE] / LEONARDO DONATO EQ[UITE] ET DI[VI] M[ARCI] PRO[CURATORE] / GYMNASII MODERATORIBUS / MDXCIIII/

[18] **L. Rossetti (ed.), *Gli Stemmi dello Studio di Padova*, Trieste 1983, p. 614, n. 2776: [HIERONY]MUS FABRICIUS [A]B / [AQUAPENDE]NTE SUPRA ORDINA/RIUS.**

[19] **P. Damini, *The Miracle of the miser's heart*, Church of San Canziano, Padua. For an up-date on the artist see *Pietro Damini 1592-1631 - Pittura e Controriforma*, Atti della giornata di studio, Padova 29 settembre 1993, "Bollettino del Museo Civico di Padova", (back copy) LXVI, 1977, 167 pp.**

[20] **G. Sterzi, *Giulio Casseri anatomico e chirurgo (1552 c. - 1616)*, "Nuovo Archivio Veneto", n.s., XVIII (II), 1909, extract 167 pp.**

[21] **L. Messedaglia, *L'"Iter Italicum Patavinum" di D. Cotugno - G. B. Morgagni e L'Università i Padova nel 1765*, "Atti del Reale Istituto Veneto di Scienze, Lettere ed Arti", LXXIII (II), 1913-1914, pp. 1691-1803, see p. 1771; L. Belloni, *Dominici Cotunnii Iter Italicum anni MDCCLXV*, "Memorie dell'Istituto Lombardo – Accademia di Scienze e Lettere. Classe di Lettere, Scienze Morali e Storiche", s. IV, vol. XXVII (II), 1960, extract 93 pp., see p. 64.**

[22] **E. Rigoni, *L'architetto Andrea Moroni*, Padua 1939, p. 35.**

[23] **G. Cagnoni, *I Teatri Anatomici dell'Università di Padova*, Degree thesis, Institute of Architecture, University of Venice, Dept. of History of Architecture, a. a. 1987-88.**

[24] **C. Semenzato, *Il Palazzo del Bo - Arte e Storia*, Padua-Trieste 1979, p. 107.**

[25] **G. Fabrici d'Acquapendente, *De visione voce auditu*, Venice 1600, p. 93.**

[26] **A. Favaro (a cura), *Atti ...*, cit., vol. II, p. 30.**

[27] ***Ibidem*.**

[28] **G. Favaro, *L'insegnamento anatomico di Girolamo Fabrici d'Acquapendente*, in *Monografie storiche sullo Studio di Padova*, Venice 1922, pp. 107-136; for example, during a lesson in 1589, Acquapendente imitated the pronunciation of his German students to such a point that they lodged a formal protest.**

[29] **The church was demolished in 1808.**

[30] **B. Franzina Bartolomeo, *Collaudatio mortuorum quorum anatomen publice professus est Patavii Perillustris et Excellentissimus Iulius Casserius Placentinus ... in templo Servorum anno 1614 mense Martii die octavo*, Patavii 1614.**

[31] **A. Favaro (a cura), *Atti ...*, cit., vol. I, p. 186.**

[32] **G. Favaro, *Gabriele Falloppia modenese (MDXXIII - MDLXII). Studio biografico*, Modena 1928, pp. 226-227.**

[33] **A. Favaro (a cura), *Atti ...*, cit., vol. II, p. 142-143; the same happened during the academic year 1599-1600, *ibidem*, p. 206 and G. Favaro, *L'insegnamento anatomico di Girolamo Fabrici d'Acquapendente*, in *Monografie storiche sullo Studio di Padova*, cit., p. 113.**

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A Program of STENSEN III for Opening Day on 20th October 2006

Opening Ceremony 17:00-17:15

Opening **Masataka Murakami** President of Organizing committee for STENSEN III
Welcome **Noboru Mizuno,** President of National Institute for Physiological Sciences
Messages from
Japan Salivary Gland Society
Yasunobu Okada President of Physiological Society of Japan
Angelo Volpi Science & Technology Attache of Embassy of Italy in Tokyo
Masaru Ishikawa Dupty Mayor of Okazaki City

Opening Talk 17:15-17:40

The work of Fabricius ab Aquapendente (Harvey's Teacher) in the light of the recently restored Tabulae Pictae: its influence in the development of modern anatomy in Europe and in Japan.

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In the dedication of his De Visione, Voce, Auditu, Fabricius ab Aquapendente (1533-1619) reports that he had in preparationg an atlas of human and animal anatomy of which, in 1600, he had ready more than 300 tables (the Tabulae Pictae) all in colour and life-size. The painted tables, lost after Fabricius's death, were traced in 1909 by Giuseppe Sterzi (1876-1919) in the Marciana, the former state library of the Venetian Republic. In the Marciana there are 169 large oil-painted illustrations collected in eight files, while the rest (43) are in 3 volumes containing 5 Fabricius's published works. Most of them are true masterpiece of figurative art. The Tabulae are unlabeled and are the work of many artists still unknown. The evaluation of the Tabulae Pictae under the anatomical profile, fully endorses both the great admiration of Fabricius's contemporaries, and Sterzi's statement that they represent the most important anatomical work of the XV-XVII centuries. There are, in fact, many priorities that will be reported here. Fabricius is the man who introduced Aristotelian anatomy at Padua. He was the first to describe, inter alia, the sensitivity of pupil to light, the disappearance of the ductus arteriosus and of the umbilical vessels, and the discoverer, in fowl, of the lymphatic organ which now bears his name (bursa of Fabricius). His research program greatly influenced his students, among who there were Julius Casserius (1552-1616), Adrianus Spigelius (1578-1625), Johannes Veslingius (1598-1646), William Harvey (1578-1657) and many others from all Europe. Even if Casserius became Fabricius's fierce academic rival, and Harvey, on the valves of the veins, reached conclusions opposite to those of his erstwhile teacher, all their published works are based on Aristotle's philosophy. Unlike Leonardo's drawings and Eustachius's engravings, that also were lost for centuries and, therefore, could not influence the development of the new anatomical science, many discoveries contained in Fabricius tables that during his lifetime were freely available (1) are incorporated in many books published by Anatomists that had been his pupils. In fact, it is through the "Syntagma Anatomicum", a book originally published in Venice by Veslingius (Johannnes Wesling), who belonged to Fabricius's school and was one of his successors in the chair of Padua, that Western Anatomy first entered Japan (2, 3). Moreover, it has been said that most of the illustrations of Kulmus's' "Ontleedkundige Tafelen", the book that gave rise to "the dawn of Western medicine in Japan", were taken just from the "Syntagma Anatomicum" (4).

[References]
1) Ongaro G. Fabrici: dai manoscritti alla stampa. In: Rippa Bonati M and Pardo-Tomàs J, eds. Il Teatro dei Corpi: le Pitture Colorate di Fabrici d'Acquapendente. Milano: Mediamed, 2004:17-30.
2) Ogawa T. History of Medicine. (in Japanese) Tokyo: Chuokoron-Sha, 1964.
3) Bowers JZ. Western medical pioneers in feudal Japan. Baltimore: J Hopkins, 1970:66
4) Luyendijk- Elshout AM: Ontleedinge (Anatomy) as underlying principle of Western Medicine in Japan. In: Beukers et al. Eds. Red–Hair Medicine. Dutch–Japanese medical relations. Amsterdam: Rodopi, 1991: 32.

Opening Concert 17:40-18:40

Program for Lute Concert in Okazaki on 20th October 2006

Terrell Stone – lute
8 course lute by Paul Thomson, 1989

Lute Music from the Court of Francis the 1st

Prelude	Pierre Attaingnant
Regi Seculorum	Claude de Sermisy/Albert de Rippe
Basse dance '*La brosse'*	Pierre Attaingnant
D'amour je suis desheritée	Jean Richafort/P. Attaingnant
2 branles	Pierre Attaingnant
C'est Boucane	Anon/P. Attaingnant
4 Branles de Burgogne	A. LeRoy
Fantasia XVIII	A. de Rippe
Pavane '*Veneziana'* e Gaillard '*La roque'*	P. Attaingnant
Fortune laisse	P. Attaingnant
O passi sparsi	Costanzo Festa/A. de Rippe
Gaillarde *piedmontese*	G. Morlaye

Pause

Paduan Lute Music from the 16th Century

Reccerchar	A. Rotta
Pasa'e mezzo, Saltarel, Padovana *ala villana*	
Gentil Madonna	
Rose e viole	
La rocha'l fuso	
Recerchare	J. Barbetta
Baletto de Rusia, Baletto deto *del Capello Cinganesco*	
Aria Prima	
Padoana Terza, deta *la Lubiana*	
Aria Seconda	
Padoana Ottava deto *Zo per la Brenta*	
Baletto de Ruscia deto *Duda*	
Almande	from the Herold ms.
Galliarda	
Balletto *Diomedi*	
Courante	
Volte *quant ie voy ce bel oeil*	
Pavana *Lachrime*	
Bergamasca	

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PROGRAM NOTES

Pierre Attaingnant (*c* 1494 - late 1551 or 1552)
French printer and publisher. Son-in-law and heir of the printer-engraver Philippe Pigouchet (fl. 1490-1514). Beginning with a collection of chansons dated April 4, 1527/28, he invented and introduced a new method of printing music in which the staff-segments and notes were combined, so that both could be printed in a single printing. Royal privileges protecting his music books were granted or renewed three times, about a year after the first book was printed, in 1531, and in 1537. His publications include several books of pieces in lute tablature or keyboard score, seven books of Masses, fourteen books of motets, and over thirty-six books of chansons, plus numerous re-editions. New music by French composers from the time of Francis I dominates most of these books, and, in contrast to the usual practice of the times,

very little material comes from the publications of his national and foreign competitors. It is rather unlikely that Attaingnant composed the lute music in his collections and probably appropriated music by famous lutenists such as Pierre Blondeau.

Claudin de Sermisy (*c*1490 - 1562)
French composer. Possibly a choirboy at the Sainte Chapelle in Paris; a singer there in 1508, when he was appointed a singer in the Royal Chapel of Louis XII. With Francis I he travelled to Italy in 1515 and he was among the musicians who delighted their hearers when Francis and Henry VIII met at the Field of the Cloth of Gold in 1520. He became *sous-maître* of the Sainte Chapelle in 1530. In 1533, Claudin was made a canon of the Sainte Chapelle (where he had served briefly in 1508), thus being assured a substantial salarya canon there in 1533, and finally attaining the rank of choirmaster in 1547. He and Louis Hérault shared the post in 1547 and retained it under Henry II.

Claudin (as he is usually known) published three books of motets, eleven Masses and a Passion, but is best known for the 160 or so chansons which came out in many printed anthologies, including Attaingnant's first collection of 1528 where he is represented by no fewer than seventeen pieces of the thirty-one. These constitute the quintessential French chansons—lyrical miniatures with attractive melodies carefully declaiming the words in mainly syllabic fashion, and a chordal idiom without much contrapuntal elaboration in a basic 4-part texture. Many of the texts are by contemporary poets of the royal circle; he set 22 of Clément Marot's text- more than any other composer did – and the initial results of their collaboration, which appear anonymously in Attaingnant's *Chansons nouvelles* (1528), antedate by four years the first literary edition of the same poems in the *Adolescence clémentine*.

Guillaume de Morlaye (*c*1510 - after 1558)
French lutenist editor and composer. Said to have maintained a variety of commercial interests; in 1548 he was involved in the slave trade and between 1549 and 1553 he dealt in engravings. He was a student of Albert de Rippe, whose music he published with his own in six volumes between 1553 and 1558 under a ten year royal privilege. He also published dances and his own arrangements for voice and lute of Certon's psalm-settings; he wrote for guitar as well as lute.

Albert de Rippe (Alberto da Ripa) (*c*1480 - 1551)
Italian lutenist and composer. He was in the service of Ercole Gonzaga, Cardinal of Mantua, on 12 February 1529, when he played before Henry VIII. Three months later he was in the service of Francis I; with the exception of a visit to Rome in 1531, he appears to have remained at the French court until his death. A renowned virtuoso whose death provoked tributes from such well-known poets as Marot and Ronsard. Like other virtuosos of the 16th century, he appears to have been reluctant to publish his music. Six volumes of his music were published posthumously by his pupil Morlaye; the surviving order to the printer to produce 1500 copies of one of them is testimony to the popularity of the lute and of Alberto da Ripa's music in sixteenth century France.

Adrien Le Roy (*c*1520 - 1598)
French publisher/printer, composer, lutenist and writer. From 1551 he ran the firm of Le Roy and Ballard in partnership with his brother-in-law Robert Ballard, of which he was artistic director and whose productions included many volumes of chansons and *airs de cour*. The firm was virtually without competition in France from 1551 to 1598. He was a friend of many composers, including Lassus, most of whose works he printed; he was author of long-popular pedagogical books for plucked string instruments. Le Roy had great skill in arranging vocal music for the lute, and his *Instruction ...* (reprinted 1574) describes how to do this, using Lassus' chansons as examples, as well as giving instruction in playing. He was a composer of chansons, accompanied songs, and pieces for cittern, lute, and guitar.

Costanzo Festa (*c*1480 - 1545)
Italian composer. He served the d'Avalos family on Ischia around 1510 and then seems to have studied with Mouton in Paris. In 1517 he entered the service of the Papal chapel in Rome as a singer, remaining there until his death. He was one of the few native Italians in the choir, which was at this time dominated by musicians from northern Europe.
He was one of the principal composers (and again one of the few native Italians) in the generation of early madrigalists. Festa took up the fashion for *note nere* madrigals (i.e. with black notes, thus faster crotchet movement). His style sometimes harked back to the chansons of Josquin; some such madrigals are written for very low voice ranges, suggesting solo performance with instrumental support. Festa contributed two ceremonial madrigals to the Duke of Florence's wedding entertainment in 1539.

Jean Richafort (*c*1480 - *c*1547)
Franco-Flemish composer. When Pope Leo X met with Francis I in 1516 to devise the Concordat of Bologna, the pope demonstrated his graciousness by rewarding many members of Francis' entourage. He made two of the leading French composers, Longueval and Mouton, apostolic notaries, and he gave lesser gifts to various other singers, among them Claudin de Sermisy and Richafort. Amaster contrapuntist following Josquin Desprez's example, Richafort was among those who attempted to deepen and enhance the relationship between words and music

Antonio Rotta (?-1549)
Although biographical information concerning Antonio Rotta is rather scarce, we are able to acquire intriguing insights regarding the activities of Rotta thanks mostly to archival documents. We

know for example that Rotta lived in *contrada Rudena* near the Basilica of St. Anthony and became quite wealthy as a lutenist. Further documents attest to a duel Rotta had, for unkown motives, with a certain Agostino Lavandaro. It seems that Rotta lost the swordfight resulting in rather serious injury and was unable to carry on his teaching activities for more than a month, which led him to take legal action against Lavandaro for medical expenses, and lost income. The Court records show that in 1532 Rotta had 32 lute students (most of them foreign) and gave lessons daily to many of them. Do to his intense teaching activity it is not surprising that he published only one book of lute music. In 1546, Rotta's INTABOLATURA DE LAUTO was published in Venice. The INTABOLATURA is a well-organized book containing groups of dances (mostly in form of pass'e mezzo - gagliada - padoana), vocal intabulations (motetts, madrigals and french chansons) and recercare. The appearance of pieces from the INTABOLATURA in important non-Italian anthologies for the lute (edited by Pierre Phalèse in France and Hans Gerle in Germany) attest to the popularity and diffusion of Rotta's music throughout Europe during the mid 16th century.

Julio Cesare Barbetta (c. 1540 – after 1603)

Most of the biographical information we have concerning Julio Cesare Barbetta is gleaned from his 4 extant publications for lute: IL PRIMO LIBRO / DELL'INTAVOLATURA / DE LIUTO… Venice, 1569; NOVAE / TABULAE MU/SICAE… Strasbourg, 1582; INTAVOLA/TURA DI LIUTO… Venice, 1585; and INTAVOLATURA / DI LIUTO / DELLE CANZONETTE / A TRE VOCI Venice, 1603. On each of the title pages he invariably includes the adjective *Padoano* after his name proudly attesting to his origins. We also learn from these editions that Julio Cesare Barbetta Padovano enjoyed friendships and acquaintances with dignitaries and merchants both foreign and local. In the 1585 publication used for this recording Barbetta explains that he was persuaded by his patrons and friends to compose pieces "*nel modo da moderni usato*". The collection includes an assortment of characteristic dance music coming from "*diverse nationi*" such as France, Germany, England, Poland, Russia, and Slovenia in addition to Italy.
Barbetta was perhaps the greatest advocate of his time for the development of the seventh course on the lute, an endeavor that was credibly facilitated by the presence in Padua of excellent lute makers.

The Herold Manuscript (1602)

The title page of the Herold Manuscript states that it was copied in Padua by Christoph Herold (Christophori Herholdess) in 1602. Christoph Herold was born in Halle in 1578, studied jurisprudence at the University of Leiden from 1598 to 1601 transferring to the University of Padua in 1601 where he earned his Doctoral degree in 1603. While at the University of Padua, he became the leader of the assembly of German law students known as "natio gemanica iuristarum". During his studies at the Leiden University, he probably became the student of the well-known Netherlands composer-lutenist Joachim van den Hove (1567 – 1620) who taught lute in Leiden to several students at the University. Several pieces in the Herold Manuscript are by van den Hove or share concordances with pieces published by van den Hove. The musical contents of the Herold Manuscript demonstrate a wide variety of national influences representing music from England, Germany, France, the Netherlands and Italy. Interestingly, the last piece of the manuscript is the *Balletto de Russia / deto Duda* by Julio Cesare Barbetta.

Terrell Stone began his musical studies in the United States dedicating himself to the study of the lute since 1974. He studied lute as an internal student at the "Schola Cantorum Basiliensis" in Basel, Switzerland with Eugene Dombois and Hopkinson Smith and in Paris, France with Frank Eyler. He earned his diploma in lute with highest marks at the Italian State Conservatory "F. E. Dall'Abaco" in Verona under the guidance of Orlando Cristoforetti. For many years he has performed as a soloist and has participated in important music festivals in North and South America and in Europe and in the Middle East. He is also very active as a chamber musicians realizing *basso continuo* on the lute, archlute and theorbo.

Stone has recorded for television, radio and recording companies and has over thirty recordings to his credit. His solo recordings include a compact disc of the solo music of Giuseppe Brescianello for gallichone, a 3 CD set of the music of Silvius Weiss for baroque lute from the Warsaw manuscript RM 4137, and most recently, a CD containing music of 16th century Paduan lute composers recorded in the world famous Anatomical Theater of the University of Padova.

Stone has resided in Italy for over 25 years and has taught lute at the Conservatory "Santa Cecilia" in Rome, Italy, at the Conservatory "N. Piccini" in Bari, Italy and is presently Professor of lute and President of the Early Music Department at the Conservatory "A. Pedrollo" in Vicenza, Italy.

In addition to his performing, recording and teaching activities, he has edited several modern editions of music for lute and has conducted research and has written scholarly articles concerning early music. Stone devised the computer program *'Tastar de Corde'* for writing lute tablature and its' transcription to modern notation.

Acknowledgement

This concert was planned by organizing committee of the 3rd International Symposium on Salivary Glands in Honor of Niels Stensen (STENSEN III, 20-24 October 2006, Okazaki Japan) and financially supported by Institute of Italian Culture Tokyo and the participants of STENSEN III.

Bond graph expression on an epithelial transport system

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Membrane transport systems can be modeled by bond graphs. In the simultaneous transport equation deduced from the graph, every flow becomes a function of all driving forces that have reciprocal coefficients as same form as Onsager's phenomenological equations.

Key words: causality of coupling, across and through variables

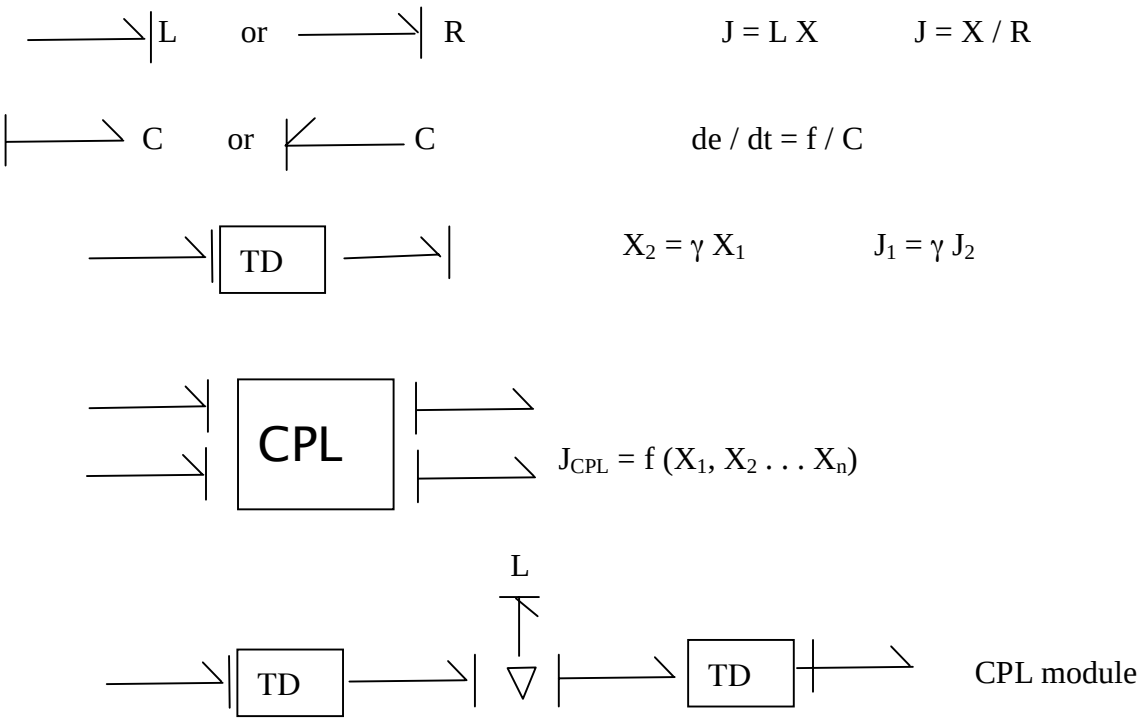
Oster et al. (ref. 1) introduced bond graphs in network thermodynamics. The bond graph is very useful for a representation of thermodynamic systems in which Eulerian (stationary) co-ordinate frame is used. But it is inadequate for a representation of mechanical systems in which Lagrangian (moving) co-ordinate frame is used. This paper is limited to use the bond graph in thermodynamic systems like as membrane transport systems. The membranes are assumed to be dissipative subsystems in which power dissipates, and fluid compartments are capacitive subsystems in which power is stored. The dissipative subsystems are represented by bond graphs from consideration of *coupling causality*.

The bond graph is composed with bonds, thermodynamic elements and connection elements. A bond, a half-arrow, can express a power with its direction and its causality. Each power is expressed by a product of through variable (f or J) and conjugated across variable (e or X). The causality is expressed by appending to a bond, a vertical bar, called a causal stroke, with following meaning: an element adjacent to the vertical bar is governed by an across variable. The through variable is measured at one point in a circuit, and is additive at a parallel connection and called KCL (Kirchhoff current law) variable. The across variable is measured by attaching the instrument to two connection points on the component, and is additive at a series connection and called KVL (Kirchhoff voltage law) variable (table 1). In a thermodynamic system, through variables are a current, a volume flow, a mass flow, a reaction rate and etc., and the across variables are a voltage, a pressure, a chemical potential, a chemical affinity and etc. In a mechanical system, the through variables are a force, and the across variables are a velocity.

Elements in bond graphs (Fig.1) are a parallel junction, a series junction, a capacitor C, a disspative conductor L or resistor R and a generalized transducer TD.

	Through variable (KCL)	Across variable (KVL)
Eulerian (stationary) co-ordinate frame	Current i=dQ/dt Solute flow f or J = dn/dt Reaction rate dξ/dt Volume flow dV/dt	Volt V=E or ΔE Potential μ or Δμ Affinity A Pressure Δp 、-Δπ
Lagrangian(moving) co-ordinate frame	Force F	Relative velocity v or Δv

Table 1. Bond graphs variables



Series junction (KVL junction)

Parallel junction (KCL junction)

Fig. 1. Elements of bond graphs and their causalities

Electrophysiological analysis of the afferent activity from the submandibular salivary gland in the rat.

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Previous histochemical studies have shown that the sensory nerves in rat salivary glands contain both substance P and calcitonin gene-related peptide (CGRP), and most of the CGRP containing nerve fibers contain substance P (1,2). Such sensory nerves are seen around or in close contact with small ducts and blood vessels in the submandibular gland (1, 3), and substance P containing nerve fibers are also present around its major duct (4). However, functional role of the sensory nerves has not studied yet.

In the present study, we analyzed afferent neural activities in the sympathetic and parasympathetic nerve supply to the submandibular gland of rats. Our recent histochemical study in rats has shown that the sensory nerves travel in both the sympathetic and parasympathetic nerve to the submandibular gland (5).

Adult male Wistar rats weighing 280-320 g were anesthetized with urethane (1g/kg, i.p.). We removed the sublingual gland and exposed the submandibular gland, its main duct, and artery. The sympathetic and parasympathetic nerve branches were separated from the artery and main duct, respectively, near the hilus of the sumandibular gland. Each nerve bundle was cut and the pripheral cut end was placed onto the bipolar recording electrode. Afferent neural activity was amplified and stored in a personal computer equipped with an analyzing system (PowerLab/8sp, ADInstruments). In some experiments, the main duct was cannulated with a polyethylene tubing and connected to a pressure-sensitive transducer. The pressure in the duct was monitored with the transducer during salivary secretion and application of back pressure to duct. We applied four kinds of stimulation to activate afferent activity; 1) mechanical pressure to the main duct, 2) mechanical pressure to the gland, 3) back pressure to the duct system, and 4) ligation of the artery to examine effects of blood flow changes.

The sensory nerves usually showed no spontaneous discharges. When we pressed the main duct with small glass rod, afferent activities could be recorded from the

parasympathetic nerve branches, but not the sympathetic nerve branches. As shown in Fig. 1, the mechanical pressure induced a train of larger impulses during stimulation (under lines). The receptive field was small confined area, and was summarized in the inset figure obtained from 21 instances. The smaller impulses were responsive to mechanical pressure to the gland.

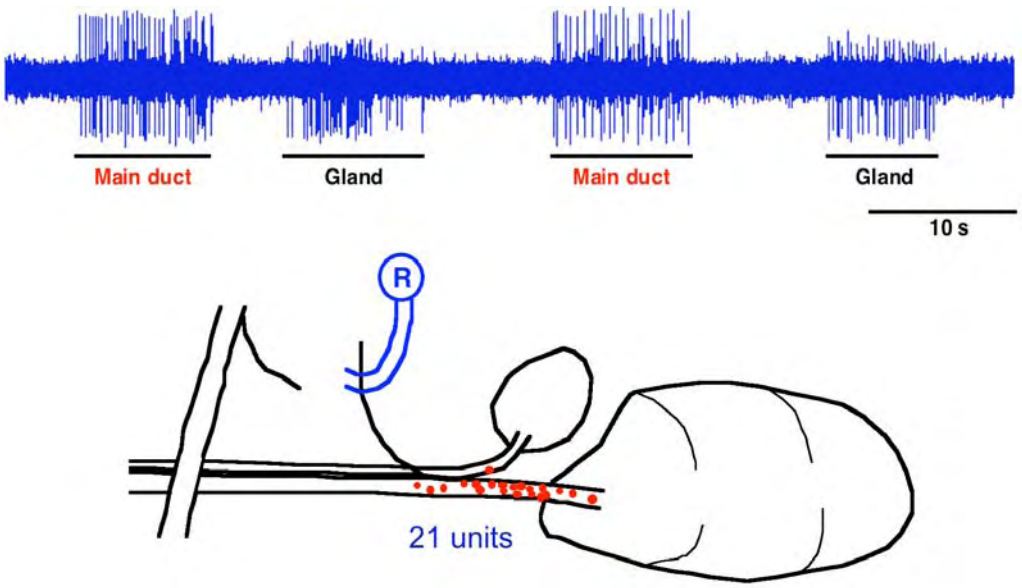

Fig. 1 Neural activity of the sensory nerve in the parasympathetic nerve supply. Mechanical pressure to the main duct induced a train of large impulses. Receptive fields located near the hilus of the submandibular gland.

When we pressed or stroked the surface of the gland with a glass rod, sensory nerves both in the sympathetic and parasympathetic nerve supplies were responsive. These nerves also responded to back pressure to the duct by manual injection of saline into the duct cannulae via the pressure-sensitive transducer. Fig. 2 shows example of recordings obtained from the parasympathetic nerve supply. The threshold back pressure to yield impulse discharges was 75.8 ± 18.9 mmHg (mean ± SD, n=13) and 68.9 ± 18 mmHg (n=13) for the sensory nerves in the sympathetic and parasympathetic nerve supplies, respectively. These values were higher than the maximum secretory pressure (61.1 ± 7.6 mmHg, n=13), which was measured during continuous electrical stimulation (3V, 0.5ms, 10Hz) of the chorda tympani (the parasympathetic secretory nerve). When the outlet of the duct cannula was closed, the duct pressure reached the maximum level, the maximum secretory pressure, in a few min after beginning of the

electrical stimulation. At the maximum secretory, we usually observed swelling of the gland.

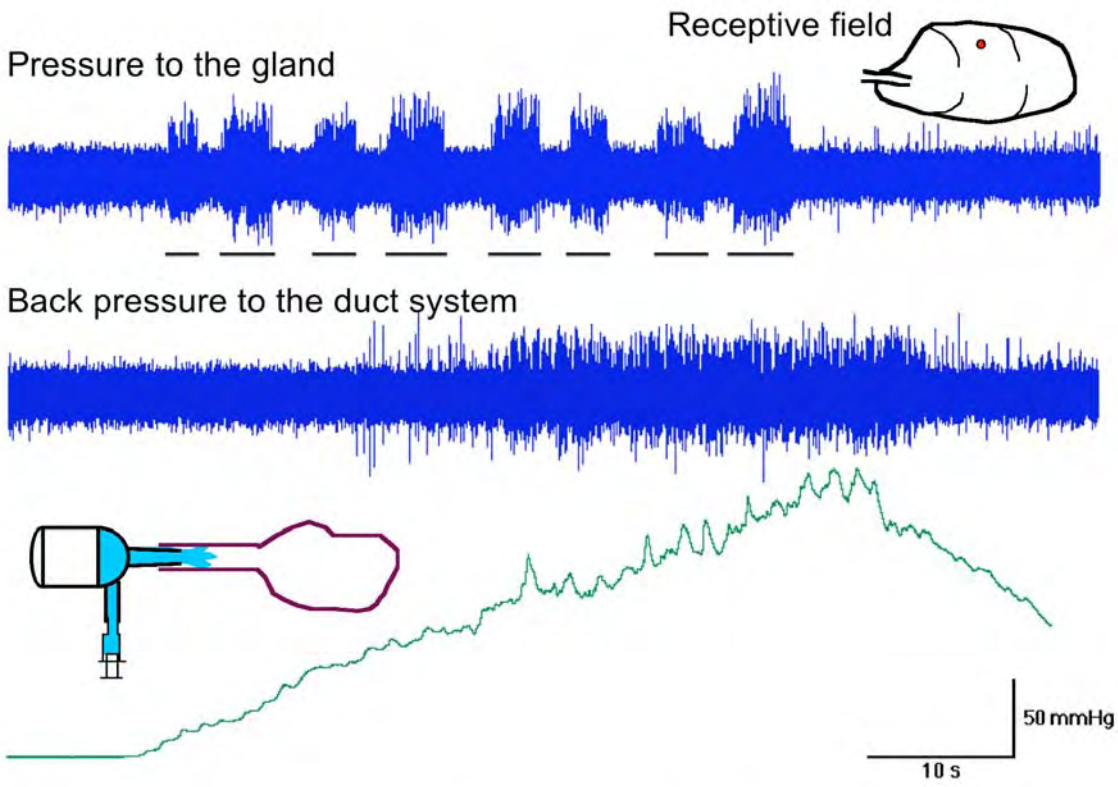

Fig. 2 Neural activity of the sensory nerve in the parasympathetic nerve supply. Mechanical pressure to the gland and back pressure to the duct induced impulse discharges.

When we ligated the artery to the submandibular gland, the sensory nerves in the sympathetic nerve supply (12 out of 18 nerve bundles) showed impulse discharges within 1 min (Fig. 3). On the other hand, the sensory nerves in the sympathetic nerve supply were hardly responsive. One out of 8 nerve bundles responded slightly. The sensory nerve in the sympathetic nerve supply also discharged in 1 min after attached a small balloon (50 ml) to the tracheal cannula, suggesting that the discharges are due to hypoxia.

Our previous study shows that sensory nerves to rat submandibular gland have various sizes of cell soma (5). This suggests that the sensory neurons recorded in the present study comprise various fiber diameters and this may affect the height of impulses. We usually recorded large impulses from the nerves innervating the main duct, whereas small impulses were observed in the nerves sensitive to ligation of the artery. Thus, different kind of sensory nerves may relate to the main duct, gland, and artery.

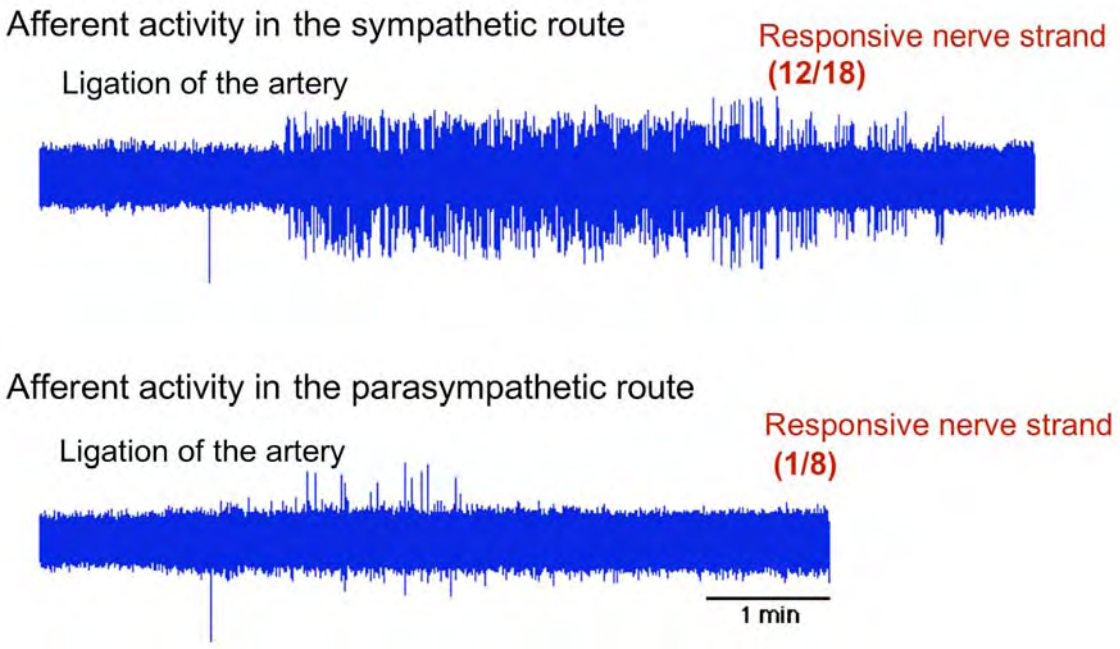

Fig. 3 Neural activity after ligation of the artery to the submandibular gland.

The sensory nerves responsive to the back pressure to the duct (Fig. 2) needed relatively high pressure to produce impulse discharges. This suggests that such nerves would be activated when salivary stone block passage of the duct, and conduct pain. We could not find a possibility that the sensory nerves are concerned with a role to monitor and control flow rate of saliva.

Afferents in the sympathetic nerve supply may monitor hypoxia of the gland. The afferent activity may relate to reflex control of blood flow of the submandibular gland (6). Further electrophysiological study is needed to demonstrate this reflex.

In conclusion, the sensory nerves in the parasympathetic nerve supply innervate the submandibular gland and its main duct, whereas those in the sympathetic nerve supply innervate the gland and its artery. The nerves innervating the duct are responsive to relatively strong duct pressure rather than the maximum secretory pressure. The nerves innervating the artery may contribute control of blood flow changes.

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Oxidative modification of serum albumin via paracellular route of rat submandibular gland.

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Introduction

Human serum albumin is the most abundant protein in the circulatory system. One of structural characteristics in this protein is one reactive free sulfhydryl group in position 34, which can react with several oxidants such as cystine, oxidized glutathione, nitric oxide, hydrogen peroxide and so on[1)]. Albumin is a mixture of reduced form and oxidized forms in extracellular fluid such as serum and interstitial fluid[2)]. The reduced form has one free sulfhydryl group. The oxidized forms are composed of at least three kinds of compounds. The major form is disulfide mixed with cystine or oxidized glutathione. One of minor forms is products oxidized higher than mixed disulfide, such as sulfenic, sulfinic and sulfonic acids. Moreover, another minor form is S-nitrosoalbumin which is bound with nitric oxide. Recently, the sulfhydryl group in human albumin has been reported to be highly oxidized in aging and renal dysfunctions, having attracted attention for an extracellular marker of oxidative stress[3-5)]. However, the oxidization mechanism including site within organ still is not clear.

The isolated rat salivary gland[6)] allow us to sample intercellular fluid, venous effluent and saliva, thus to understand the site of oxidization through capillary, across vascular wall or through paracellular route. In this study, to clarify the oxidization mechanism of serum albumin in living system, we isolated the rat submandibular gland and perfused with the perfusate containing commercial human albumin. The collected samples were analyzed by a special system of high-performance liquid chromatography (HPLC) which could separate albumins into reduced and oxidized forms[3,4)].

Methods

Tissue preparation and perfusion: The isolation and perfusion of rat submandibular gland was followed with the procedure of Murakami *et al*[6)]. In brief, Wistar male rats (9-10 week old) were anesthetized by intraperitoneal injection of pentobarbital sodium. Then, the submandibular gland was exposed surgically. The attached sublingual gland was removed after ligation of the feeding artery and draining vein. The extra-lobular main duct from the submandibular gland was cannulated with a polyethylene tube for

the collection of saliva. The artery distal to the glandular branch was cannulated with a stainless steel catheter and the vein from the gland was cut. The gland was isolated, and transferred to an organ bath at 37°C. The gland was perfused with HEPES buffer (pH 7.40) for c.a. 10 min to steady condition of the gland. Then, the gland was perfused with HEPES buffer containing 2% human serum albumin (fraction Ⅴ, Calbiochem Behring Co., USA) and a secretory stimulant for secretion of saliva. This study consisted of three experiments: (1) 1.0 μM carbachol was used as muscarinic stimulation for saliva secretion; (2) The isolated gland treated with 300 μM *N*-nitro-*l*-argininemethylester (*l*-NAME) which inhibited synthesis of nitric oxide was stimulated with 1.0 μM carbachol; (3) 0.5 μM carbachol-0.5 μM isoproterenol was used as muscarinic and beta-adrenergic stimulation for saliva secretion. After onset of secretory stimulation, intercellular fluid, venous effluent and saliva were collected to microfuge tubes. Each sample was immediately stored at –80°C until HPLC measurements.

HPLC measurements: The HPLC system consisted of a Model CCPM double-plunger pump and a Model FS-8000 fluorescence detector (excitation wavelength, 280 nm; emission wavelength, 340 nm) in conjunction with a Model SC-8020 system controller, all from Tosoh Co., Japan. For ion-exchange column, a Shodex-Asahipak ES-502N (DEAE-form for ion-exchange HPLC, Showa Denko Co., Japan) column was used at 35 ± 0.5°C. Measurements were carried out by solvent gradient elution with increasing ethanol concentration from 0 to 10% in 0.05 M sodium acetate-0.40 M sodium sulfate (pH 4.85) at a flow rate of 1.0 ml/min. Samples were injected to the HPLC system without chemical modification for detection of reduced form and oxidized forms of albumins except for detection of *S*-nitrosoalbumin. For detection of *S*-nitrosoalbumin, sample was mixed with 1.0 mM *N*-ethylmaleimide during 10 min before injection to the HPLC system. All data were transferred to personal computer for data analysis. The HPLC profiles were subjected to numerical curve fitting with software for simulation (Peakfit, version 4, SPSS Science, USA). Concentrations of total and each fraction of albumin in each sample were obtained from calculated area on HPLC profile. All data were shown in mean ± SEM.

Results and Discussion

Human albumins were able to detect in intercellular fluids, venous effluent and saliva from isolated rat submandibular gland. Under 1.0 μM carbachol stimulation during 60 min, the concentrations of total albumin in intercellular fluids, venous effluent and saliva were 315.5 ± 2.5, 195.1 ± 12.7 and 1.0 ± 0.3 μM, respectively (n = 5). The concentration in saliva was approximately 0.05-0.7% of albumin in the perfusate. Despite the evidence that the rat salivary gland has no intrinsic secretion system of

human albumin, small amount of the albumin in saliva was detected. Additionally, variation in the concentrations was large, which indicated that albumin concentration in saliva might depend on individual differences. These facts suggested that the human albumin in saliva might be passed through the paracellular pathway such as tight junction in rat submandibular gland. Mazariegos *et al.*[7] investigated alternation of tight junctional permeability in the rat parotid gland by administration of several tracers of different molecular weight *in vivo*. They showed that some tracers of the tight junctional permeability increased after isoproterenol stimulation. In this study, concentration of total albumin in saliva under isoproterenol stimulation was measured by HPLC method. Secretion-perfusate ratio showed a tendency to increase exponentially according to decline of flow rate (Fig. 1). This evidence also supported that albumin passed through tight junction in submandibular gland.

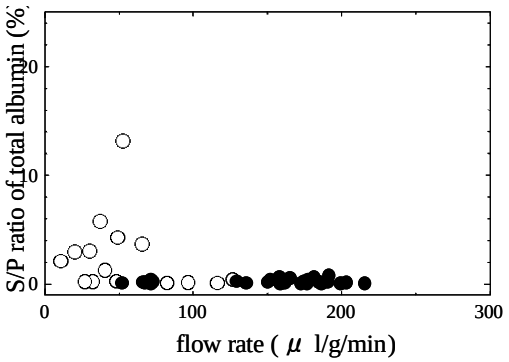

Fig. 1. Secretion/ perfusate (S/P) ratio (%) of total albumin in saliva as a function of flow rate. Salivary secretion was stimulated with 1.0 μ M carbachol (solid circle) or 0.5 μ M carbachol -0.5 μ M isoproterenol (open circle).

In time course in saliva secretion by 20 min, concentrations of total albumin in saliva were 0.7 ± 0.2, 0.5 ± 0.1 and 0.7 ± 0.2 μM during 0-20 min, 20-40 min and 40-60 min from onset of carbachol stimulation, respectively (n = 10). Redox states in albumin in the same time course were measured. Fraction of oxidized form which was mixed with sulfenic acid, sulfinic acid and sulfonic acid, was increased as compared with that form of albumin in the perfusate. The oxidized forms were 9.5 ± 2.1, 15.6 ± 4.1 and 13.5 ± 3.6% of total albumin in saliva during 0-20, 20-40 and 40-60 min from onset of 1.0 μM carbachol stimulation, respectively (n = 10). The well known pathway for sulfenic acid formation is the reaction of a thiol with oxidants such as hydrogen peroxide and peroxynitrite[8]. In this study, increase of the oxidized form which was mixed with sulfenic acid, sulfinic acid and sulfonic acid suggested that reactive oxygen species such as hydrogen peroxide oxidized directly free sulfhydryl group of albumin in intercellular spaces in salivary gland.

In this study, fraction of *S*-nitrosoalbumin which was bound with nitric oxide could be detected in saliva. In time course in saliva secretion by 20 min, the *S*-nitrosoalbumins were 16.6 ± 3.6, 14.3 ± 3.5 and 13.3 ± 3.9% of total albumin in saliva during 0-20, 20-40 and 40-60 min from onset of 1.0 μM carbachol stimulation, respectively (n = 10). To confirm with existence of *S*-nitrosoalbumin in saliva, the salivary gland was treated with an inhibitor on synthesis of nitric oxide (*l*-NAME).

When the gland was treated with *l*-NAME, S-nitrosoalbumin was hardly able to be detected in saliva samples. Fig. 2 shows time course of saliva flow rate in the salivary gland treated with *l*-NAME at 1.0 μM carbachol stimulation. During 30 min from onset of secretory stimulation, the flow rate was at the same level between group treated with *l*-NAME and control group. It was suggest that nitric oxide might hardly affected secretion volume of saliva at carbachol stimulation.

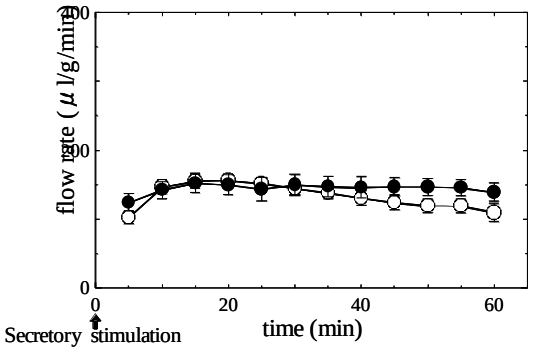

Fig. 2. Time course of saliva flow rate in the salivary gland treated with *l*-NAME at 1.0 μM carbachol stimulation (mean ± SEM). Group treated with *l*-NAME (300 μM in perfusate, open circle, n = 9) and contorl group (no *l*-NAME in perfusate, solid circle, n = 14) were perfused with same condition except treatment of *l*-NAME.

In conclusion, several evidences strongly suggested that the human albumin in saliva might be passed through tight junction in the rat submandibular gland and oxidative modification of the albumin might be occurred during the paracellular pathway.

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Electrophysiological study of the inhibitory inputs from the forebrain and brainstem to the superior salivatory nucleus in rats

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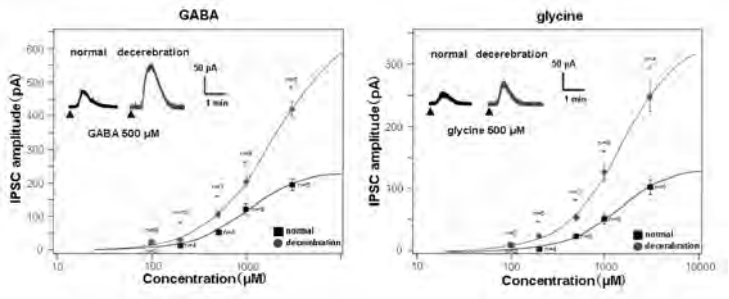
Introduction

It is well known that salivary secretion is reflexly induced by oral sensory stimulation such as taste and mechanical stimulation, and the fundamental structures for reflex salivary secretion are located in the lower brainstem. The superior salivatory (SS) nucleus is the primary parasympathetic center of submandibular and sublingual salivary secretion, and is located in the lateral reticular formation of the medulla oblongata in various animals (1). Many neuroanatomical studies (2, 3) and *in vivo* experiments (4, 5) suggest that the nucleus innervating superior salivatory neurons involves the brainstem and forebrain, and salivary secretion is controlled not only by lower centers but also by higher centers. Recently, we have showed that SS neurons receive glutamatergic excitatory, and GABAergic and glycinergic inhibitory synaptic inputs in rats (6). However in many studies on salivary secretion, researchers usually focus on the excitatory aspect of secretory neural mechanisms, and it has not been examined whether SS neurons receive inhibitory inputs from higher and lower centers with respect to inhibitory salivary control. In this study, we compared inhibitory synaptic inputs in SS neurons of normal and decerebrate rats and demonstrated that these origins exist in both higher and lower centers using normal and decerebrate rats.

SS neurons were labeled by retrograde axonal transport of a fluorescent tracer. In

some rats, brainstem was cut at the precollicular level. Whole-cell patch-clamp recordings were obtained from labeled neurons in sagittal brainstem slices.

Currents evoked by exogenous GABA and glycine application

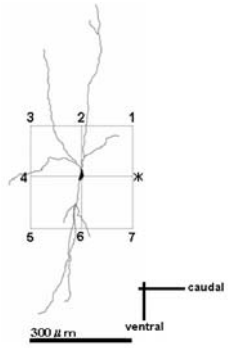


Concentration-response curves of GABA and glycine in normal and decerebrate SS neurons

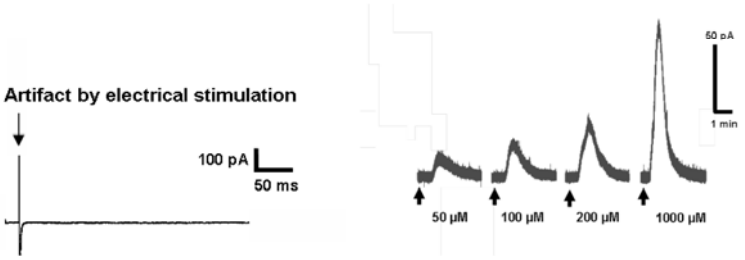
In all decerebrate SS neurons, exogenous GABA and glycine application produced larger amplitude of inhibitory currents as compared with normal SS neurons, suggesting that $GABA_A$ and glycine receptors at the postsynaptic membrane were up-regulated due to decreased neurotransmitter release from the higher centers after decerebration (7).

Inhibitory postsynaptic currents (IPSCs) evoked by electrical stimulation

IPSCs were evoked by electrical stimulation near the recording cell. To evaluate the IPSC quantitatively, stimulation points were decided by morphological observation of SS neurons.

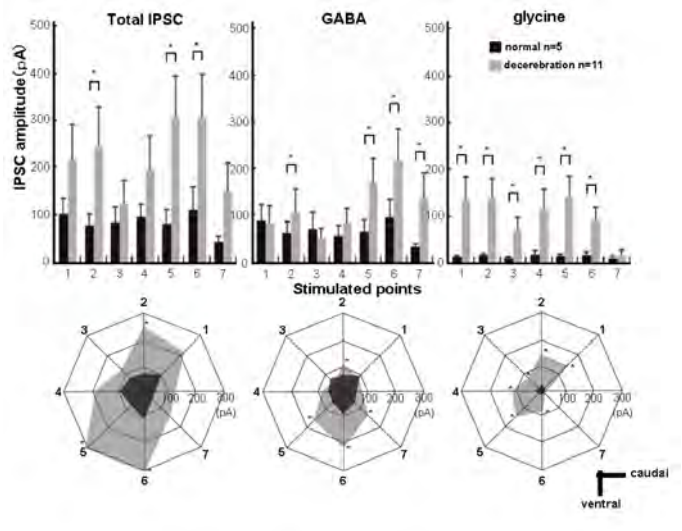


Dendrite morphology of SS neurons and electrical stimulation sites



Characteristics of decerebrate SS neurons which showed no IPSCs by electrical stimulation

All normal SS neurons invariably showed IPSCs. In contrast, 17 % of decerebrate SS neurons displayed no IPSCs. In these neurons, to confirm the presence of GABA receptors, we applied exogenous GABA into the recording chamber. Larger currents were induced than normal SS neurons at various concentrations.



Characteristics of decerebrate SS neurons which displayed enhanced IPSCs by electrical stimulation

Eighty-three percents of decerebrate SS neurons displayed enhanced IPSCs than those of normal SS neurons in all points. In decerebrate SS neurons, the peak amplitude of total IPSCs was enhanced in all points compared to normal SS neurons. In the GABAergic component, the peak amplitude of IPSCs in dorsal and ventral regions was larger than that of normal SS neurons. In the glycinergic component, the peak amplitude of IPSCs was enhanced at many points.

These results suggest that most SS neurons receive descending inhibitory synaptic

inputs from higher centers, with 17 % receiving inhibitory inputs exclusively from the higher centers, and 83 % having inhibitory inputs from higher and lower centers.

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Electrophysiological study on the descending excitatory synaptic inputs to the superior salivatory nucleus in the rat.

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Introduction

It is well known that copious salivary secretion is observed during feeding behavior. During this behavior, the superior salivatory (SS) nucleus which is the primary parasympathetic center of submandibular and sublingual salivary secretion would be activated by the following inputs; oral sensory inputs via the brainstem (lower centers) and descending inputs from the forebrain (higher centers) such as the feeding center. Generally it is believed that the salivary secretion is reflexly evoked by oral sensory inputs. However, descending inputs from higher center may be more important than oral sensory inputs. Anderson *et al* (1) reported the followings: Human parotid secretion is induced by chewing of acryl bite block in constant rhythm (1 Hz, 10 s) while the salivation is not induced by empty clenching and grinding their teeth. This results mean that only oral sensory inputs does not evoke the salivation and the inputs from the higher centers are essential for the salivation during feeding. In this study, we examined whether SS neurons receive the excitatory input from higher center electrophysiologically.

Synaptic inputs to SS neurons

First, we studied the components of the synaptic inputs to SS neurons in rats (2). SS neurons innervating the salivary glands were retrogradely labeled by injecting fluorescent tracer into the chord-lingual nerve. Whole-cell patch-clamp recordings were performed from the labeled cell in the brainstem slice. Synaptic currents were evoked by electrical stimulation near the recording cells (Fig. 1). Fig. 1Aa shows a typical example of total excitatory postsynaptic currents (EPSCs). Application of CNQX (non-NMDA type glutamate receptor antagonist) suppressed the fast component of the total EPSCs (Fig. 1Ab). Subsequent additional of CPP (NMDA type glutamate receptor antagonist) completely abolished the remaining EPSCs (Fig. 1Ac). Therefore NMDA

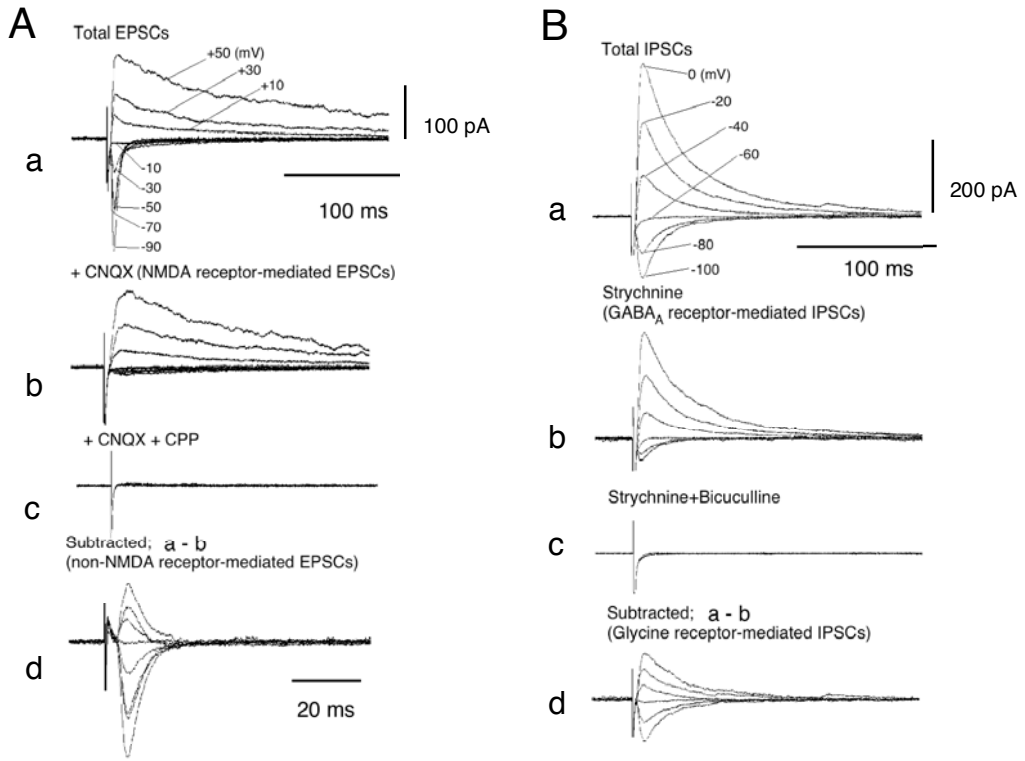

Fig. 1 Excitatory and inhibitory postsynaptic currents evoked by electrical stimulation SS neurons innervating the salivary glands. (Modified from Mitoh *et al*., Brain Res. 999, 2004.)

receptor-mediated EPSCs are shown in Fig. 1Ab. Fig. 1Ad shows non-NMDA receptor-mediated EPSCs which were obtained by digital subtraction of EPSCs in Ab from EPSCs in Aa. Fig. 1B shows a typical example of inhibitory postsynaptic currents (IPSCs). From the voltage-dependency and pharmacological characteristics, the inhibitory synaptic inputs consist of GABAergic (Fig. 1Ba) and glycinergic (Fig. 1Bd) components. These results suggest that SS neurons have glutamatergic excitatory synaptic inputs and GABAergic and glycinergic inhibitory synaptic inputs. To examine the descending excitatory synaptic inputs, we compared the excitatory responses to the SS neurons of the normal and decerebrate rats.

The Origin of the synaptic inputs to SS neurons

Matsuo (3) shows that many nuclei of autonomic nervous system innervate SS neurons by the injection of horse radish peroxidase (HRP) into SS nucleus. In the lower centers, the labeled cells were observed in the parabrachial nucleus, spinal trigeminal nucleus, solitary nucleus, and pontine and medullary reticular formation. In the higher centers, the labeled cells were found mainly in the bed nucleus of the stria terminalis, hypothalamic paraventricular nucleus, central nucleus of the amygdala, and lateral

hypothalamic area. This result is similar to that obtained by the injection of virus into the submandibular gland of sympathetically ganglionectomized rats (4). Functionally, these higher centers are related to feeding, drinking, thermoregulation. These lower centers are related to taste and visceral sensation, and general sensation of the oral region. There are some notable points. First, it is possible that these nuclei have direct connection to SS neurons because HRP is used as a tracer. Second, these centers include excitatory and inhibitory neurons.

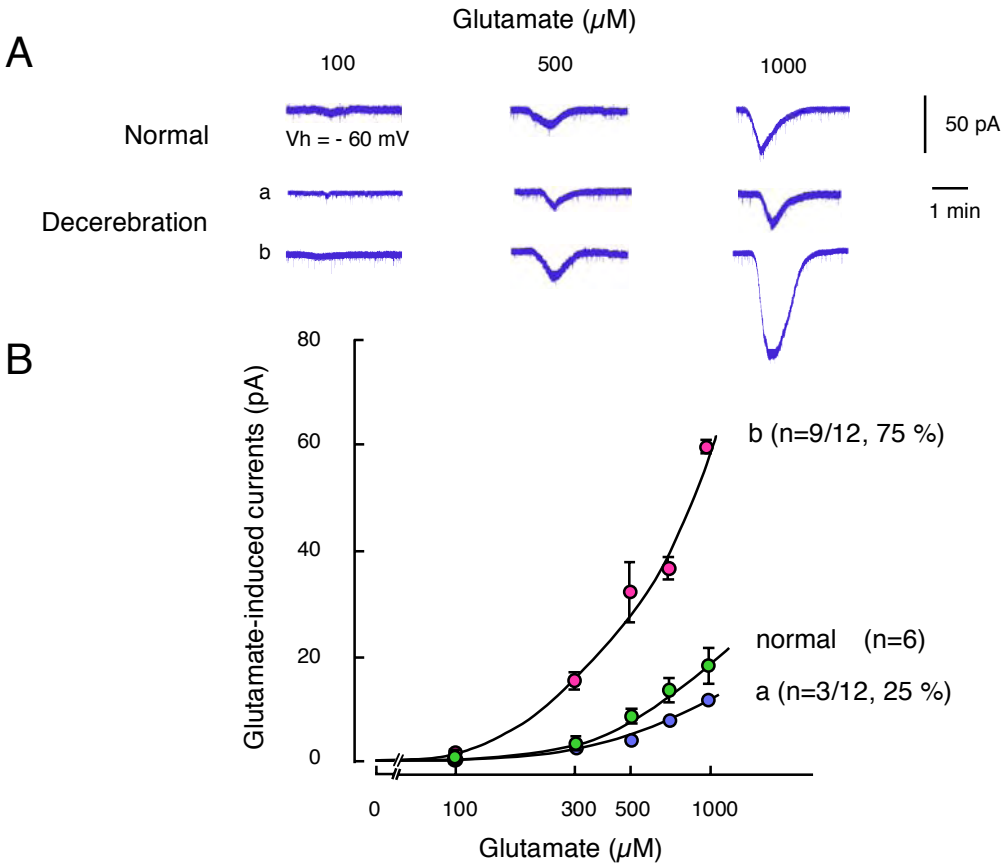

Fig. 2 Glutamate-induced currents recorded from normal and decerebrate SS neurons

Descending excitatory synaptic inputs from the higher center

We focused on the excitatory input and studied whether SS neurons receive actually excitatory inputs from the higher centers. For this purpose, we used decerebrate rats which brainstem was cut at the precollicular level. The amplitude of the excitatory currents evoked by the application of exogenous glutamate was compared between normal and decerebrate SS neurons (Fig. 2). Fig. 2A shows typical traces of glutamate-induced currents recorded from normal and decerebrate SS neurons. In many SS neurons (n=9/12, 75 %) of the decerebrate rats, the amplitude of glutamate-induced

currents was increased as compared with the normal SS neurons. This suggests that glutamate receptors at the postsynaptic membrane were up-regulated due to decreased neurotransmitter release from the higher centers after the decerebration (5). Thus, such SS neurons receive descending excitatory inputs from the higher center. In some SS neurons (n=3/12, 25 %) the amplitude of glutamate-induced currents was unchanged, suggesting they have hardly excitatory synaptic inputs from the higher centers.

Conclusion

Many SS neurons innervating the salivary glands receive the glutamatergic excitatory inputs from the higher centers. It is suggested that not only the reflex inputs from the lower centers but also the descending excitatory inputs from the higher centers participate in the regulation of the salivary secretion.

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Role of the feeding center for submandibular salivary secretion during feeding behavior in the rat.

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It is well known that vigorous salivary secretion is evoked during feeding behavior, especially during chewing of food. This salivation is largely attributed to afferent inputs from taste receptors and mechanoreceptors in oral mucosa and periodontal tissues (1). The afferent inputs may activate not only the sympathetic and parasympathetic salivary centers in the spinal cord and medulla, respectively, but also the forebrain structures such as the feeding center and cortical masticatory and taste areas. The activation of the forebrain may produce descending excitatory influence on the salivary centers (2), and be involved in the so-called masticatory-salivary reflex. To test this hypothesis, we recorded submandibular salivary flow during chewing of various textures of foods, using normal and feeding center-destroyed rats.

For this examination animals were placed on a food deprivation schedule and allowed access to various foods for 3 hours per day for one week. Water is available ad lib. The foods were food pellets, power diet, and 3 types of mashed diet (power diet : water = 1:1.5, 1:1, and 1:0.5). After the training schedule, the animals were anesthetized and the duct of left submandibular gland was cannulated with polyethylene tubing for recording salivary flow rates, and a pair of stainless electrodes was inserted into bilateral masseter muscle for recording EMG activities. The salivary flow rate was monitored by a pressure-sensitive transducer. Signals of the salivary flow and EMG activities were amplified and stored in a personal computer equipped with an analyzing system (PowerLab/8sp, ADInstruments). When the feeding center (lateral hypothalamus) was destroyed, DC current (0.2 mA, for 20 s) was applied to the feeding center through a stainless wire electrode (200 μm). After recovery from anesthesia, the recording experiment was performed. After recording, the brain was removed and lesion was verified histologically.

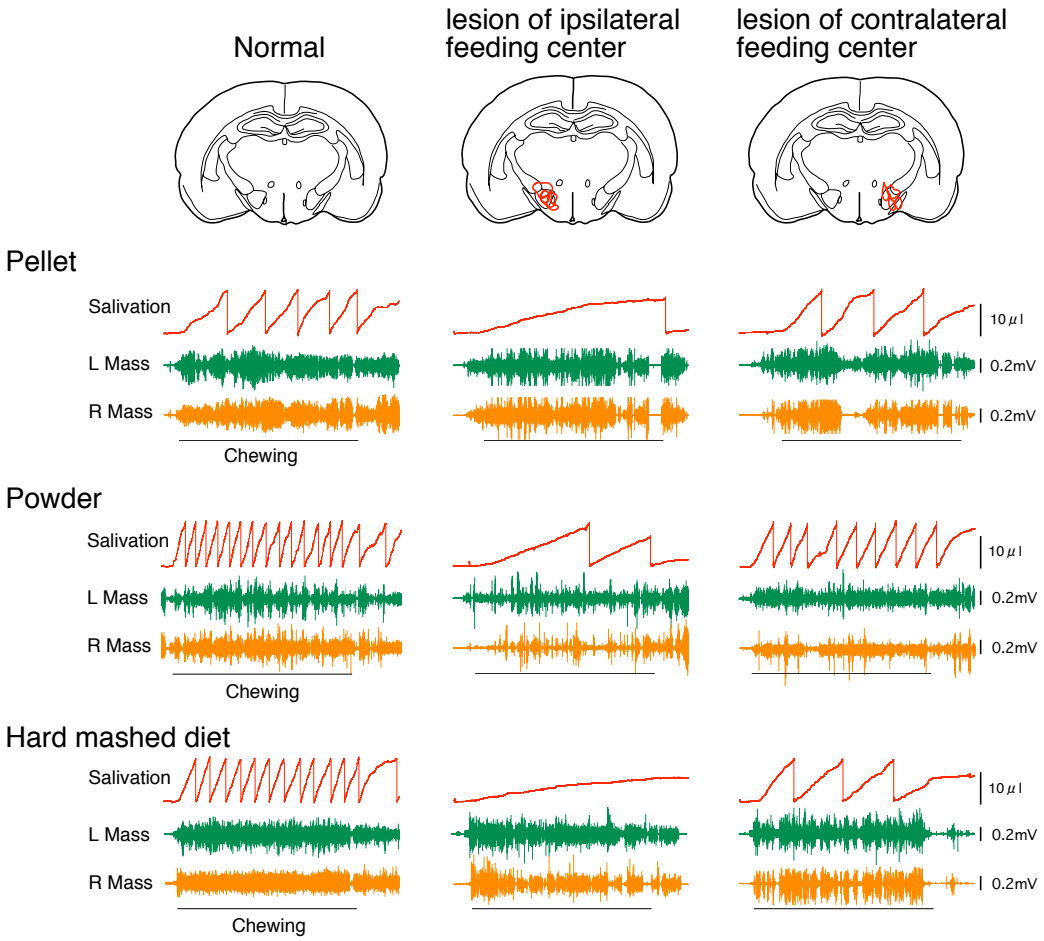

Fig. 1 Submandibular salivary secretion in normal and feeding center-destroyed rats. When the ipsilateral feeding center was destroyed, salivation decreased markedly (center panel).

Fig. 1 shows examples of recordings during chewing of pellet, powder, and mashed diet obtained from normal, and feeding center-destroyed rats. The rat can chews food particles on both sides at once (3), and may secrete saliva equally from both sides of salivary glands. The recorded right and left masseter muscles activated in a bilaterally symmetrical fashion, and constant salivary flow rates were observed during chewing (food was given for about 3 min) (4). From the recordings, we compared salivary flow rate, food consumption, and EMG activity for 3 min between normal and lesioned rats. As to salivary flow, destruction of ipsilateral feeding center reduced by about 80 % salivary output during chewing of various foods (Fig. 2A). Destruction of the contralateral feeding center yielded about 40 % reduction in salivary flow as compared with normal rats. In normal rats, the average salivary flow rates for 3 min were in the order power diet > hard mashed diet > medium mashed diet > food pellet > soft mashed

diet. Similar order was observed in the lesioned rats.

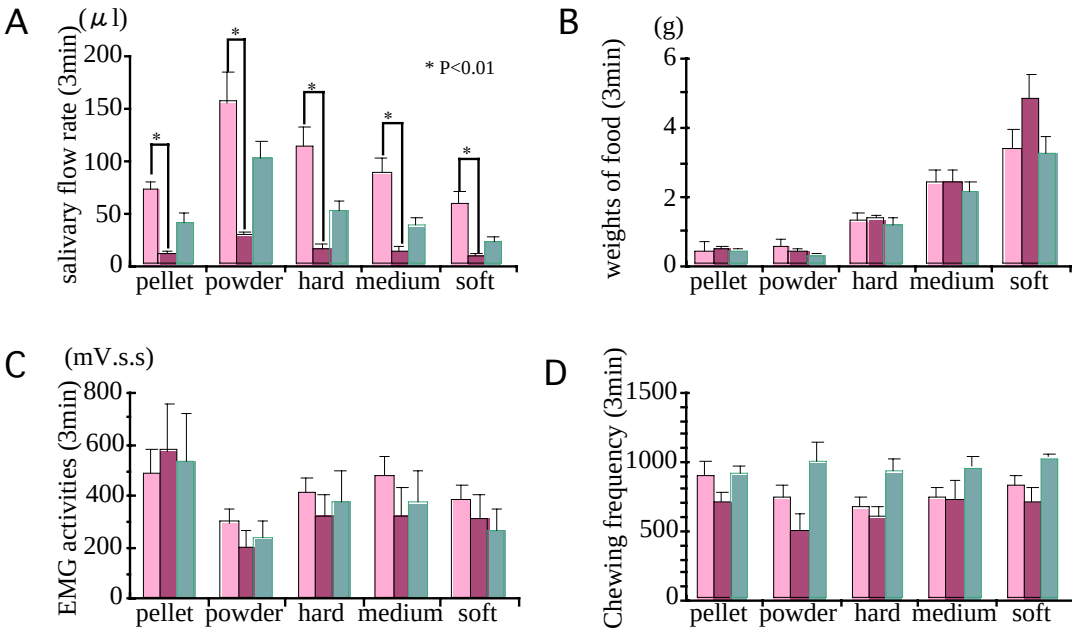

Fig. 2 Comparison of salivary flow, food consumpsion, EMG activity, and chewing frequency. Each bar indicates average and SE value obtained from normal (, n=6), ipsilaeral feeding center-destryoed (, n=4), and contralateral feeding center-destroyed rats (, n=4).

In contrast, no significant differences were observed in food consumption, total EMG activities, and total chewing cycles for 3 min (Fig. 2B-D). This finding suggests that swallowing rate and movement of the oral structure such as jaw and tongue did not largely alter by destruction one side of the feeding center. However, and destruction of the feeding center reduced salivation, and the recorded submandibular saliva was not returned to the oral cavity. So, even the oral cavity of "normal rats" in the present experiment is different from that of intact rats. In fact, the feeding center-destroyed rats showed more frequent prandial drinking than normal rats. It is likely that our used starved rats were hungry enough to eat food continuously for 3 min, even if salivation was reduced.

The present study showed that destruction of ipsilateral feeding center markedly reduced submandibular salivary secretion in response to chewing, without considerable changes in movement of the jaw and food consumption. The result suggests that

descending effects from the feeding center are essential for salivation during chewing, and participate in main neural network to evoke the masticatory-salivary reflex.

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Secretagogues stimulate phosphorylation of MARCKS in parotid acinar cells

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Abstract

It is well known that activated PKC phosphorylates myristoylated alanine-rich C kinase substrate (MARCKS). We investigated the effects of secretagogues on phosphorylation of MARCKS in rat salivary gland cells. MARCKS were detected in rat parotid and sublingual acinar cells by western blotting analysis. In parotid acinar cells, the β-adrenargic agonist isoproterenol (IPR) stimulated phosphorylation of MARCKS. Myristoylated PKC peptide inhibitor partially inhibited on IPR-induced amylase release. These findings suggest that phosphorylation of MARCKS is involved in secretory function induced by the activation of β-adrenargic receptors in rat parotid acinar cells.

Introduction

Myristoylated alanine-rich C kinase substrate (MARCKS) is a major cellular substrate for protein kinase C (PKC). MARCKS has been demonstrated to be implicated in cell motility, phagocytosis, membrane trafficking and mitogenesis. Phosphorylated MARCKS (p-MARCKS) has also been reported to be involved in secretory functions such as catecholamine release in bovine adrenal chromaffin cells (1), noradrenaline release in SH-SY5Y human neuroblastoma cells (2), mucin release in human airway epithelial cells (3) and glucose-induced insulin secretion in rat pancreatic islets (4). In rat parotid acinar cells, the activation of β-adrenergic and muscarinic cholinergic receptors provokes exocytotic amylase release. We investigated the effects of secretagogues on phosphorylation of MARCKS in rat parotid acinar cells.

Methods

Sprague-Dawley rats (male, 200-250 g) were intraperitoneally anesthetized, and parotid, sublingual and submandibular glands were removed. The acinar cells were isolated using trypsin and collagenase. Lysates were obtained by homogenizing with Tris-HCl buffer (pH 7.5) containing 2% IGEPAL. For preparing membrane and cytosolic fractions, rat parotid acinar cells were homogenized with HEPES buffer (pH 7.2) containing 0.3 M sucrose and 1 mM PMSF, and centrifugated at 100,000 *g* for 60 min. Secretory granules of the rat parotid glands were purified using Percoll gradient.

The isolated granules were treated with MOPS buffer (pH 6.8) containing 0.4 mM PMSF and centrifuged at 100,000 *g* for 60 min. The pellet was collected as the secretory granule membrane fraction.

Protein expression: Proteins were separated on a 7.5% SDS-PAGE gel. Immunoblot analysis was performed with anti-MARCKS (Santa Cruz, diluted 1:200) or anti-MARCKS phpsphoSer152/156 (Chemicom, diluted 1:2000) antibodies and detected with the ECL. The intensity was determined by ImageJ (NIH).

Amylase release: Cell suspensions were stimulated by isoproterenol (IPR, 1 μM), carbachol (CCh, 10 μM) and phorbol-12-myristate-13-acetate (PMA, 1 nM) at 37°C for 15 min. When the effect of myristoylated PKC peptide inhibitor (myr-φPKC peptide: 100 μM) was examined, cells were pretreated this drug for 30 min and then agonists were added. Amylase activities in the medium and in acinar cells homogenized with phosphate buffer (pH 6.9) with 0.01% Triton X-100, released and total amylase activities, respectively, were measured according to the method of Bernfeld (5).

Protein concentrations were determined by the method of Bradford (6).

Results

In western blotting analysis, MARCKS and p-MARCKS were detected in parotid and sublingual acinar cells, but not in submandibular acinar cells (Fig. 1). The expressions of MARCKS and p-MARCKS in membrane fraction were detected more clearly than that in cytosolic fraction in parotid acinar cells (Fig. 2). In addition, the expressions of MARCKS and p-MARCKS were also detected in secretory granule membrane (Fig. 2).

In rat parotid acinar cells, PMA, CCh and IPR stimulated phosphorylation of MARCKS time-dependently. These agonists had no effect on total amount of MARCKS (Fig. 3, 4 and 5). In western blotting analysis, we detected the bands cross-reacted with p-MARCKS antibody, and these intensities became strong in a time-dependent manner. These results suggest that phosphorylation of MARCKS is stimulated by PMA, CCh and IPR in rat parotid acinar cells.

It has been demonstrated that myr-φPKC peptide inhibits phosphorylation of MARCKS in human foreskin fibroblasts (7) and insulin secretion in rat pancreatic islets (8). We next examined the effect of myr-φPKC peptide on amylase release in parotid acinar cells. Myr-φPKC peptide partially inhibited on IPR-induced amylase release in parotid acinar cells. This inhibitor less inhibited PMA- and CCh-induced amylase release and had no effect on non-stimulative amylase release (Fig. 6). These results suggest that phosphorylation of MARCKS is involved in exocytotic amylase release in rat parotid acinar cells.

Discussion

Phosphorylation of MARCKS was induced by the β-adrenargic agonist IPR as well as PMA and CCh in rat parotid acinar cells (Fig. 3, 4 and 5). It has been known that activation of muscarinic receptor increases the intracellular Ca^{2+} concentration, and which activates PKC. Therefore, CCh probably stimulates phosphorylation of MARCKS via Ca^{2+} signaling. On the other hand, activation of β-adrenargic receptor increases intracellular cAMP concentration. Therefore, an isoform(s) of PKC appears to be activated via cAMP pathway.

IPR-induced amylase release was partially inhibited by myr-φPKC peptide in rat parotid acinar cells (Fig. 6). This ovservation suggests that phosphorylation of MARCKS is involved in regulation of secretory function.

The hypothesis has been reported that amylase release mechanism consists of two sequential steps, docking and fusion, in rat parotid acinar cells (9). Docking and fusion are induced by activation of cAMP signaling and Ca^{2+} signaling, respectively. Taken together, phosphorylation of MARCKS appears to be involved in both docking and fusion on amylase release in rat paritod acinar cells.

In conclusion, IPR induces phosphorylation of MARCKS and myr-φPKC peptide partially inhibited on IPR- and PMA-induced amylase release in rat parotid acinar cells.

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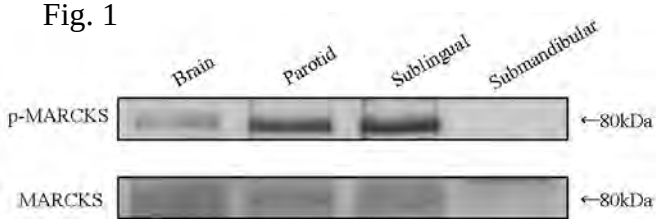
Fig. 1



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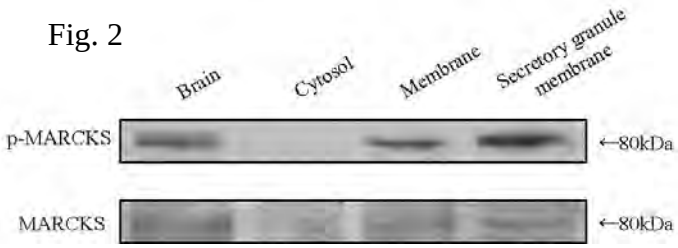

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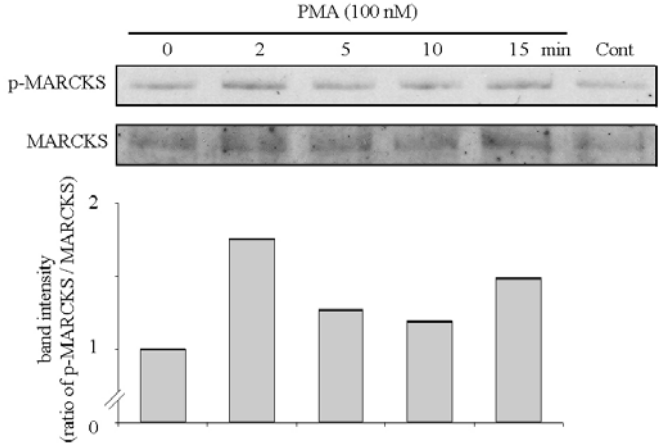

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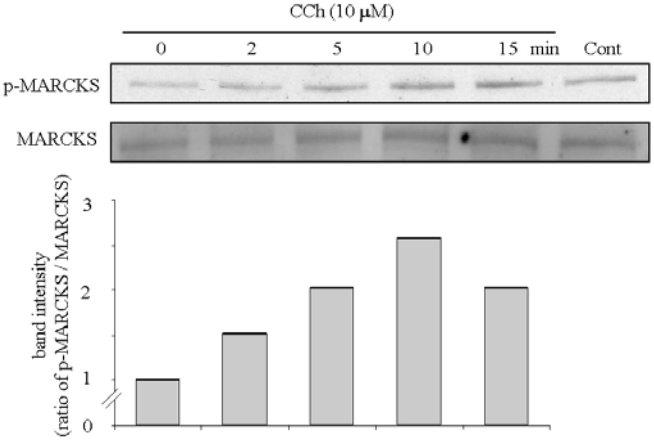

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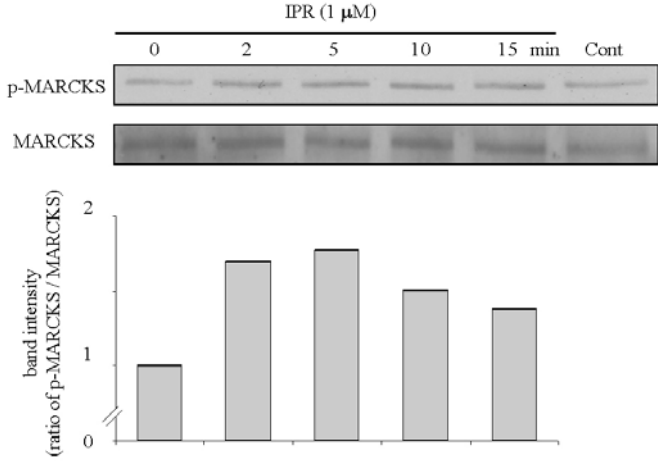

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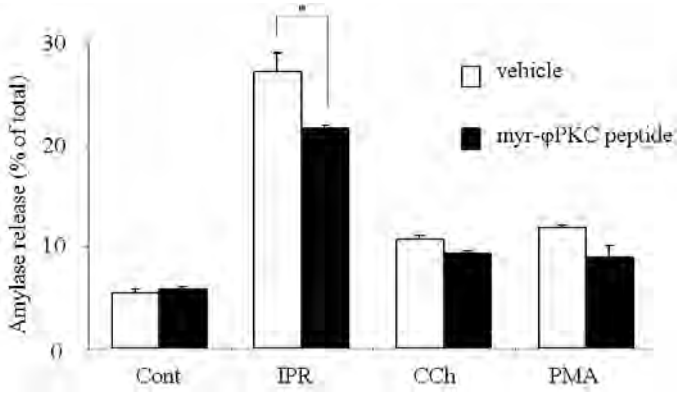

Expression and localization of aquaporin-6 in rat parotid acinar cells

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Abstract: We investigated the expression and localization of aquaporin-6 (AQP6) in rat parotid acinar cells. Expressions of AQP6 mRNA and protein were detected by RT-PCR and western blotting, respectively. Localization of AQP6 in apical membrane and secretory granule membranes was confirmed by using laser scanning confocal microscopy and immunoelectron microscopy in ultra thin cryosection. β-agonist stimulated accumulation of AQP6 at the luminal side in the cells. These results suggest that AQP6 is involved in regulation of secretory functions in parotid acinar cells.

Introduction

Aquaporines (AQPs) are proteins with six trans-membrane domains that were originally identified as a water channel in plasma membrane in 1992 (1). So far, 13 isoforms (AQP0-12) have been identified in mammalian, which are expressed in various epithelial tissues (2). AQP6 has been found in kidney (3) and considered to function not as a water channel but as an anion channel (4). We here demonstrate the expression and localization of AQP6 in rat parotid acinar cells.

Materials

Rat parotid acinar cells were prepared by using trypsin and collagenase A as previously described (5). mRNA of AQP6 was detected by RT-PCR. Secretory granules of the rat parotid glands were isolated by Percoll-gradient centrifugation as previously described (6). Western blotting was carried out by using anti-AQP6 antibody (Chemicon, Temecula, CA).

For laser scanning confocal microscopy and immunelectron microscopy in ultra thin cryosection, frozen 6 mm slices obtained from liquid nitrogen-treated parotid glands were attached MAS-coated slide glass and dried 60 min. Immunofluorescence labeling was carried out using rabbit polyclonal anti-AQP6 antibody (1:100) and mouse monoclonal anti-ZO-1 antibody (1:200). For fluorescent

labeling, Alexa-488 anti-rabbit IgG (1:200) and Alexa anti-mouse IgG (1:200) were used. Tissues were embedded by ProLong® Gold antifade reagent and observed by MRC-1024UV (BIO-RAD Laboratories Inc., CA). For ultra-thin cryosection, the samples floated on a drop of the anti-AQP6 polyclonal antibody (1:50). Subsequently, the sections were transferred onto a drop of 10-nm colloidal gold conjugated with anti-rabbit IgG (1:50) and incubated at 37°C for 1 hour. The bound antibodies were fixed with 1% glutaraldehyde in 120 mM phosphate buffer (pH 7.2) for 10 min, osmicated, dehydrated, and then embedded in LR white resin. The tissue was observed by HITACHI H-7100 electron microscopy.

Results

When mRNA expression of AQP6 was examine by RT-PCR, 262 base-pared band was detected in parotid acinar cells by RT-PCR, of which size was coincident with the band in the kidney medullae epithelial cells used as the positive control. When protein expression of AQP6 was examined by western blotting, ~33 kDa protein band immunoreacted with anti-AQP6 antibody was detected in parotid acinar cells, which was coincident with the 33 kDa protein band in kidney medullae epithelial cell membrane used as the positive control. The protein band was dominantly detected in membrane fraction but less in cytosolic fraction in parotid acinar cells, suggesting that AQP6 is dominantly located at the membrane.

Localization of AQP6 protein in rat parotid gland was showed by laser scanning confocal microscopy. When AQP6 was labeled with anti-AQP6 antibody and green fluorescence, AQP6 was mostly observed in nearby tight junction of basolateral side of acinar cells, although AQP6 was weakly observed in cytoplasmic area of acinar cells. When ZO-1, a marker protein of tight junction, was labeled with anti-ZO-1 antibody and red fluorescence, ZO-1 was detected in nearby AQP6, indicating that AQP6 localizes in nearby tight junction.

To confirm the subcellular distribution of AQP6 in parotid acinar cells, and immunoelectron microscopy in ultra-thin cryosection was carried out. In plasma membrane, gold particles were localized nearby tight junction area. In cytoplasm, gold particles as AQP6 were localized at secretory granule membrane.

In isolated secretory granules, gold particles as AQP6 were clearly ordered in parotid secretory granule membranes. In the granule membrane of secretory granules, anti-AQP6 antibody specifically recognized 33 kDa band by western blotting, confirming the expression of AQP6 in secretory granule membrane of rat parotid acinar cells.

In the parotid gland of the rat stimulated by the β-agonist isoproterenol, reaction with anti-AQP6

antibosy was observed to accumulate at the luminal side in the cells in confocal microscopy. These observations suggest that AQP6 is accumulated in luminal side of parotid acinar cells by β-agonist stimulation.

Discussion

In the renal epithelial cells, AQP6 was localized in intracellular small vesicle membranes, but less in the plasma membrane (3, 7). In contrast, AQP6 localizes in plasma membrane of parotid acinar cells. Beitz et al. (8) have suggested that N-terminus of AQP6 is critical for trafficking of the protein in MDCK cells. Therefore, N-terminal sequence of AQP6 in rat parotid acinar cells appears to be different from that in rat kidney.

It has been demonstrated that AQP6 functions not as a water channel but as an anion channel in response to acidic pH or Hg^{2+} activation in *Xenopus Laevis* oocytes expressing AQP6 (4). In HEK239 cells expressing GFP-tagged AQP6, AQP6 has also been demonstrated to function as an anion chanel with the halide permeability sequence: $NO_3^- > I^- >> Br^- > Cl^- >> F^-$ (9). Saliva secreted from the parotid gland contains anions such as Cl^-, I^- and F^- (10). Therefore, AQP6 appears to contribute to secretion of such anions.

We also demonstrated that AQP6 localized in secretory granule membrane in parotid acinar cells. In various exocrine cells including parotid acinar cells, the secretory granule is formed from the condensing vacuole, which buds off from the *trans* face of the Golgi complex. In the condensing vacuole, secretory proteins exist as dilute form. In a subsequent packing process, the proteins are condensed. During this process, it is conceivable that transport of ions and water through the secretory granule membrane is necessary for the protein condensation. Therefore, AQP6 appears to contribute to osmoregulation in the granules.

In this paper, anti-AQP6 antibody positive reactions were observed diffusely in luminal area of parotid acinar cells by β-adrenergic receptor activation, which stimulates exocytotic secretion of amylase. In exocytosis, the fluidity of the primary secretion has been considered to be important for the discharge of granule contents from exocytosed secretory granules (11). It is likely that AQP6 both in secretory granule membrane and apical membrane contributes to exocytotic release in parotid acinar cells.

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Effects of actin-related drugs on exocytosis in parotid acinar cells.

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Objectives

In parotid acinar cells, activation of β-adrenergic receptors provokes exocytotic amylase release. Since apical actin network undergoes reorganiztion during the regulated exocytotis, regulation of actin cytoskeletal structure is considered to be involved in the exocytosis. Actin-related drugs have been used to investigate the relation between amylase release and reorganiztion of actin cytoskeleton. In this paper, we investigated the effects of actin-related drugs on amylase release and actin reconstruction in rat parotid acinar cells.

Introduction

F-actin consists of monomeric (depolymeric) G-actin and polymeric F-actin. It has been reported that involvement of F-actin reorganization (polymerization and depolymerization) in exocytosis of exocrine acinar cells. In parotid acinar cells, cortical F-actin cytoskeleton localizes under apical and plasma membranes. The cortical actin under apical membrane has been thought to function as a barrier to exocytosis. If the Ω-shaped F-actin beneath apical membrane is involved in exocytosis, exocytosis in parotid acinar cells needs actin reorganization, depolymerization of cortical actin beneath apical membrane and polymerization around secretory granules. However, it is unknown how F-actin is involved in reorganization in exocytosis in parotid acinar cells.

For investigation of actin turnover and regulation, actin-related drugs have been used. Cytochalasin D, a kind of fungus toxin, caps barbed end (polymerizing end) of F-actin and facilitates depolymerizing F-actin. Jasplakinolide, the toxin of marine sponge *Jaspis Jonstoni*, binds lateral side of F-actin and stabilizes turnover. Y-27632, Rho-kinase inhibitor, blocks Rho-kinase ROCK-I or II activity and cytoskeletal signaling via Rho-ROCK cascade. To investigate of involvement F-actin reorganization in exocytosis

in parotid acinar cells, we examined amylase release and laser scanning microscopy of parotid acinar cells stimulated by β-agonist in the presence of actin-related drugs.

Methods

Parotid acinar cell isolation: Parotid acinar cells were isolated from male 6 weeks Sprague-Dawrey rat using collagenase A (75 U/ml) and hyalronidase (50 U/ml). Filtrated acinar cells through nyron mesh were diluted in 6 ml Krebs-Ringer bicarbonate solution containing 0.5% BSA and 2 mg trypsin inhibitor.

Amylase release: Diluted parotid acinar cells were pre-incubated for 10 min with or without of actin-related drugs and subsequently stimulated by 1 μM isoproterenol for 15 min. Amylase activities in the mediums and the cell lysates were assayed by the method of Bernfeld (1995). Amylase release was described as a ratio of activity released from the cells.

Laser scanning confocal microscopy: The cells prepared as described above were fixed using 10% phosphate neutralized formarine. Cells were washed by PBS (pH7.4) and stained using Alexa 488 phalloidin. The stained cells were attached on a glass-based dish and embedded with Prolong Antifade.

Results

1) Effects of actin-related drugs on amylase release

When cells were treated without or with actin-related drugs and subsequently stimulated by 1 μM isoproterenol for 15 min, amylase release was partially inhibited in the presence of Cytochalasin D (1 μM), jasplakinolide (1 μ M) or Y-27632 (10 μM) as shown in (a).

2) Effects of actin-related drugs on actin reoconstruction

F-actin in rat parotid acinar cells was observed in confocal microscopy. F-actin and nuclear were indicated with Alexa-488 conjugated phalloidin (green) and with To-pro-3 iodide (blue), respectively. In the absence of actin-related drugs, F-actin was observed in cortical layer of parotid acinar cells (b). When the cells were stimulated with isoproterenol, Ω-shaped F-actin was observed beneath apical membrane (c).

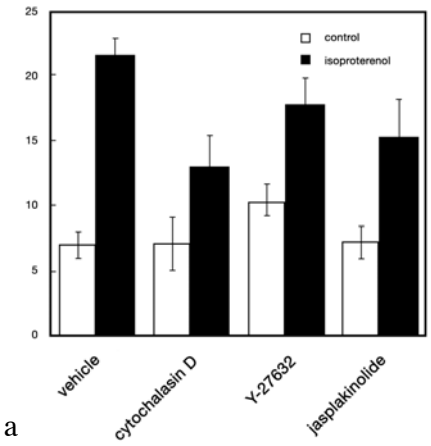
In the cells treated by cytochalasin D, fluorescence intensity of F-actin in cortical layer was reduced and breaking filament structure was observed (d). When the cells were stimulated with isoproterenol, Ω-shaped F-actin was not observed beneath apical membrane (e).

In rat parotid acinar cells treated by jasplakinolide, fluorescence intensity of F-actin in cortical layer was reduced and aggregation of cortical F-actin occurred (f). When the jasplakinolide-treated cells were stimulated with isoproterenol, observation of F-actin was not changed and Ω-shaped F-actin was not observed beneath apical membrane (data not shown). In apoptosis, nuclear fragmentation occurred and fragments stained by propidium iodide detected by flow cytometry. Japlakinolide-treatment increased nuclear fragments (g).

In Y-27632-treated cells, fluorescence intensity of F-actin in cortical layer was not changed (h). When the cells were stimulated with isoproterenol, Ω-shaped F-actin beneath apical membrane was observed (i).

Discussion

In the cytochalasin D-treated cells, cortical actin broken was observed, indicating that cytochalasin D induced facilitation of depolymerizing F-actin. In the cells, neither isoprotrenol-induced amylase relase nor Ω-shaped F-actin were observed, suggesting that alteration of F-actin inhibits amylase release. On the other hand, isoproterenol-induced amylase release was partially inhibited in the presence of Y-27632, but cortical actin and Ω-shaped F-actin were not affected. These results suggest that amylase release inhibition by actin-related drugs is caused by not only detriment of actin regulation but also other factors, and ROCK-I or II appear to regulate other signaling in parotid acinar cells. Furthermore, jasplakinolide partially inhibited isoproterenol-induced amylase release. However, apoptosis occurred in the jasplakinolide-treated cells. Therefore, the relationships between jasplakinolide-induced apoptosis and stabilization of F-actin should be studied.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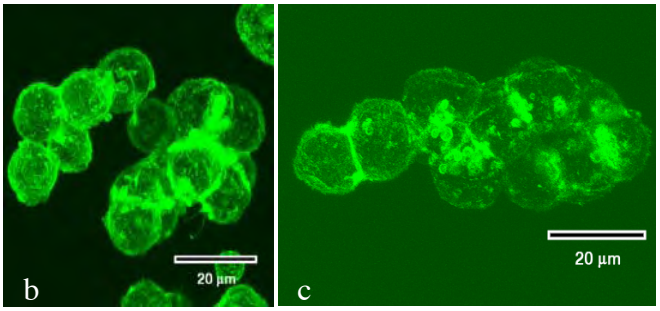
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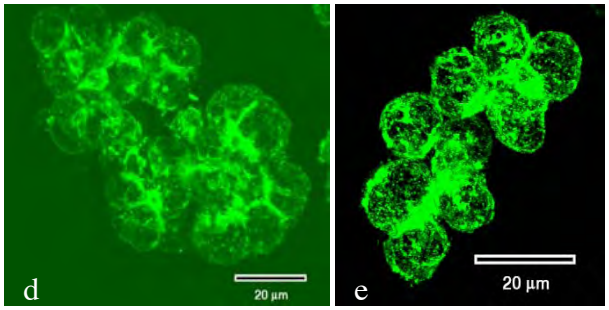
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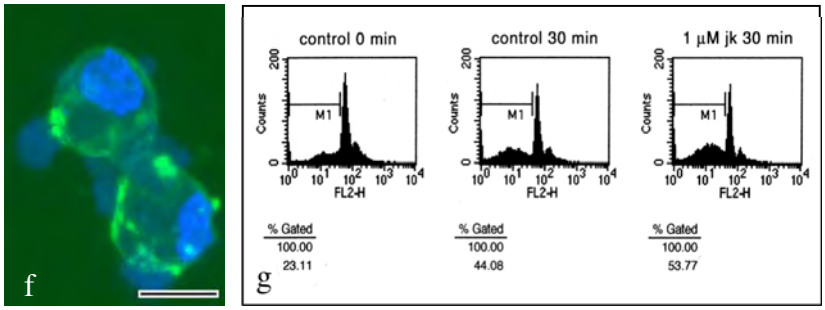
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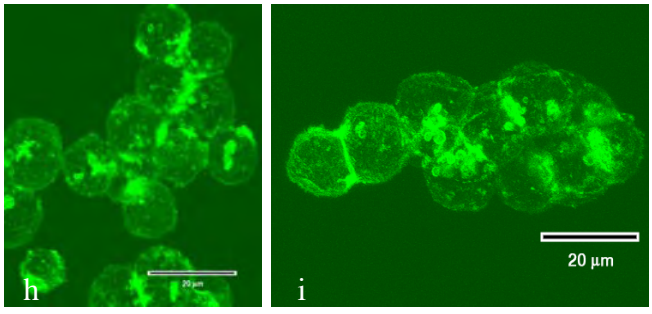
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A modular approach to computational modelling of epithelial electrolyte transport

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Systems biology is an exciting and rapidly emerging field which attempts to combine genome, transcriptome and proteome data in order to synthesize a 'complete' description of a biochemical or physiological process at a cellular, tissue or organismal level. A crucial element of this approach is the development and subsequent refinement of a computational model of the process based on mathematical descriptions of the individual components and their interrelationships.

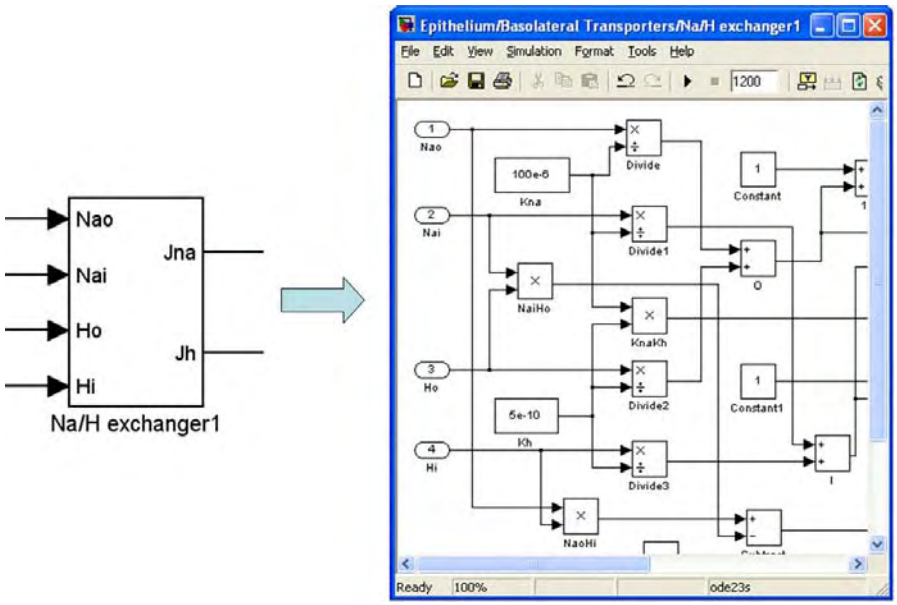
Although the systems biology concept is applied mainly to metabolic and signalling pathways at present, a very similar approach has been used sporadically to characterize epithelial transport processes for many years. Computational models, based on knowledge of the individual channels and transporters that are active in a particular epithelium, have successfully simulated many experimental results that have been obtained in the laboratory. Where differences have been observed between model predictions and experimental observations this has often led to improvements in the model which progressively result in a better match between prediction and observation.

What is lacking at present is a user-friendly software framework to enable transport physiologists, who may not have the time or training to write simulation programs from scratch, to assemble single-cell and epithelial transport models and test the behaviour of these models in simulated experiments. It should be possible, for example, to build a library of modules that describe the properties and behaviour of known channels and transporters, many of which are expressed in numerous different epithelia. A module representing an NHE1 Na^+/H^+ exchanger, for example, could then be 'plugged' into the basolateral membrane of an epithelial cell model in such a way that the Na^+ and H^+ fluxes that it generates realistically respond to and influence the electrochemical gradients that drive it. As more is learned about the kinetics and regulation of the individual channels and transporters, modules such as this could be gradually improved and updated.

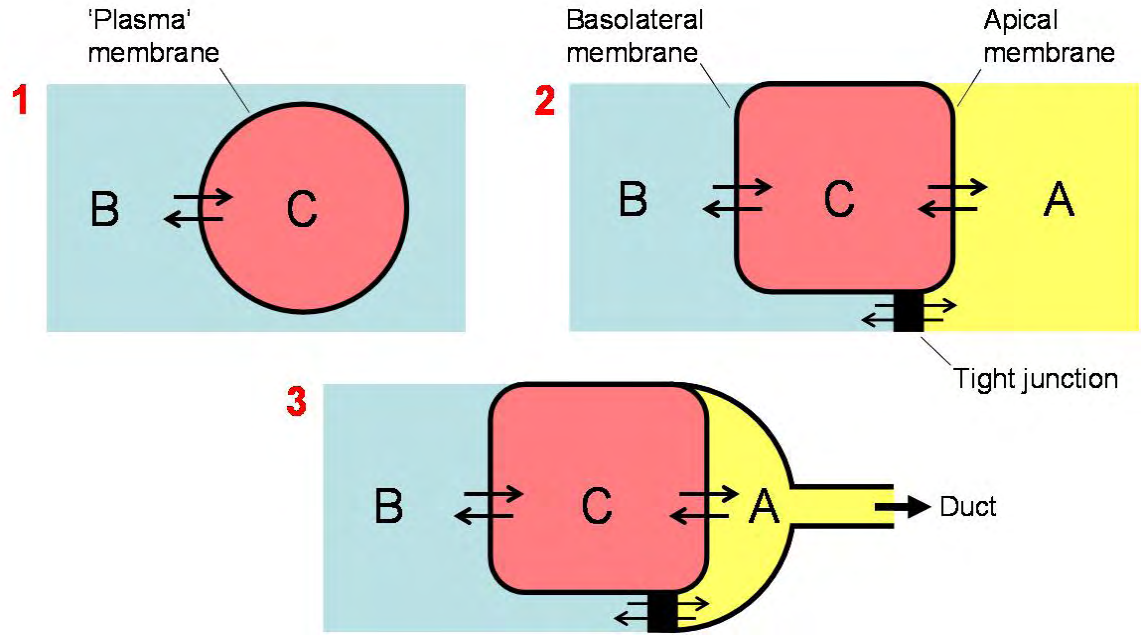
This presentation illustrates our attempt to modularise an existing, highly successful computational model of electrolyte transport by pancreatic duct epithelium (Sohma Y et al. J Membr Biol 176,77-100, 2000). Our longer-term aims are: 1) to develop a generic, user-friendly software environment which enables researchers to build, test and refine mathematical models of epithelial water and electrolyte transport; 2) to assemble and maintain a library of modules representing specific channels and transporters that can be 'plugged' into a number of predefined experimental scenarios.

We currently use Simulink software for building and running simulations in the MATLAB programming language (www.mathworks.com). Simulink enables non-experts to 'write' programs for computational biology using blocks and connecting wires. In our Simulink models, channels and transporters are represented by modules which calculate fluxes on the basis of the concentration gradients (and membrane potentials if electrogenic).

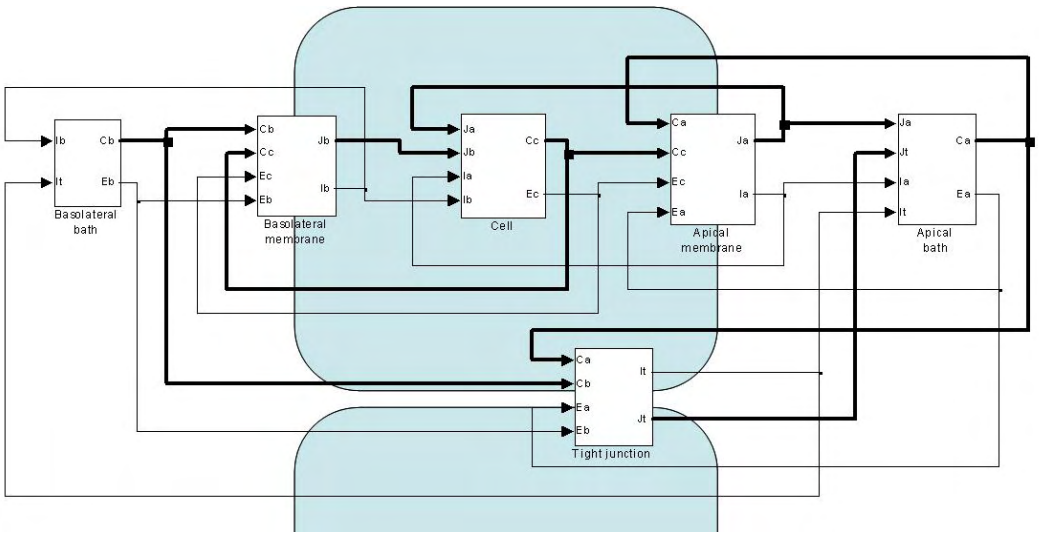
For example, a simple Na^+/H^+ exchanger module can be represented in Simulink like this (left):



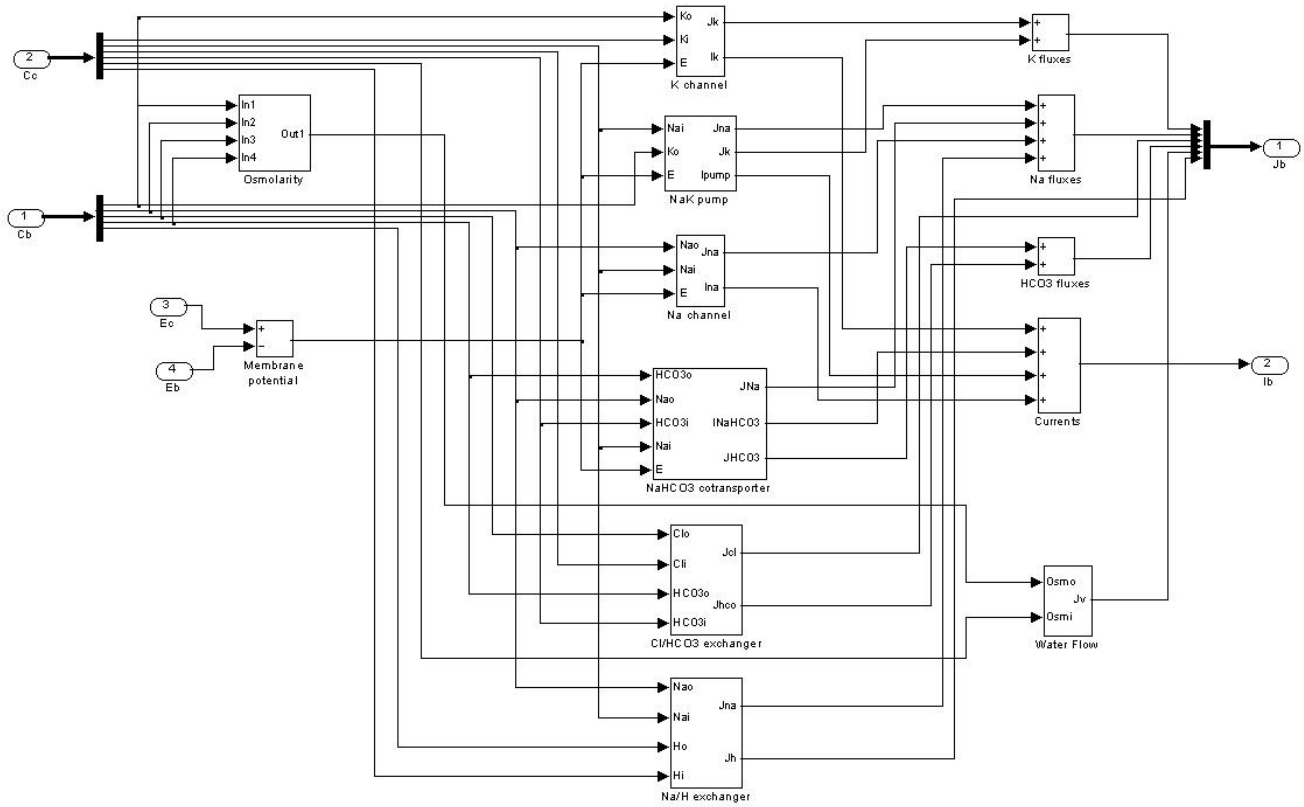
where the inputs Nao, Nai, Ho and Hi are the extracellular (o) and intracellular (i) concentrations of Na^+ and H^+, and the outputs Jna and Jh are the calculated net fluxes of the two ions. Double-clicking on the module in Simulink reveals (right) the calculation used to determine the fluxes of Na^+ and H^+. The formulae and kinetic parameters can be updated as and when further information becomes available.



The alternative model scenarios illustrated above represent: (1) isolated cells bathed by a large volume of constant composition, (2) epithelial cells bathed on both surfaces by large volumes of constant composition, (3) secretory cells bathed on the basolateral side by a large volume of constant composition, but secreting into a small fixed volume of variable composition from which the secretion drains away. (A, apical; B, bath/basolateral; C, cell)

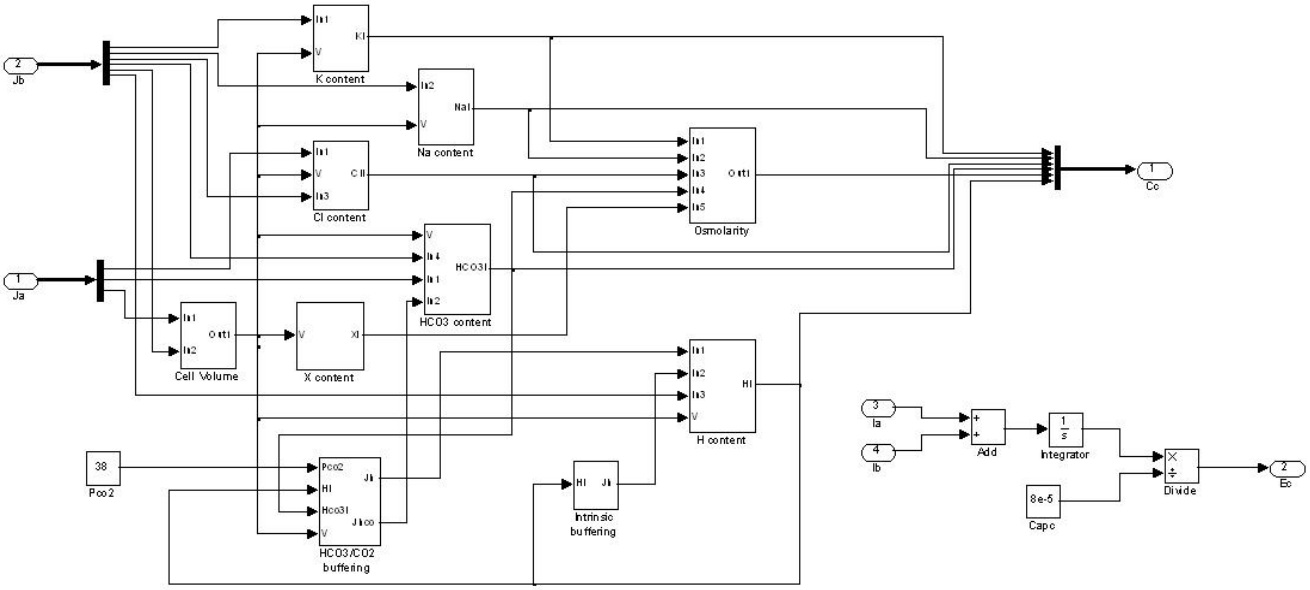


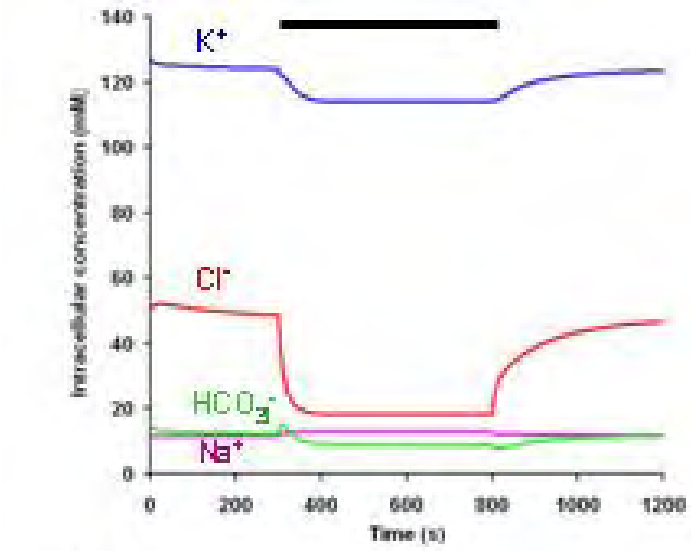
For a simple epithelium bathed on both sides with large volumes of solution, such as the pancreatic duct model shown above, the apical, basolateral and tight junction transporters are grouped into 'subsystems'. The inputs to the subsystems are the ion concentrations (C) and electrical potentials (E), and the outputs are the calculated fluxes (J) and currents (I) for all the transported ions. Double-clicking on the basolateral membrane subsystem reveals all the individual channels and transporters which are connected to the relevant concentrations and potentials on their input sides and to the flux and current combiners on their output sides:

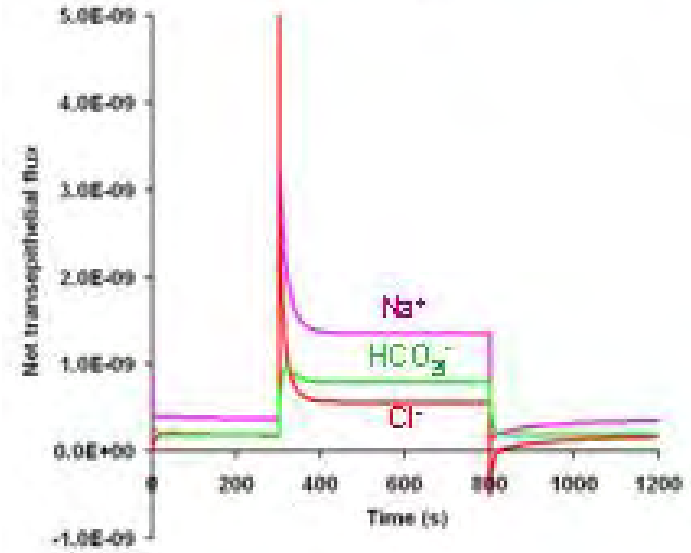


The three fluid compartments are also represented by subsystems. In this case the apical and basolateral subsystems contain fixed concentrations for the various ions but the electrical potentials can either be fixed (voltage-clamp mode) or allowed to vary (open-circuit mode). The concentrations, volume and electrical potential in the cell subsystem are constantly recalculated on the basis of the fluxes and currents

crossing the apical, basolateral and tight junction barriers. Double-clicking on the cell subsystem reveals the integrators that keep a running total of the cellular content of each ion. Intracellular ion concentrations are calculated by dividing content by cell volume, which in turn varies as a result of osmotic water flow into or out of the cell.







Simulations (e.g. left) are run for a specified period of time, starting from a set of initial estimates for the intracellular ion concentrations and membrane potentials. After a steady state has been reached, any of the transporter activities can be changed to examine the effects of stimulation, application of blockers etc. In this example, to simulate the effects of stimulating secretion (indicated by the horizontal bar), the apical membrane anion permeability and the basolateral Na^+-HCO_3^- cotransporter activity have been increased. The upper panel shows the effect of these changes on intracellular ion concentrations. The lower panel shows the effect on the net transepithelial fluxes. By comparing the model predictions with the results of real experiments, the model can be progressively improved and refined.

Regulation of the Plasma Membrane Ca^{2+}-ATPase in Parotid Acinar Cells

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Effective fluid secretion in parotid acinar cells is critically dependent on the exquisite spatio-temporal control of intracellular Ca^{2+} ($[Ca^{2+}]_i$) signals by the concomitant activation of cAMP pathways. Such signalling cross-talk is believed to be important for the precise control of apically located Ca^{2+}-dependent Cl^- channels and basolaterally located Ca^{2+}-dependent K^+ channels which maintain maximum ion and thus water movement. My previous studies in acutely isolated parotid acinar cells have demonstrated that the key molecular mechanisms for this signalling cross-talk are the PKA-mediated modulation of inositol 1,4,5-trisphosphate receptors ($InsP_3R$), that control Ca^{2+} release, and plasma membrane Ca^{2+}-ATPase (PMCA), that control Ca^{2+} clearance [1,2]. Specifically, PKA potentiates and phosphorylates the PMCA but only in the presence of $[Ca^{2+}]_i$-raising agents (2). More recently, using a convenient model for assessing PMCA activity in intact cells, activation of PKA (using 10 µM forskolin) differentially potentiated $[Ca^{2+}]_i$ clearance at the apical region compared to the basolateral region (Fig. 1).In cells pre-treated with 10 µM forskolin, it was clear that $[Ca^{2+}]_i$ clearance in the apical region of mouse parotid acinar cells occurred much faster than in the corresponding basolateral region in the same cells.

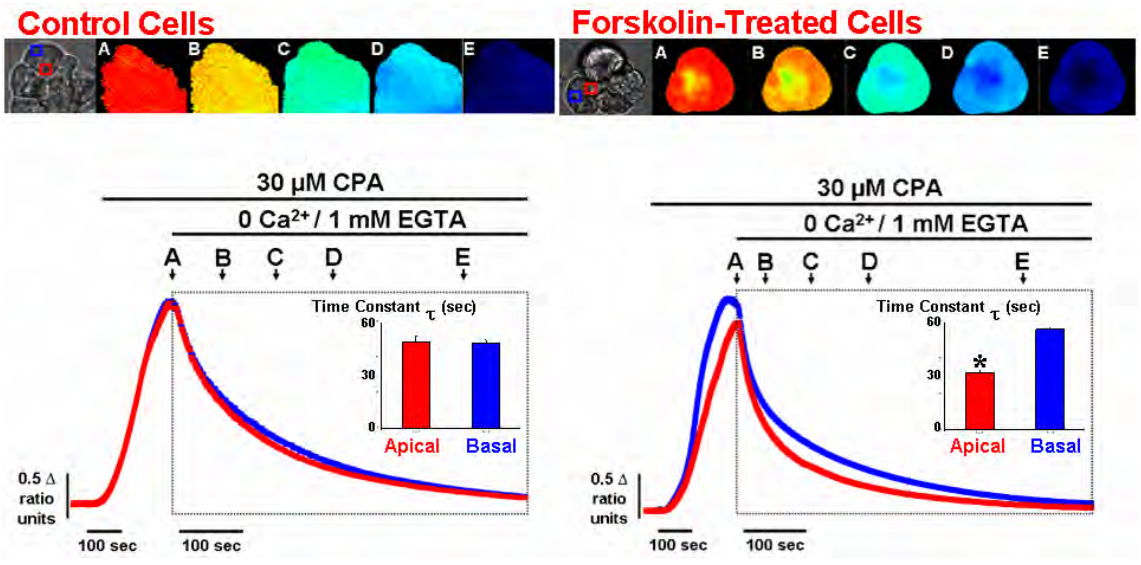

Fig 1. PKA activation differentially potentiates apical $[Ca^{2+}]_i$ clearance in acutely isolated parotid acinar cells. Fura-2-loaded mouse parotid acinar cells were treated with 30 µM CPA which elevates $[Ca^{2+}]_i$ due to ER Ca^{2+} leak and Ca^{2+} influx. Removal of external Ca^{2+} initiated the measurement of Ca^{2+} clearance that was due to PMCA activity by fitting to a single exponential decay to give the time constant (τ). Apical (red box and red trace) and basolateral (blue box and blue trace) were separately monitored (n=6, *p<0.05) either in untreated (control) or cells pre-treated with 10 µM forskolin.

This was further investigated using the immortalised parotid acinar cell line, Par-C10 cells, grown on permeable polyester transwell supports. These cells differentiated into columnar-type epithelial monolayers that formed tight junctions (3). This important property of these cells was exploited such that either the apical or basolateral sides of the cell layer could be separately perfused. Using this model it was therefore possible to selectively inhibit Ca^{2+} efflux using 1 mM La^{3+} applied to either the apical or basolateral side and therefore monitor global Ca^{2+} clearance (using the method described above) that was due to PMCA activity on the corresponding side of the epithelia (Fig. 2).

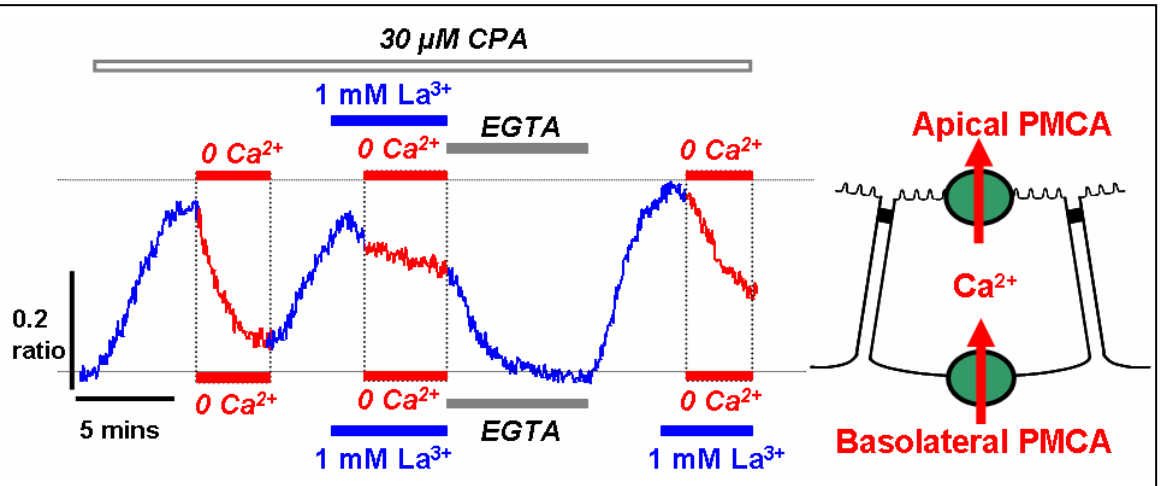

Fig 2. Functional separation of apical and basolateral PMCA activity in Par-C10 cells. Apical and Basolateral PMCA activity can be separately assessed in the immortalised parotid acinar cell-line, Par-C10, grown on transwell filters by separately perfusing the apical and basolateral sides of the cells with 1 mM La^{3+}. This functionally inhibits either the apical of basolateral PMCA enabling the corresponding PMCA activity to be functionally isolated.

Under conditions where the basolateral PMCA was inhibited by La^{3+} the rate of $[Ca^{2+}]_i$ clearance, that represented the functionally isolated apical PMCA activity, was approximately 50% of the maximum clearance (i.e. without La^{3+}). However, under conditions where the basolateral PMCA was functionally isolated (i.e. when La^{3+} was added to the apical side of the cells) the rate of $[Ca^{2+}]_i$ clearance was approximately 10% of the maximum clearance (i.e. without La^{3+}). These experiments revealed that the apical PMCA was the major route for Ca^{2+} efflux in Par-C10 cells. In addition, these data also showed that treatment with forskolin potentiated $[Ca^{2+}]_i$ clearance when the apical PMCA activity was functionally isolated (forskolin, τ=50 ± 8.6 seconds; control, τ=122.2 ± 5.0 seconds). However, treatment with forskolin failed to have any significant effect on $[Ca^{2+}]_i$ clearance when the basolateral PMCA activity was functionally isolated. These data also confirm that PKA activation differentially potentiate the apical PMCA consistent with observations in mouse parotid acinar cells.

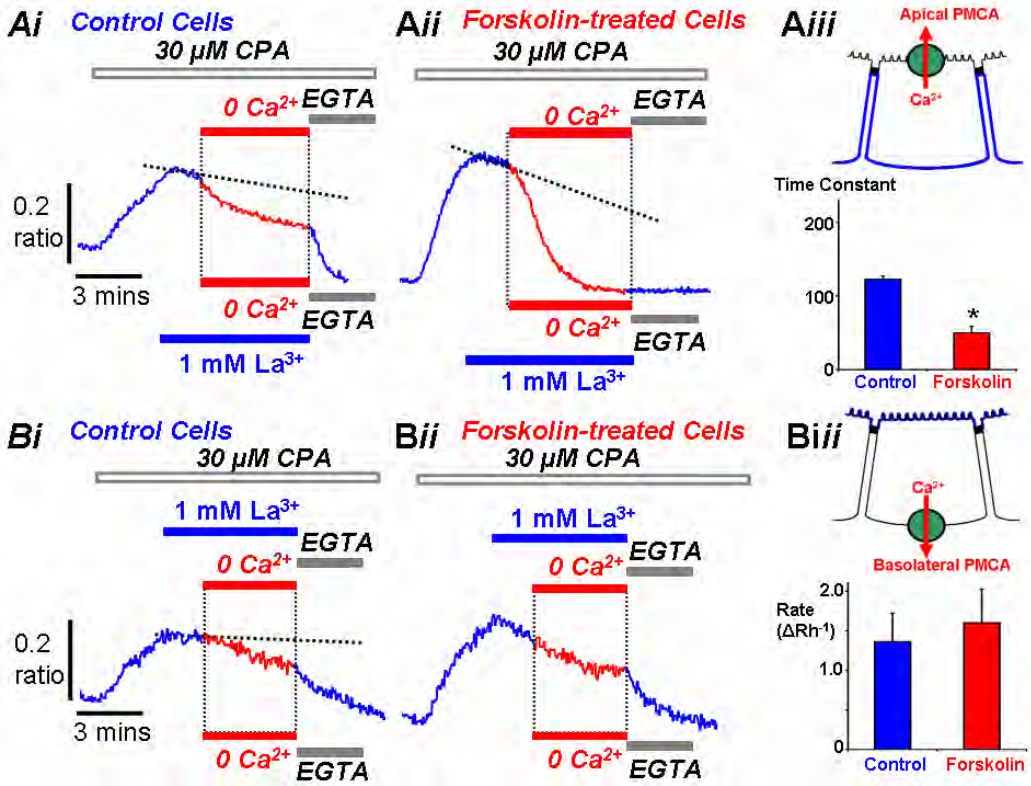

Fig 3. PKA activation differentially potentiates apical PMCA activity in Par-C10 cells. Parotid acinar cells were treated with (*Aii, Bii*) or without (*Ai*, *Bi*) 10 μM forskolin, prior to initiating the "Ca^{2+} efflux assay" (see above). La^{3+} (1 mM) was added to either the basolateral side to isolate the apical PMCA activity (*A*), or the apical side to isolate the basolateral PMCA activity (*B*). PMCA activity was quantified either by fitting to a single exponential decay to give the time constant (τ, see *Aiii*) or by fitting to a linear curve giving the change in ratio per hour (ΔRh^{-1}), when the rate was so slow that it could not be accurately fit to a single exponential decay (*Biii*). (n=6, *p<0.05)

Finally, Western blotting revealed that PMCA1, 2 and 4 are expressed in acutely isolated parotid acinar cells and Par-C10 cells, whereas PMCA3 was not expressed (data not shown). Immunofluorescence revealed that PMCA1 was distributed throughout all regions of the plasma membrane, PMCA4 was localized to the apical membrane, whereas PMCA2 was localised almost exclusively to the basolateral membrane (Fig. 4). This might suggest that PMCA4 or possibly PMCA1, but not PMCA2, might be candidates for regulation by PKA at the apical membrane of parotid acinar cells. However, *in situ* phosphorylation assays, whereby cell lysates were immunoprecipitation with specific PMCA antibodies and western blotted with the phospho(ser/thr) antibody (see Ref. 2), demonstrated that PMCA1 and PMCA2, but not PMCA4, were phosphorylated by the combined treatment with forskolin (10 μM) and CCh (1 μM CCh) (Fig.3). This therefore suggests that PMCA1 is the likely candidate to be regulated by PKA in a Ca^{2+}-dependent manner specifically at the apical membrane. This presents a functional paradox since PMCA1 was found to be distributed in all regions of the plasma membrane (Fig. 3). Interestingly, the PDZ-containing accessory proteins, ezrin and EBP50, also exhibited an apically-localised

distribution in parotid acinar cells. Since ezrin can bind EBP50, actin, and PMCA, and EBP50 can bind PKA and ezrin then these observations imply that these proteins may be involved targeting PKA either directly or in the close vicinity to the PMCA. However, direct evidence is required to substantiate such a phenomenon.

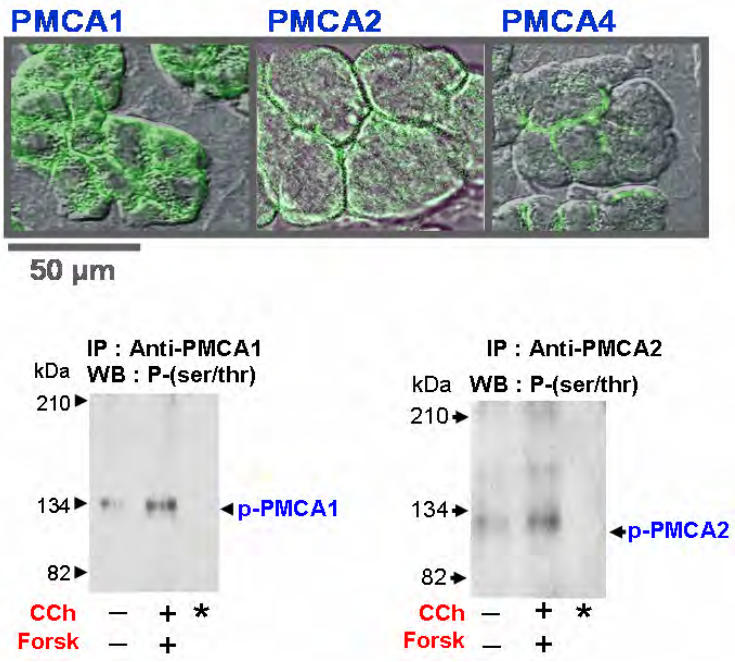

Fig 4. Immunofluorescence and in situ phosphorylation assays in mouse parotid acinar cells. PMCA1 was distributed in all regions of the plasma membrane, PMCA2 was localised to the basolateral membrane and PMCA4 was localised to the apical membrane. *In situ* phosphorylation assays showed that only PMCA1 and PMCA2 were phosphorylated following the combined treatment with CCh and forskolin.

Collectively these data suggest that PMCA is phosphorylated by PKA in a Ca^{2+}-dependent manner that differentially regulates Ca^{2+} clearance in the apical region of parotid acinar cells. This likely involves a Ca^{2+}-mediated assembly of a signalling complex that brings PKA closer to the PMCA allowing targeted regulation specifically at the apical plasma membrane. Such tight spatial regulation of Ca^{2+} efflux may represent an important mechanism for the fine-tuning of Ca^{2+}-dependent effectors at the apical membrane important for the regulation of fluid secretion and exocytosis.

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A feedback control model of fluid transport in salivary gland.

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Experiments were performed with the perfused rat submandibular gland *in vitro* to investigate the nature of the coupling between transported salt and water by varying the osmolarity of the source bath and observing the changes in secretory volume flow.

A model for control of the transport rate and osmolarity of epithelial fluid (isotonic transport) is presented by using an analogy with the control of temperature and flow rate in a shower (Fig.1). The model brings recent findings and theory concerning the role of aquaporins in epithelia [1] together with measurements of epithelial paracellular flow [2] into a single scheme. It is not based upon osmotic equilibration across the epithelium but rather on the function of aquaporins as osmotic sensors that control the tonicity of the transported fluid by mixing cellular and paracellular flows, which may be regarded individually as hyper- and hypo-tonic fluids, to achieve near-isotonicity. The system is built on a simple feedback loop and the quasi-isotonic behaviour is robust to the precise values of most parameters. Although the two flows are separate, the overall fluid transport rate is governed by the rate of salt pumping through the cell. The model explains many things: how cell pumping and paracellular flow can be coupled *via* control of the tight junctions; how osmolarity is controlled without depending upon the precise magnitude of membrane osmotic permeability; and why many epithelia have different aquaporins at the two membranes.

The model reproduces all the salient features of epithelial fluid transport [3] but also indicates novel behaviour. Isotonic transport is freed from constraints due to limited permeability of the membranes and the precise geometry of the system. It achieves near-isotonicity in epithelia in which partial water transport by co-transporters may be present. The model has been developed with a minimum of parameters, some of which require measurement, but the model is flexible enough for the basic idea to be extended both to complex systems of water and salt transport that still await a clear

explanation, such as intestine and airway, and to systems that may lack aquaporins and use other sensors.

This model has been applied to the *in vitro* rat SMG system in which hypertonic perfusions with sucrose were applied [4]. Glands were submitted to hypertonic step changes by changing the saline perfusate to one containing different levels of sucrose. The flow rate responded by falling to a lower value, establishing a new steady-state flow. The rate changes did not correspond to those expected from a system in which fluid production is due to simple osmotic equilibration, but were much larger (Fig.2). The changes were fitted to a model in which fluid production is largely paracellular, as observed in experiment [5], the rate of which is controlled by an osmosensor system in the basal membrane.

The same experiments were done with glands from rats that had been bred to have very low levels of AQP5 (the principal aquaporin of the salivary acinar cell) in which little AQP5 is expressed at the basal membrane (Fig.3). In these rats, salivary secretion rates after hypertonic challenges were small and best modelled by simple osmotic equilibration (Fig.4). In rats which had intermediate AQP5 levels the changes in flow rate were similar to those of normal rats although their AQP5 levels were reduced.

Finally, perfused normal glands were subject to retrograde ductal injection of salines containing different levels of Hg^{2+} ions (0, 10 and 100 μM) which would act as inhibitors of AQP5 at the apical acinar membrane. The overall flow rates were progressively diminished with rising Hg^{2+} concentration, but after hypertonic challenge the changes in flow rates were unchanged and similar to those of normal rats.

All these results are difficult to explain by a cellular osmotic model but can be explained by a model in which paracellular flow is controlled by an osmosensor (presumably AQP5) present on the basal membrane (Fig.5).

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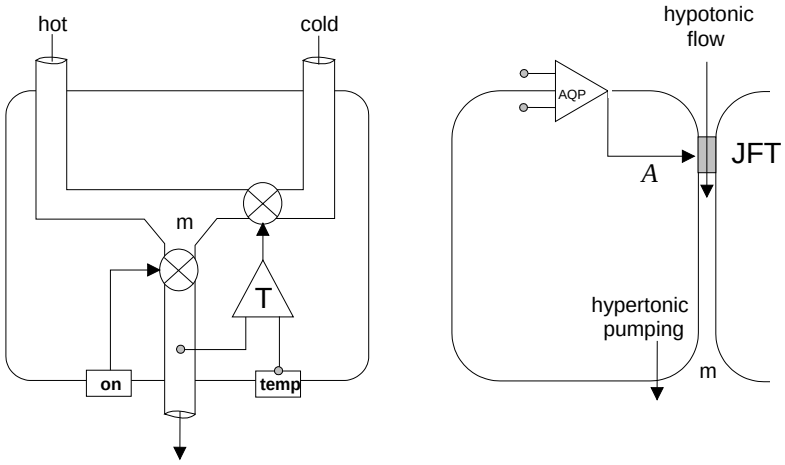
Fig.1



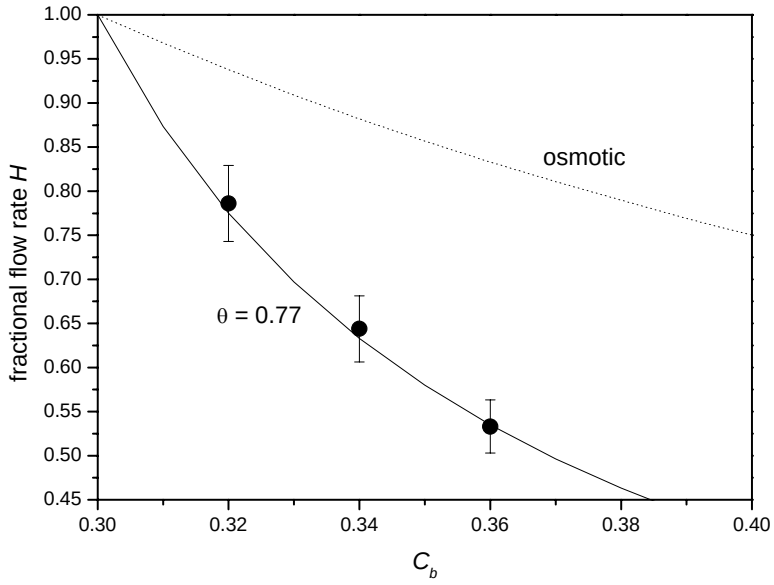
Thermostatic shower controller

Epithelial feedback model

Left. The operation of an ideal (constant pressure) shower control as a feedback circuit. The temperature control ('temp') determines the set point and the system is opened by controlling the hot water supply ('on'). The fluid streams mix in the region m. The thermostat **T** senses the exit water temperature and the amplified difference signal reduces the flow of cold water (negative feedback). In the steady-state there will be a small input difference depending on the size of the gain.

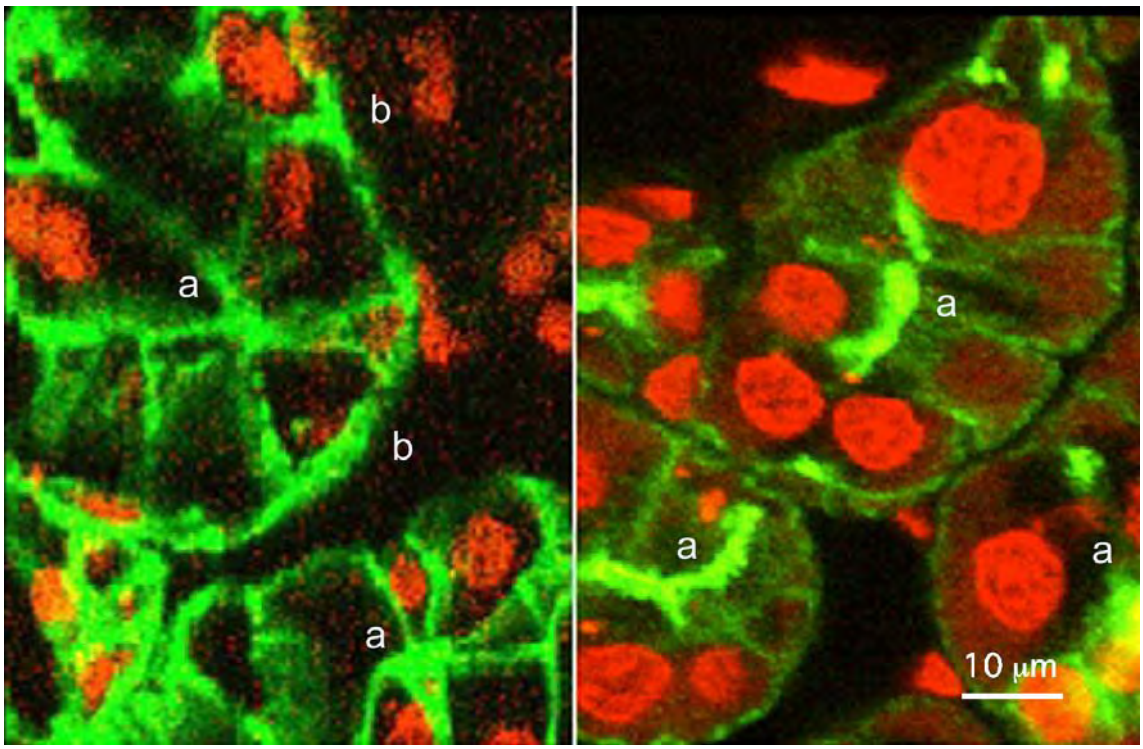
Right. A forward-facing epithelium transporting fluid quasi-isotonically. The AQP senses the osmotic difference between cell and source bath and the output signal controls the hypotonic fluid flow in the JFT system. The two fluid streams mix in the basolateral space **m** . The cell tonicity is intermediate between that of apical bath (the effective set-point) and the transported fluid and the difference in the steady-state will depend on the gain *A*.

Fig.2



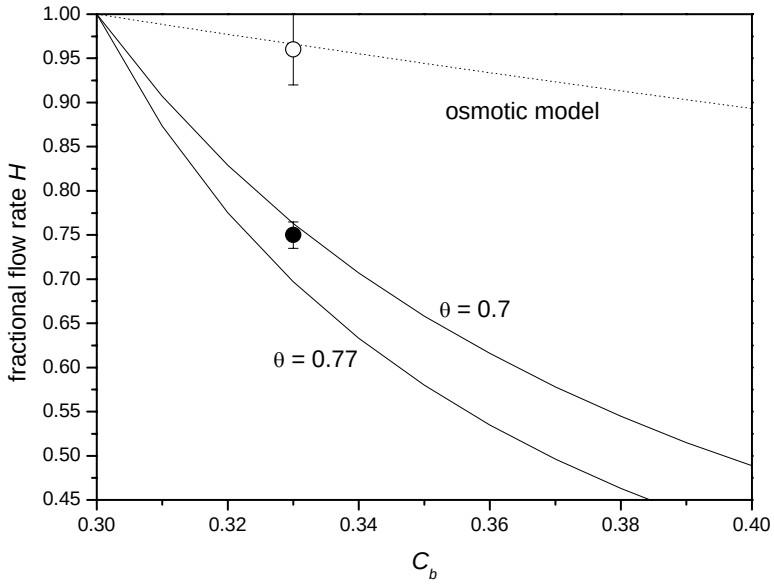
Normal SG rat preparations. Plot of the fractional flow rate (*H*) for salines with 20, 40 and 60 mOs sucrose additions. The fitted curve is that of junctions with a NaCl selectivity of 0.77. The osmotic line is the minimum value of *H* calculated for an osmotically generated fluid.

Fig.3



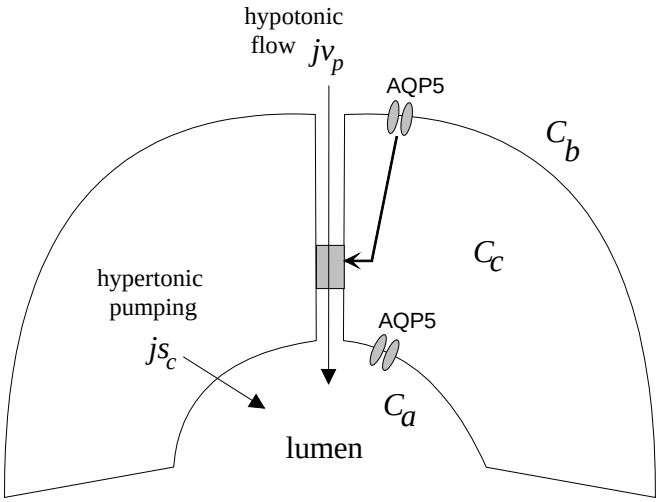
Fluorescent antibody localization of AQP5 in two sections of SMG acini from strains with different expression levels. (Left) : High expression rats in which strong staining is apparent of both apical (a) and basal (b) membranes of the acinar cells. (Right) : Low expression rats in which significant expression is apparent only at the apical (a) membrane.

Fig.4



Low AQP5 rat preparations (○) and normal controls (●). Plot of the rate ratio *H* for saline with 30 mOs sucrose addition. The lower curves are generated by the model with basic parameters and the selectivities shown. The upper osmotic curve (dotted) is generated for osmotic fluid production.

Fig.5



Major features of the sensor-feedback model applied to rat SMG. Feedback control of the junctional fluid flow system is proposed for the basal AQP5 acting as an osmosensor.

Calcium signaling mechanisms in non-excitable cells.

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In non-excitable cells, activation of phospholipase C by G-protein-coupled receptors results in release of intracellular Ca^{2+} stores and activation of Ca^{2+} entry across the plasma membrane. The intracellular release of Ca^{2+} is most commonly signaled by the second messenger, inositol 1,4,5-trisphosphate. Under physiological conditions, the release of Ca^{2+} often occurs by a cyclical, regenerative process resulting in intracellular Ca^{2+} oscillations. Early work on mechanisms of calcium revealed that Ca^{2+} entry involves signaling from depleted intracellular stores to plasma membrane Ca^{2+} channels, a process referred to as *capacitative calcium entry* or *store-operated calcium (SOC) entry* (Putney, Jr., 1997). The best characterized electrophysiological current associated with capacitative calcium entry is the calcium-release-activated calcium current, or I_{crac} (Parekh & Putney, Jr., 2005). The mechanism of activation of SOC or CRAC channels involves an endoplasmic reticulum Ca^{2+} sensor, Stim1 (Roos *et al.*, 2005; Liou *et al.*, 2005). Recently, an integral plasma membrane protein, Orai1, has been described that is essential for I_{crac} in lymphocytes ((Feske *et al.*, 2006), see also (Vig *et al.*, 2006; Zhang *et al.*, 2006). We have found that in HEK293 cells, RNAi knockdown of either Stim1 or Orai1 significantly reduces SOC entry. Yet, overexpression of Stim1 was without effect, and overexpression of Orai1 inhibited entry. However, when we co-expressed these two proteins together, huge store-operated Ca^{2+} entry and store-operated Ca^{2+} currents were observed ((Mercer *et al.*, 2006), see also (Zhang *et al.*, 2006; Soboloff *et al.*, 2006; Peinelt *et al.*, 2006)). These currents resembled I_{crac} in a number of ways, including a positive reversal potential, strong inward rectification, fast inactivation, complete inhibition by 1 μM Gd^{3+}, activation by 1 μM 2APB and complete inhibition by 30 μM 2APB. Thus these two proteins appear to completely recapitulate both the activation mechanism and permeation mechanisms for I_{crac}. In addition to Orai1, two similar proteins, Orai2 and Orai3 also support store-operated Ca^{2+} entry (Mercer *et al.*, 2006). Two laboratories have confirmed that mutations in specific sites in Orai1 change the selectivity of the large currents (Prakriya *et al.*, 2006; Yeromin *et al.*, 2006). This indicates that Orai is likely a pore-forming subunit of the SOC channel. Stim1 appears to act by redistributing within a small component of the endoplasmic reticulum, approaching the plasma membrane, but does not appear to translocate into the plasma membrane.

The current ideas about Stim1 and Orai action are consistent with a number of earlier ideas about store-operated entry; for example, the presence of a small, specialized component of the endoplasmic reticulum dedicated to communicating with plasma membrane channels; the observation that biophysical properties of CRAC channels differ substantially from conventional ion channels, predicting that they may also differ substantially in their structure; the general finding that Ca^{2+} refills the stores efficiently without resulting in a rise in global cytoplasmic Ca^{2+} and the suggested close physical association between sites of endoplasmic reticulum signaling and plasma membrane Ca^{2+} entry. One could say that the original idea of Irvine (Irvine, 1990) - conformational

coupling - is at least partially vindicated by the current model. However, it appears to be Stim1 rather than IP_3 receptors that are responsible for this coupling. A summary of the store-operated signaling pathway, incorporating Stim1 and Orais is shown in Figure 1. At present there are some general questions that will no doubt be attacked and hopefully resolved in the not too distant future: (i) Are any other players or proteins necessary for activation of capacitative calcium entry and/or I_{crac}? (ii) What is the role, if any, of plasma membrane Stim1? (iii) What is the composition of native CRAC channels (Orai homo- or heteromultimers; or combinations with TRPs)? (iv) Are there mechanisms of store-operated entry involving other modes of activation (other than Stim1) and other store-operated channels (other than Orais)? In addition, the availability of Stim1 and Orai cDNAs will lead to a detailed structural understanding of the various modes of regulation and activation of this pathway. Such information may ultimately prove of use in designing novel pharmacological reagents to aid in the treatment of a number of diseases in which the store-operated entry pathway is thought to play a role.

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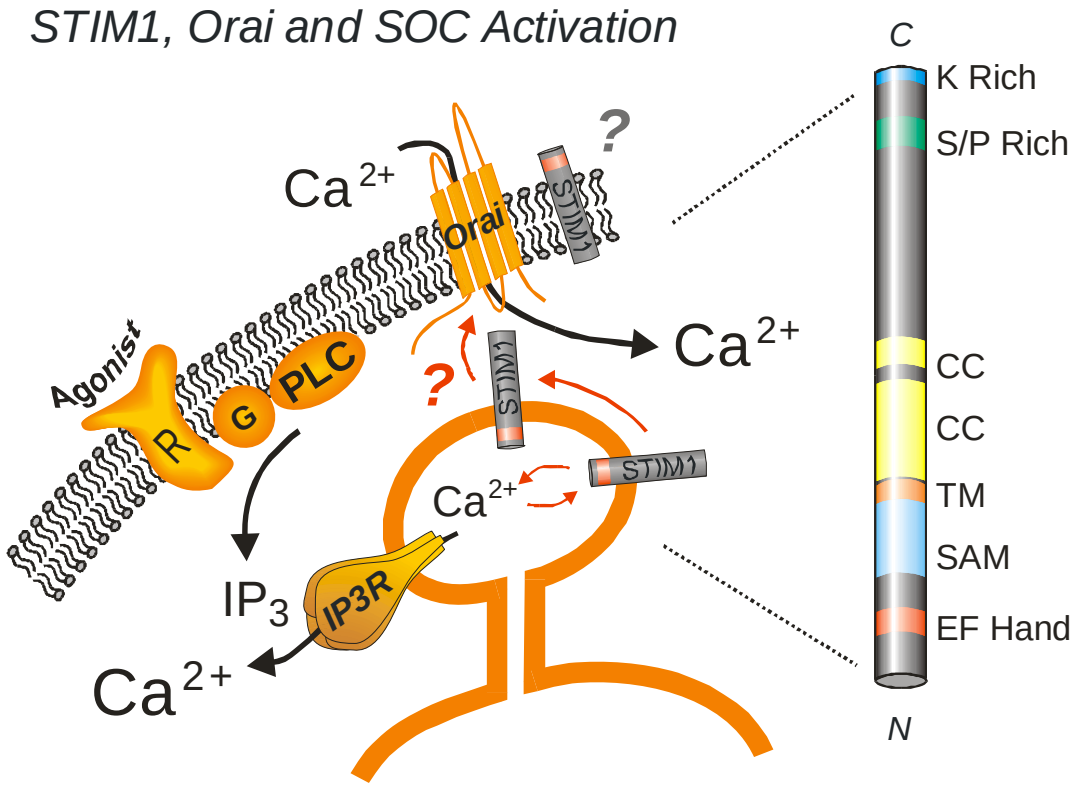

Figure 1: *Current understanding of the roles of Stim1 and Orais*. Agonist activation of a plasma membrane receptor (R) results in formation of IP_3, which activates the IP_3 receptor (IP3R) causing discharge of store Ca^{2+} from a subcompartment of the endoplasmic reticulum. Within this subcompartment, Ca^{2+} binds reversibly to an EF hand motif in Stim1; depletion of Ca^{2+} results in Stim1 without Ca^{2+} bound, which causes Stim1 to redistribute within the endoplasmic reticulum to areas near Orai within the plasma membrane. Stim1 then activates Ca^{2+}-selective Orai channels; the mechanism whereby this activation is accomplished is unknown. Stim1 is also present in the plasma membrane, although its function there is unclear.

Talk-O1

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Talk-E2

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Regulation of Cl^- secretion by muscarinic cholinergic and adrenergic stimulation in acinar cells of rat salivary glands.

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Muscarinic and α-adrenergic agonists induce fluid secretion from salivary glands via an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of acinar cells. β-Adrenergic stimulation itself does not evoke fluid secretion but modulates $[Ca^{2+}]_i$ increase-induced fluid secretion via an increase in the cytosolic cAMP level ($[cAMP]_i$). However, cellular regulation of fluid secretion from salivary gland by these agonists is still unclear. Since Cl^- secretion from acinar cells is believed to drive fluid secretion from salivary glands, we investigated the properties of $[Ca^{2+}]_i$ increase-induced Cl^- secretion from acinar cells and $[cAMP]_i$ increase-induced modulation of the Cl^- secretion to clarify the cellular regulation of the fluid secretion in parotid and submandibular glands.

The gramicidin-perforated patch recording techniques were adopted on rat parotid and submandibular acinar cells and anion current which performs intact Cl^- secretion from the cells was measured at the holding potential –80 mV. Carbachol (CCh) induced a bumetanide-sensitive oscillatory anion current in parotid acinar cells as was previously reported in submandibular acinar cells (1). The cAMP-increasing agents, forskolin + 3-isobutyl-methylxantine (IBMX), did not induce any oscillatory current but reduced the CCh-induced oscillatory anion current both in parotid and in submandibular acinar cells. Since the ionic current measured in the gramicidin-perforated patch configuration reflects the transporter activity and the ion channel activity, these results suggest that cAMP suppresses one or both of the activities in the configuration. In the conventional whole cell recording, in which the ionic current reflects not the Cl^- transporter activity but the ion channel activity, cAMP-increasing agents potentiate the Ca^{2+}-activated Cl^- channel activity of parotid acinar cells (2), and suppress the Cl^- channel activity of submandibular acinar cells (3). These suggest the difference between the mechanism of cAMP-induced suppression of the current in parotid acinar cells and that in submandibular acinar cells in the gramicidin-perforated patch configuration. In conclusion, muscarinic cholinergic stimulation induces oscillatory Cl^- secretion via $[Ca^{2+}]_i$ increase and β-adrenergic stimulation suppresses the secretion via $[cAMP]_i$ increase, both in parotid and submandibular acinar cells.

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2. Hirono C, Sugita M, Furuya K, Yamagishi S, Shiba Y. J Membr Biol. 164:197-203, 1998.
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Clinical examination using saliva: Influence of fluid secretion on the concentration of glucose in saliva: How to overcome the problem for in vivo application?

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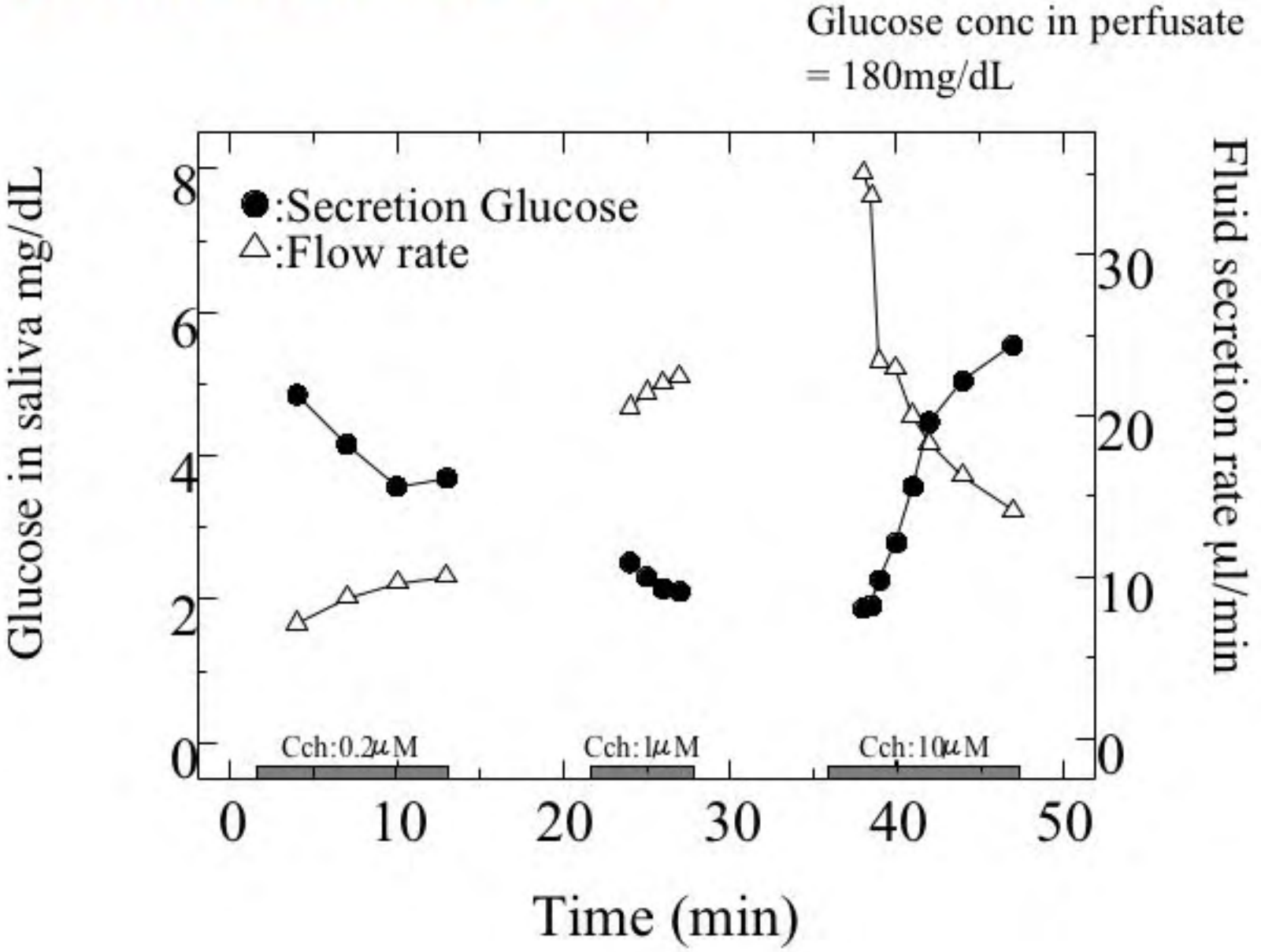
Phase I: Explorer of Basic Mechanism for Fluid secretion

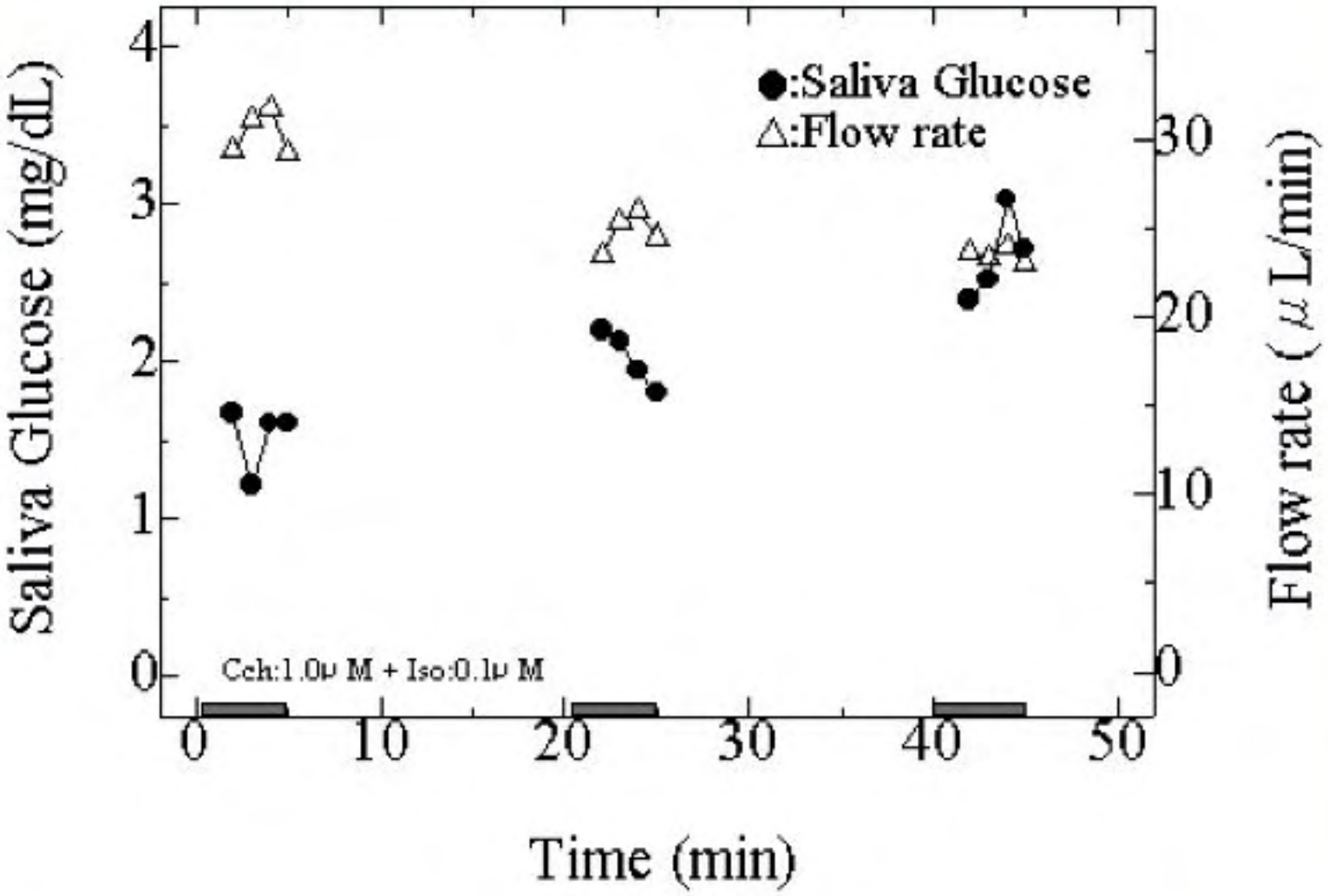
Phase II: Trials for Clinical Application for Saliva Test.

Problem is:
Salivary Concentration of Substrate
does not reflect the concentration in the blood.

Various dose of CCh

RAT SMG




●:Saliva Glucose
△:Flow rate
Cch:1.0μ M + Iso:0.1μ M
Saliva Glucose (mg/dL)
Flow rate (μ L/min)
Time (min)
0
1
2
3
4
10
20
30
40
50

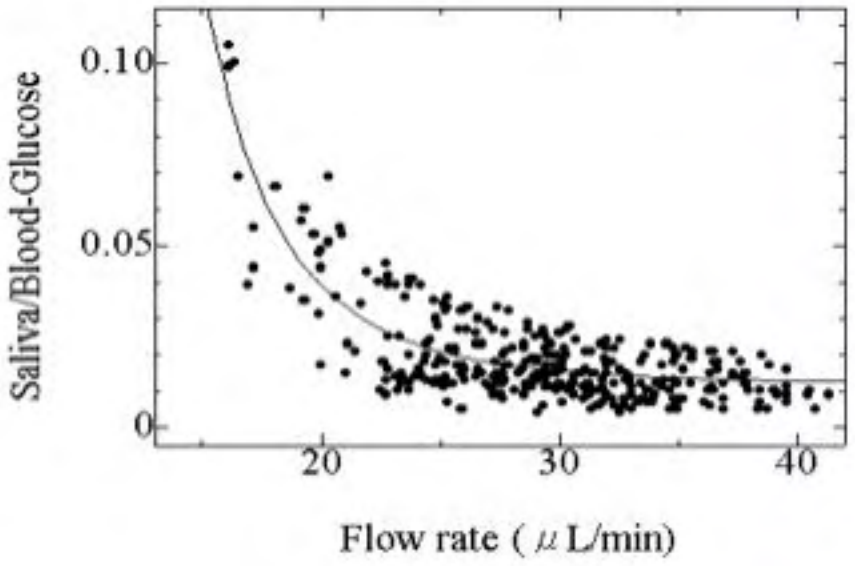


Fig. 5(a)

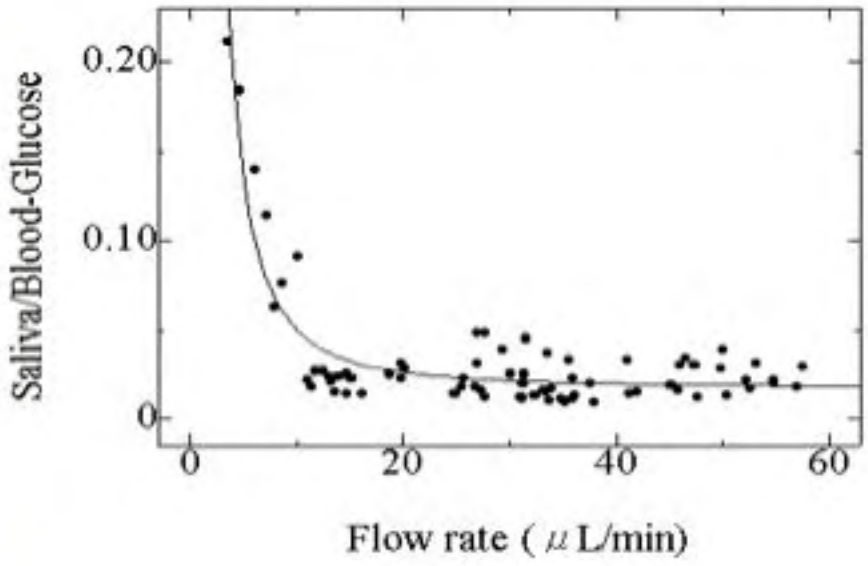


Fig. 5(b)

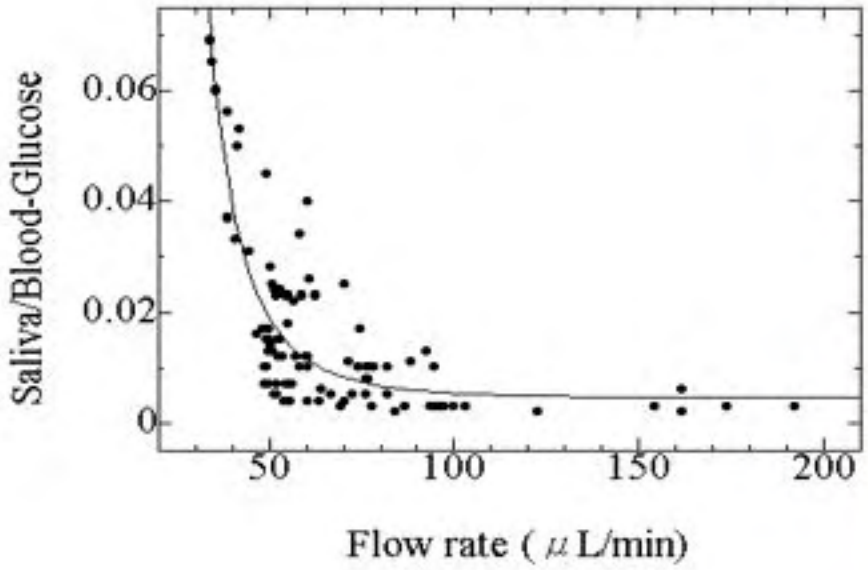


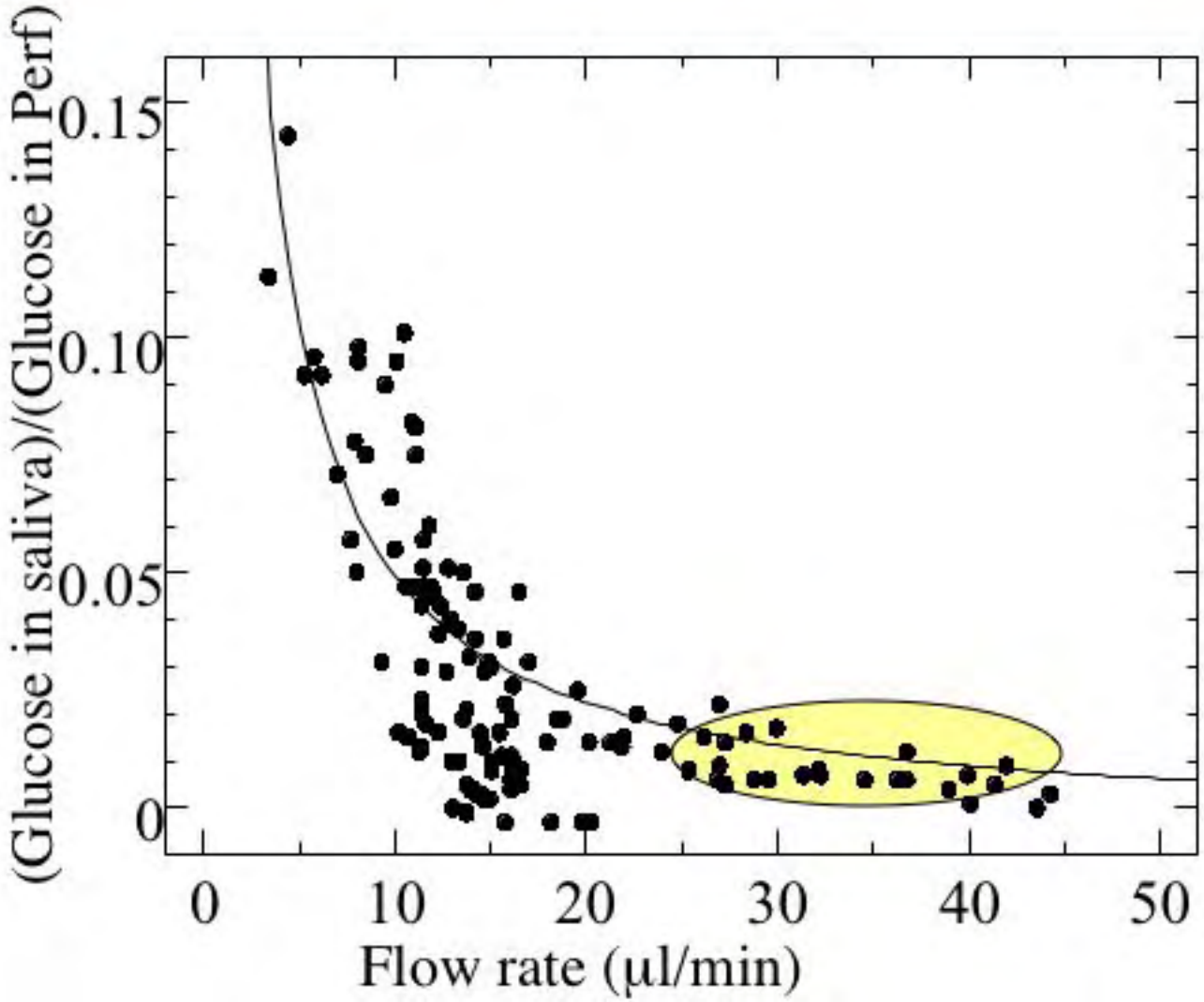
Fig. 5(c)

normal rats (a),

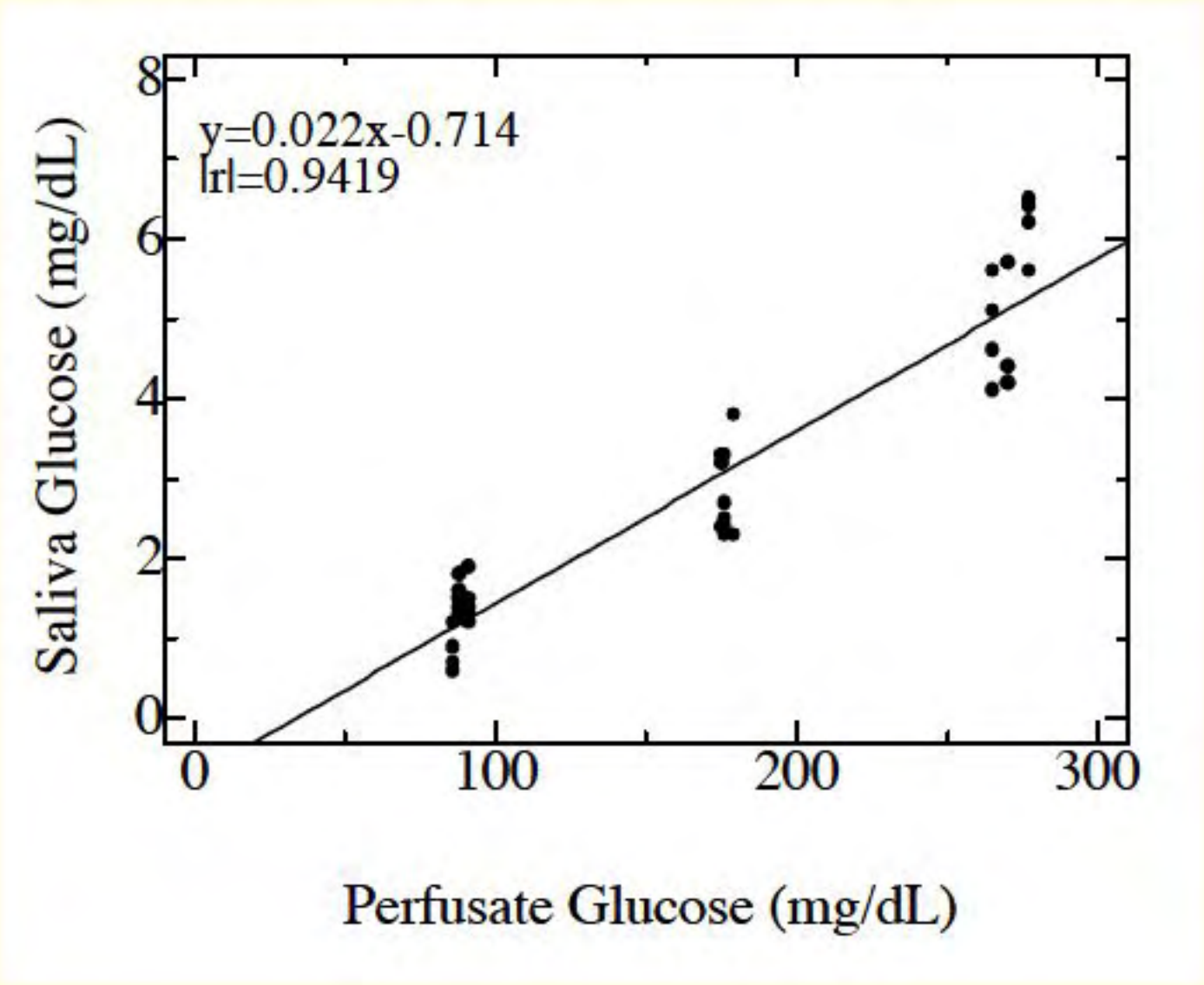
diabetus rats (b),

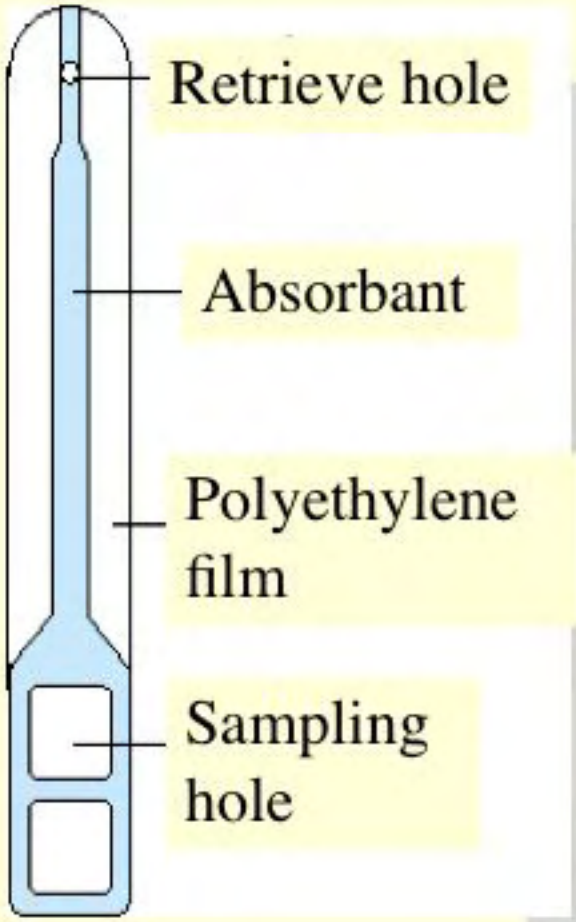
rabbits (c).

Reciprocal relationship between Glucose in saliva and flow rate



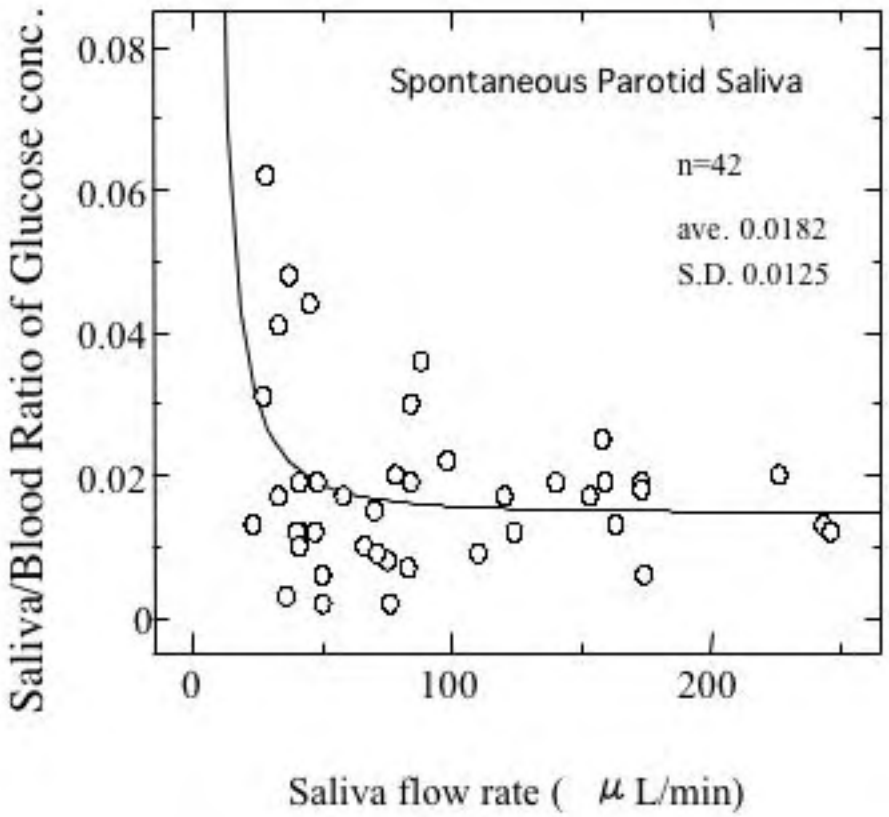
Vascularly perfused rat submandibular gland

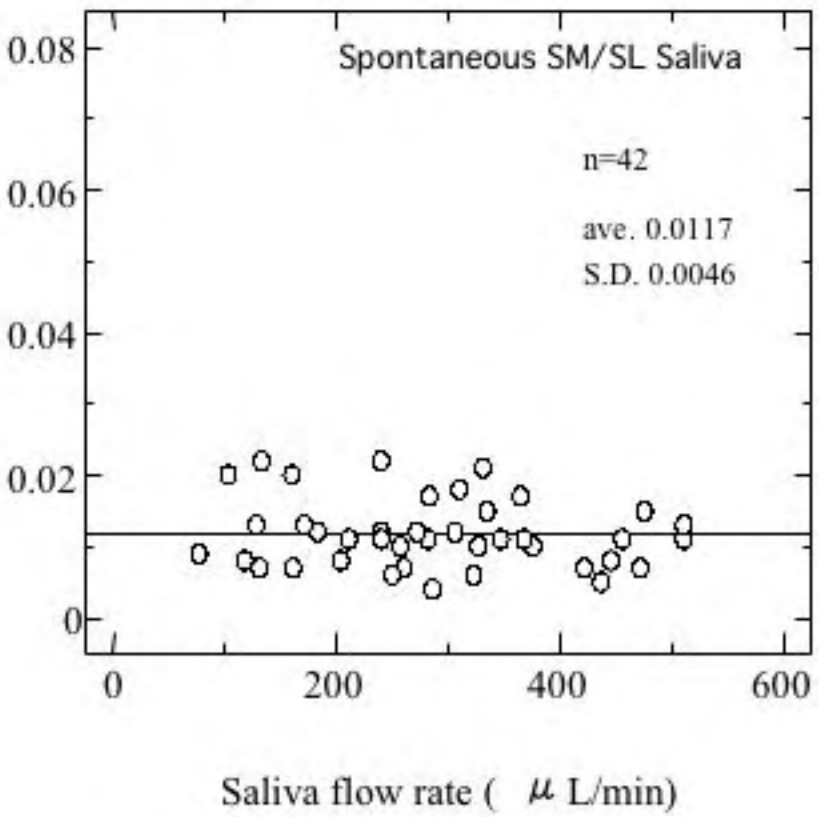



Retrieve hole
Absorbant
Polyethylene film
Sampling hole

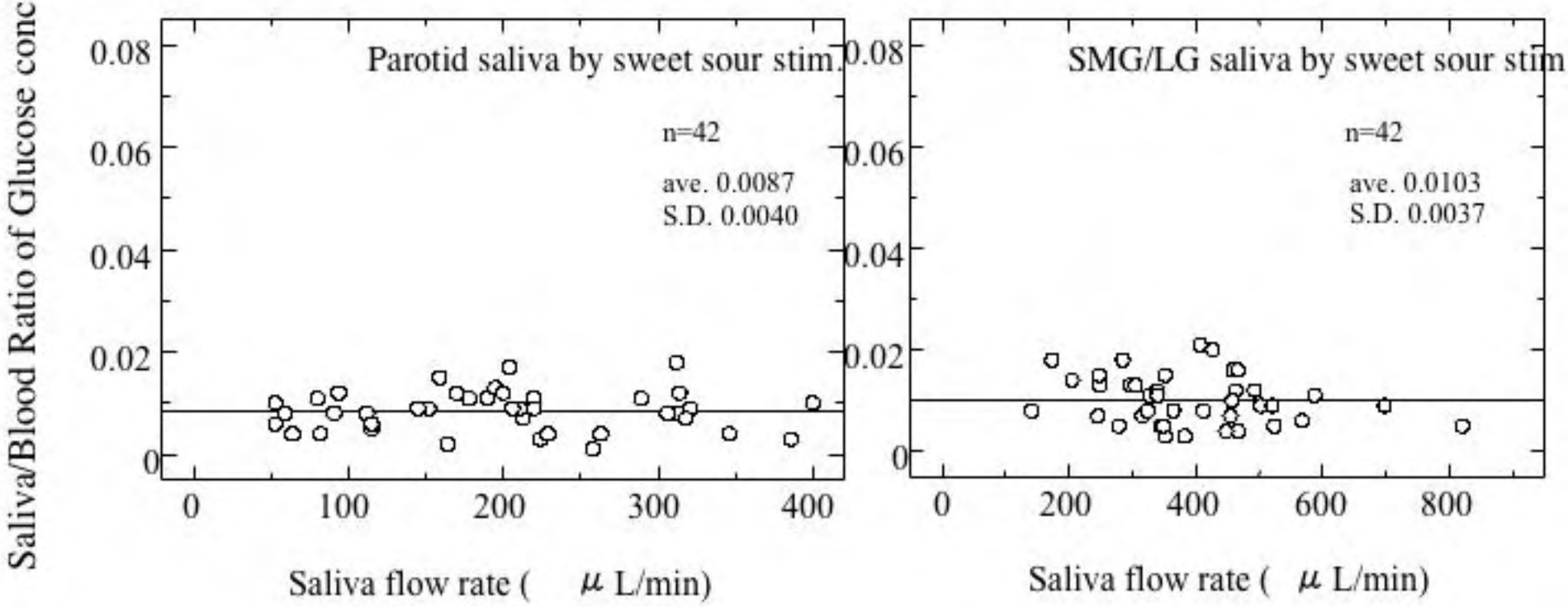

2
KING SIZE
hi-lite
ULTRA MILD
ULTRA LOW TAR & NICOTINE

Human saliva (spontaneous secretion)





Human saliva (stimulated by a sweet sour tablet)



Can the salivary glucose monitor blood glucose?

Yes. We can estimate blood glucose from salivary glucose if the salivary fluid secretion were enough high.

How to overcome the problem for in vivo application?

Measure Fluid Secretion !

Acknowledgement.

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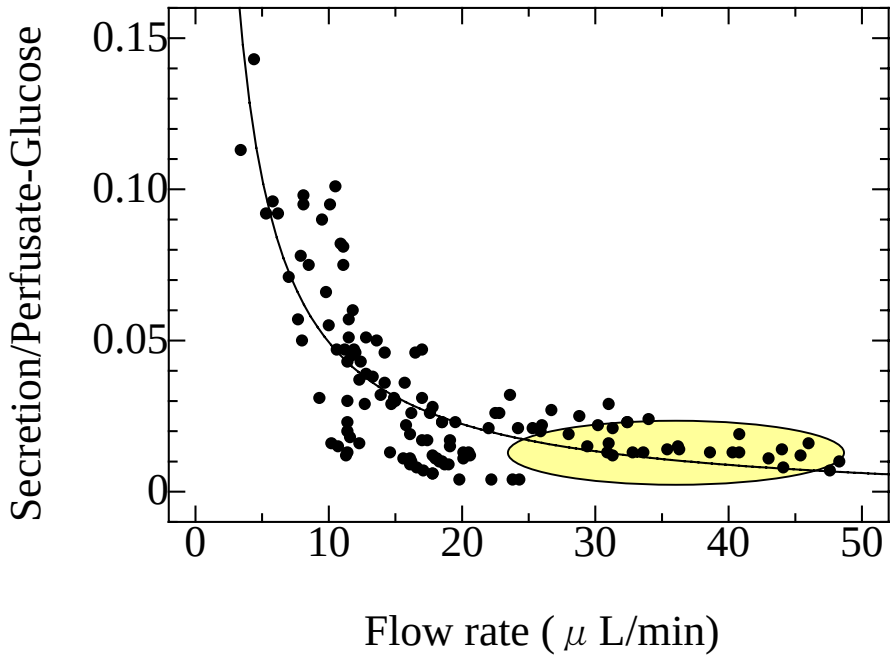
Abstract - Conclusion of Talk A1 -



We could use saliva glucose as an indicator of blood glucose when the saliva secretion rate is high enough.

Necessary Condition

- Sublingual/submandibular saliva
- Stimulation saliva

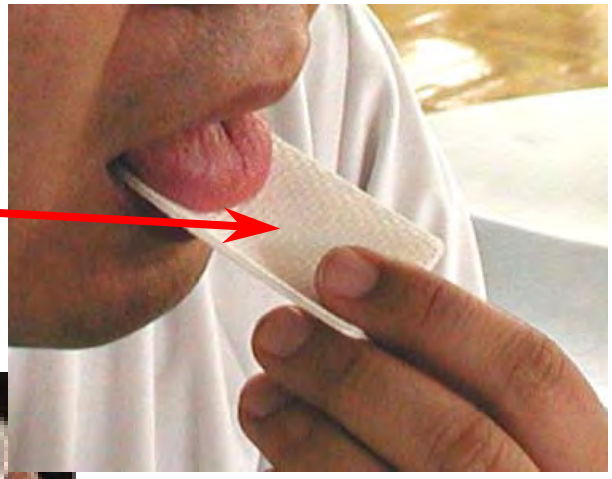
We developed a new Saliva Sampling Instrument and Biosensor, then studied relation between saliva glucose and blood glucose from result of OGTT of 16 adult volunteers.

Saliva Sampling Instrument

- **Wash Liquid**
  Cetylpyridinium Chloride
- **Saliva Sampler**
  Hydrophilic Absorbent
  (Rayon)
- **Tablet (Sweet Taste)**
  Non-sugar
- **Wiper**
  (Nonwoven Fabric)
- **Tweezers**

Saliva Sampling Method

Weight before sampling



Wiper

Under sublingual

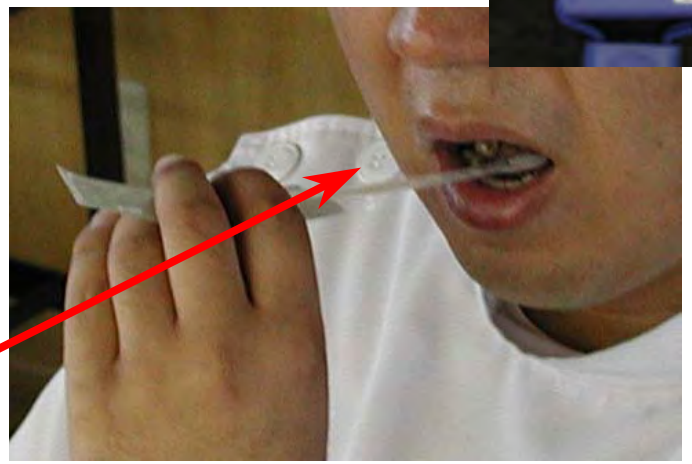
Wash Liquid

Mouth wash

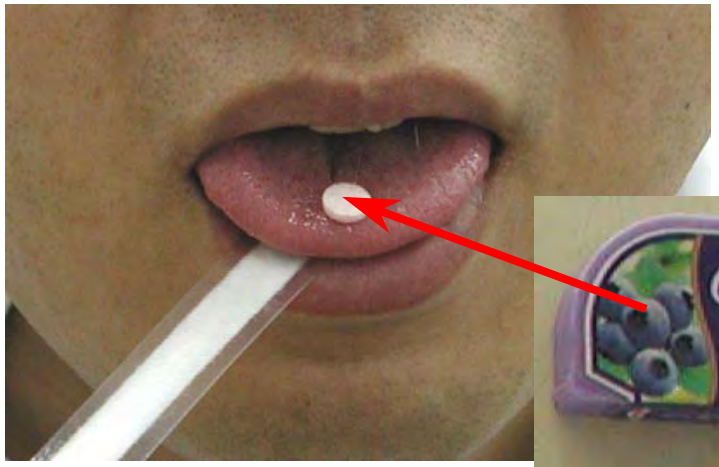
Open

Saliva Sampler

Timer start

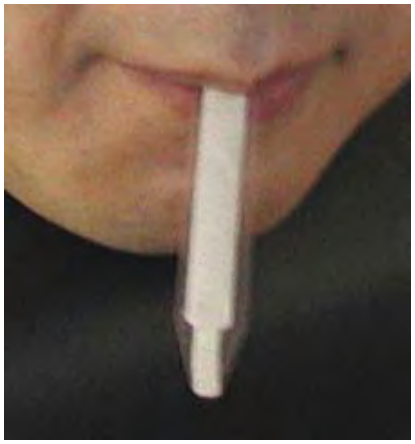


Under sublingual



Stimulant

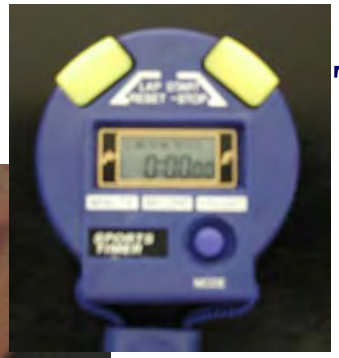
Tip of tongue



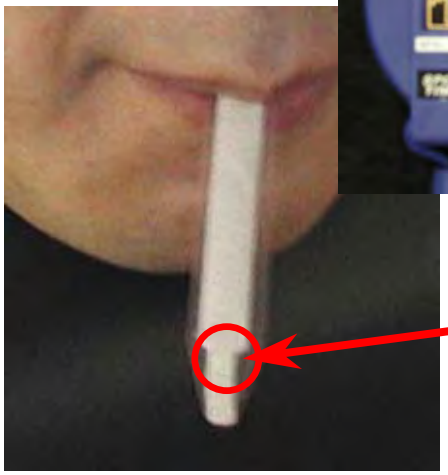
Wait

Saliva secretion rate (μL / sec) =

(Weight after sampling - Weight before sampling) / Sampling time



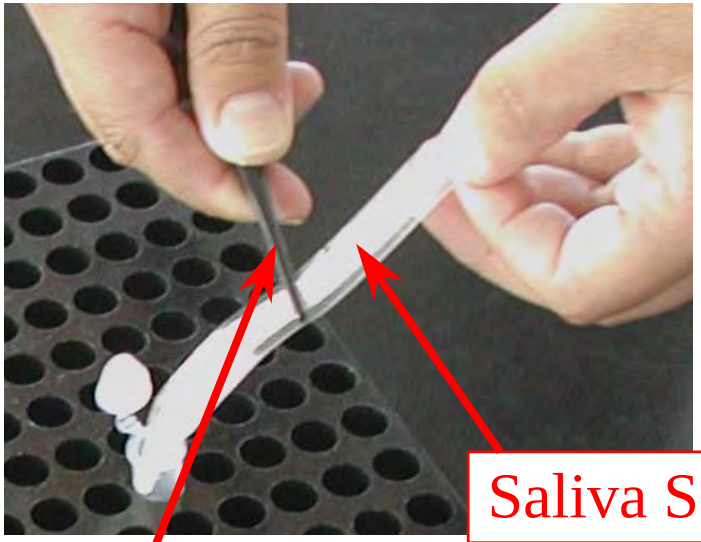
Timer stop



Green

Take out

Weight after sampling



Saliva Sampler

Tweezers

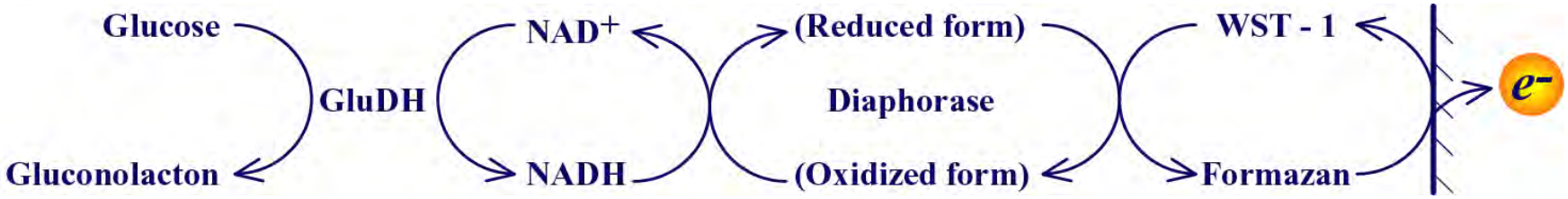
Squeeze

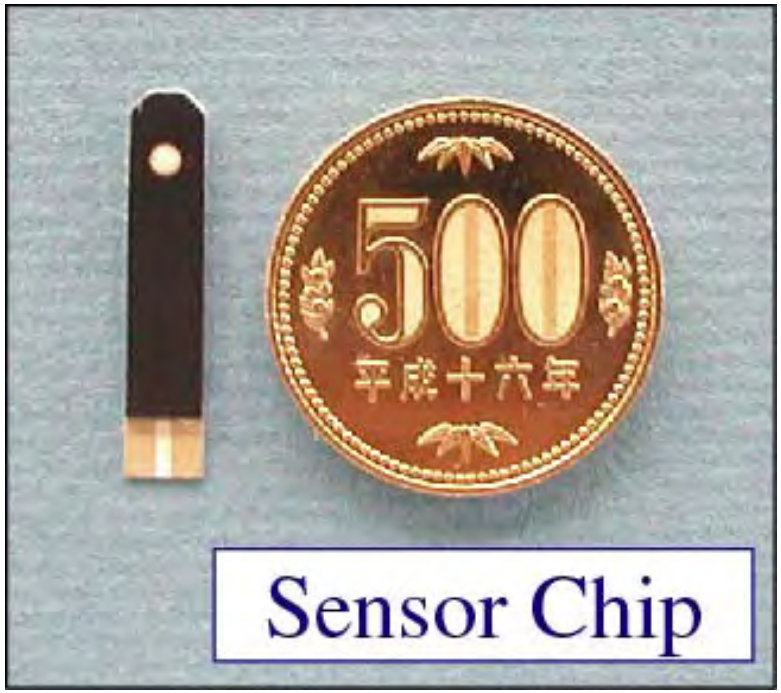
Saliva

About 200-300μL

Saliva Glucose Measurement

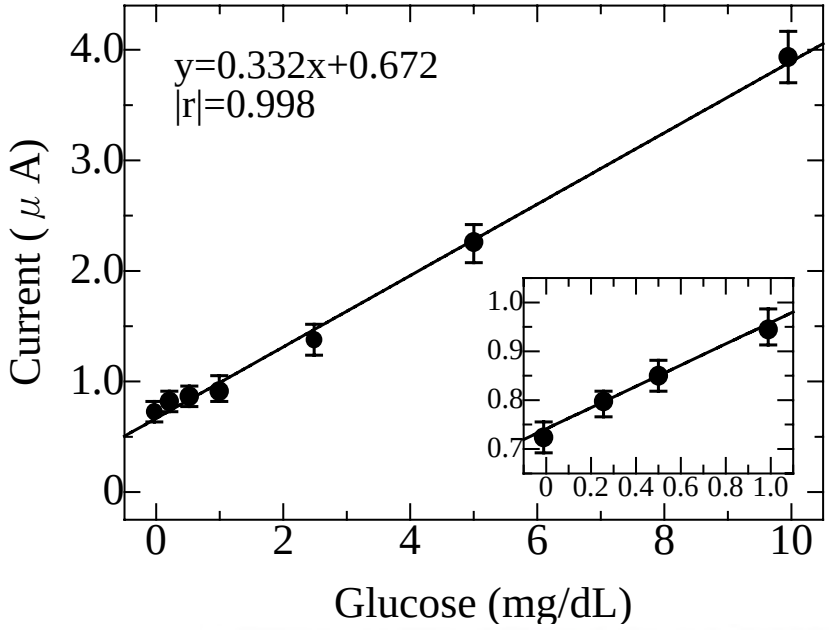
Patent No.:WO00/57166 Allowance:JP,US,EP,AU





Saliva Glucose Sensor Response

Electrode area : 12.5mm^2



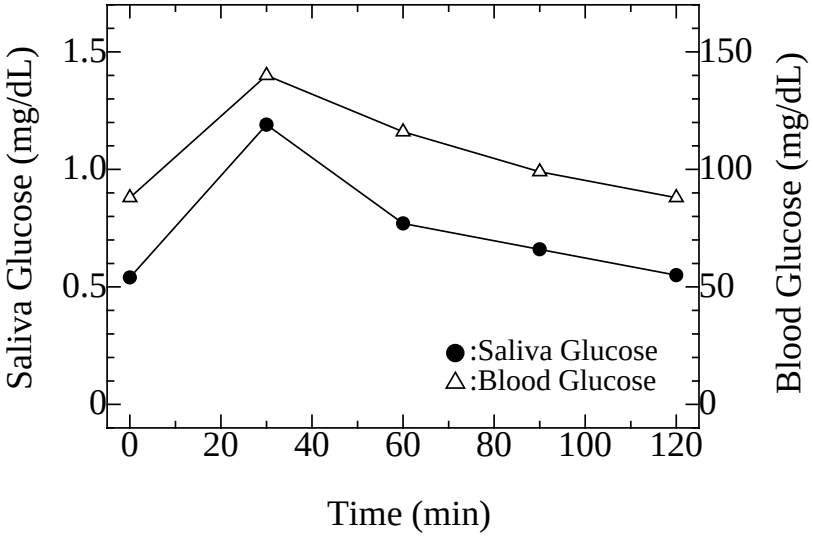
- **Minimum : 0.25mg/dL**
- **Maximum : 10mg/dL**
- **CV : Below 5%**

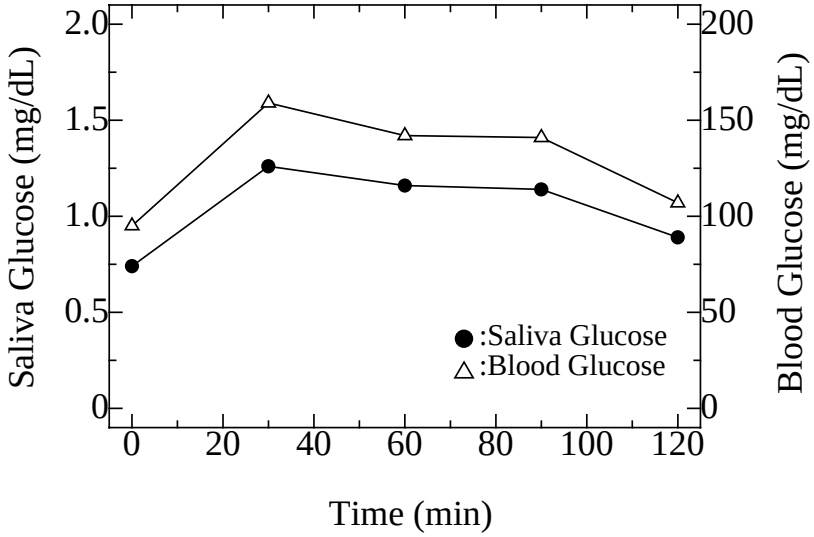
As a result presented above, it could confirm that the saliva glucose is able to be measured with the biosensor.

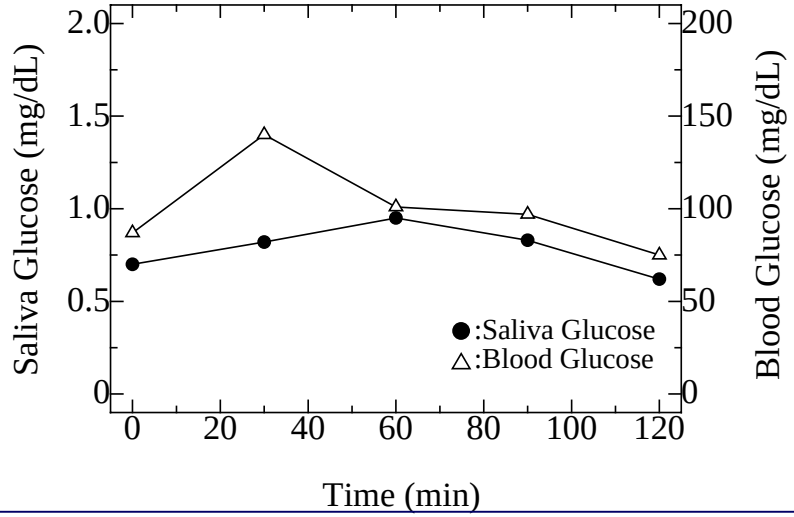
Glucose (mg/dL)	0	0.25	0.5	1	2.5	5	10
Ave. (μA)	0.737	0.808	0.855	0.953	1.357	2.256	3.937
SD	0.026	0.011	0.018	0.021	0.047	0.057	0.097
CV (%)	3.5	1.3	2.1	2.2	3.5	2.5	2.5

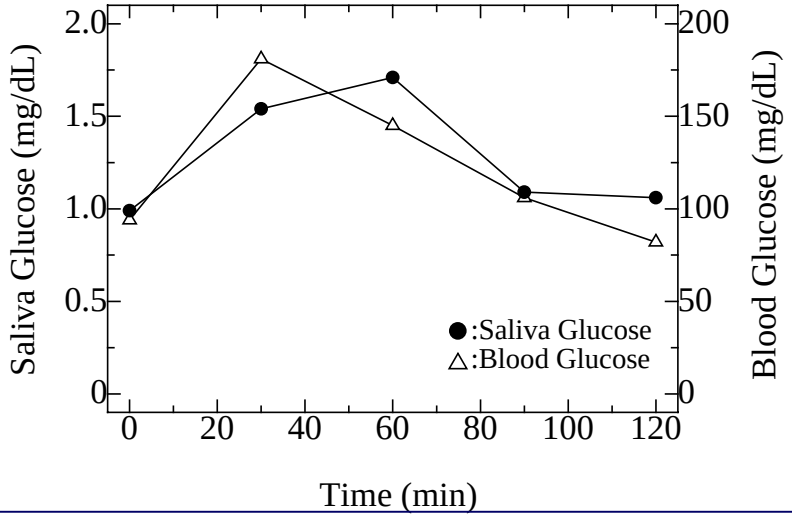
n = 10

75g OGTT
(Oral Glucose Tolerance Test)







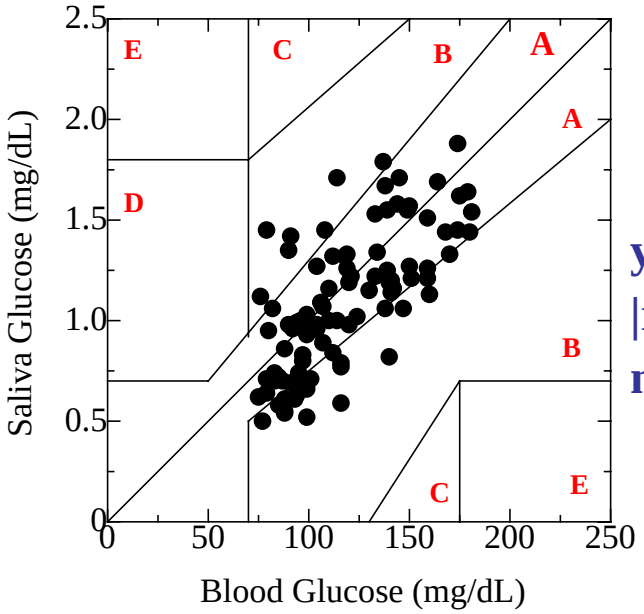


As the results of OGTT, the changes of saliva glucose and blood glucose levels occurred at the same time, or the change of saliva glucose levels was late for that of blood glucose levels.

Saliva is made form blood in salivary glands and secreted in the mouth through the duct, which probably causes that the change of saliva glucose levels occurs slower than that of blood glucose levels.

Saliva Glucose vs. Blood Glucose (Error Grid Analysis)

Saliva Glucose / Blood Glucose = 0.01



y=0.0083x+0.1154
|r|=0.7065
n=95

Correlation coefficient between saliva glucose and blood glucose was 0.7065. However, all point entered A and B zones, that is clinically acceptable for error grid analysis for clinical accuracy evaluating of blood glucose monitoring system.

Diabetes Care 10, 622-28, 1987

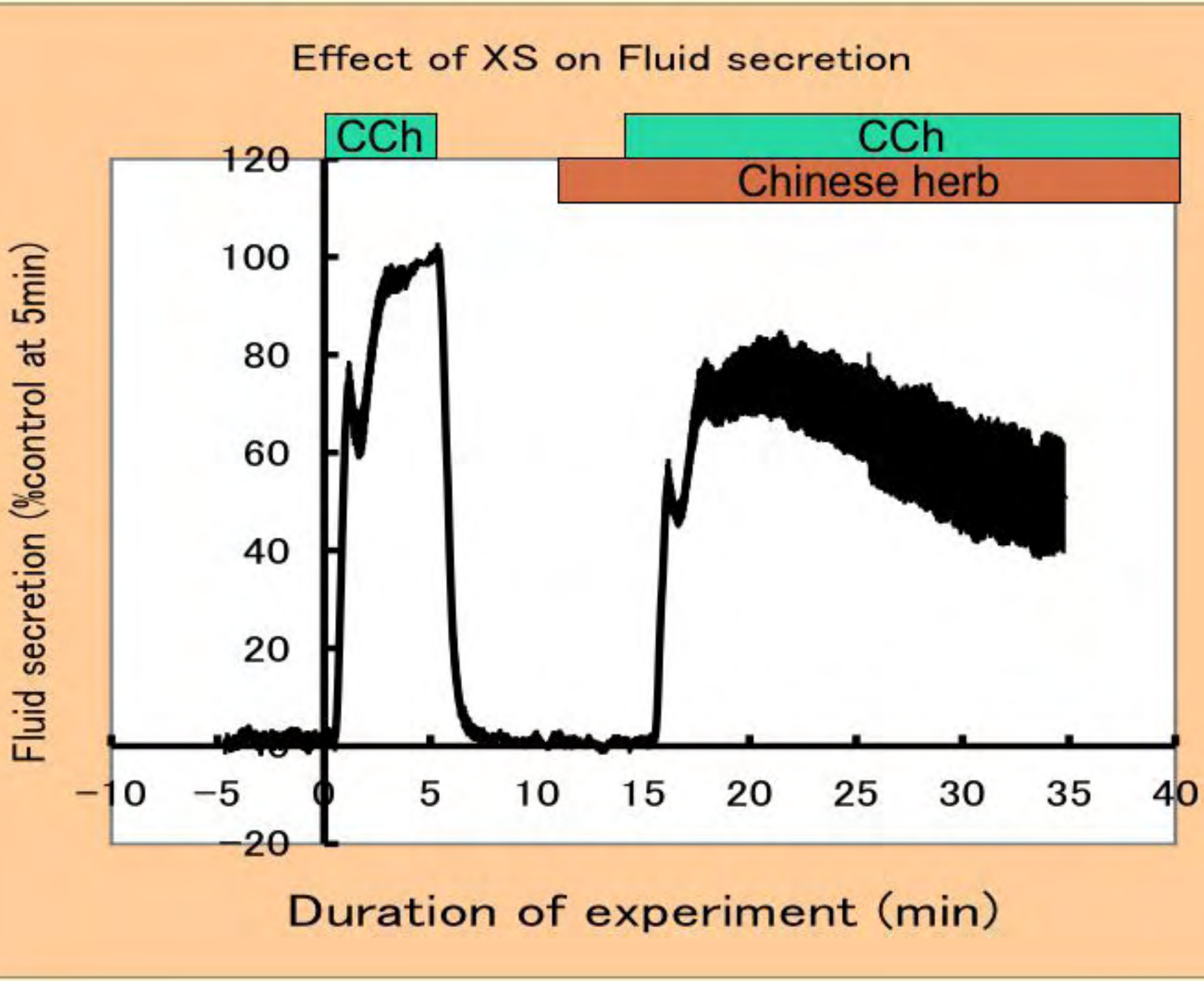
Area	Point	Average	Error Grid Analysis
A	71	75%	Clinically accurate, would lead to correct treatment decisions
B	24	25%	Would lead to benign decision or no treatment
C	0	0	Would lead to overcorrection of normal glucose levels
D	0	0	Would lead to failure to detect and treat high or low glucose levels
E	0	0	Would lead to erroneous treatment decisions

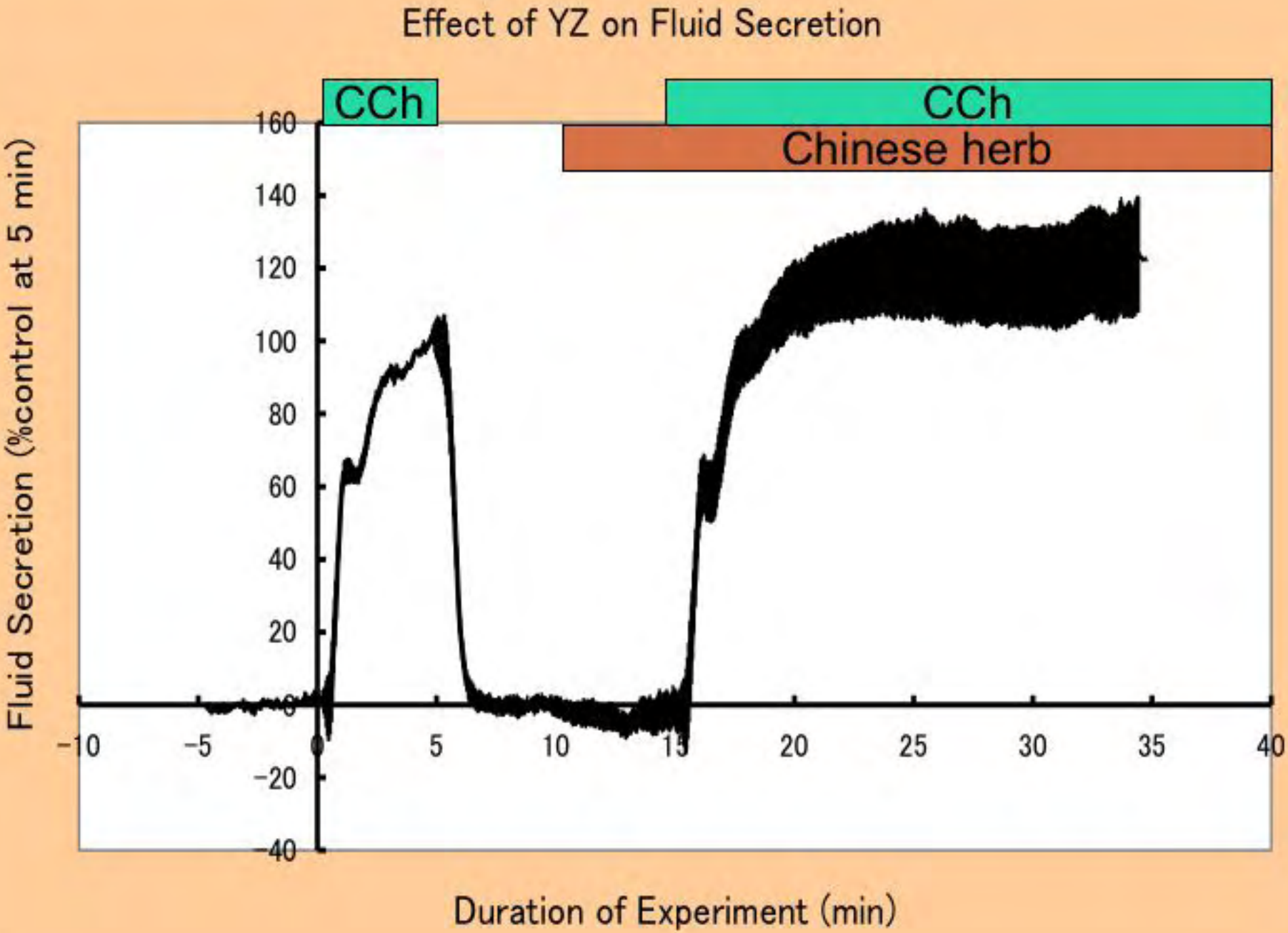
Conclusion

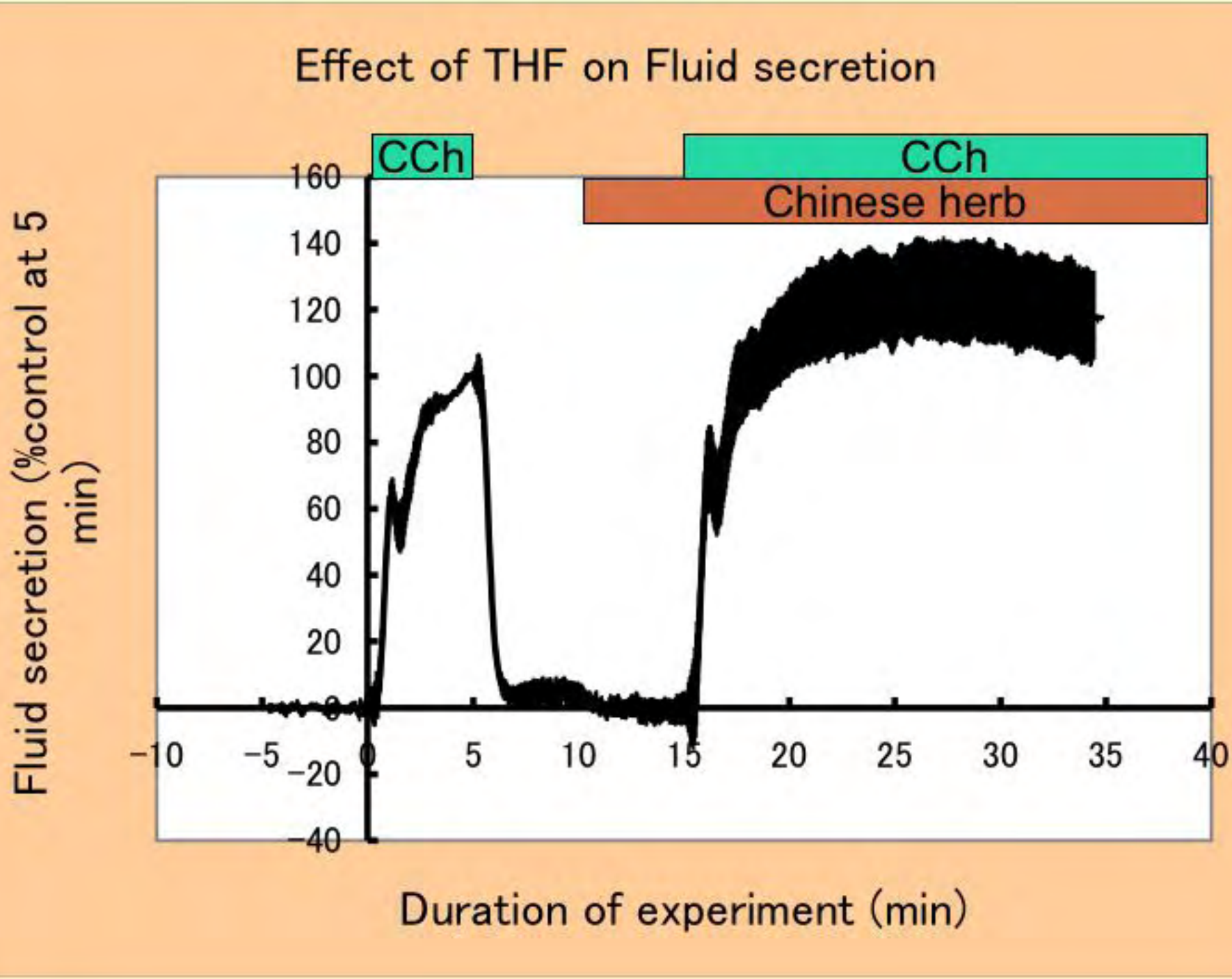
The saliva glucose measurement is difficult to be used as SMBG (Self Monitoring Blood Glucose), because the change of saliva glucose by OGTT is not corresponding to that of blood glucose.

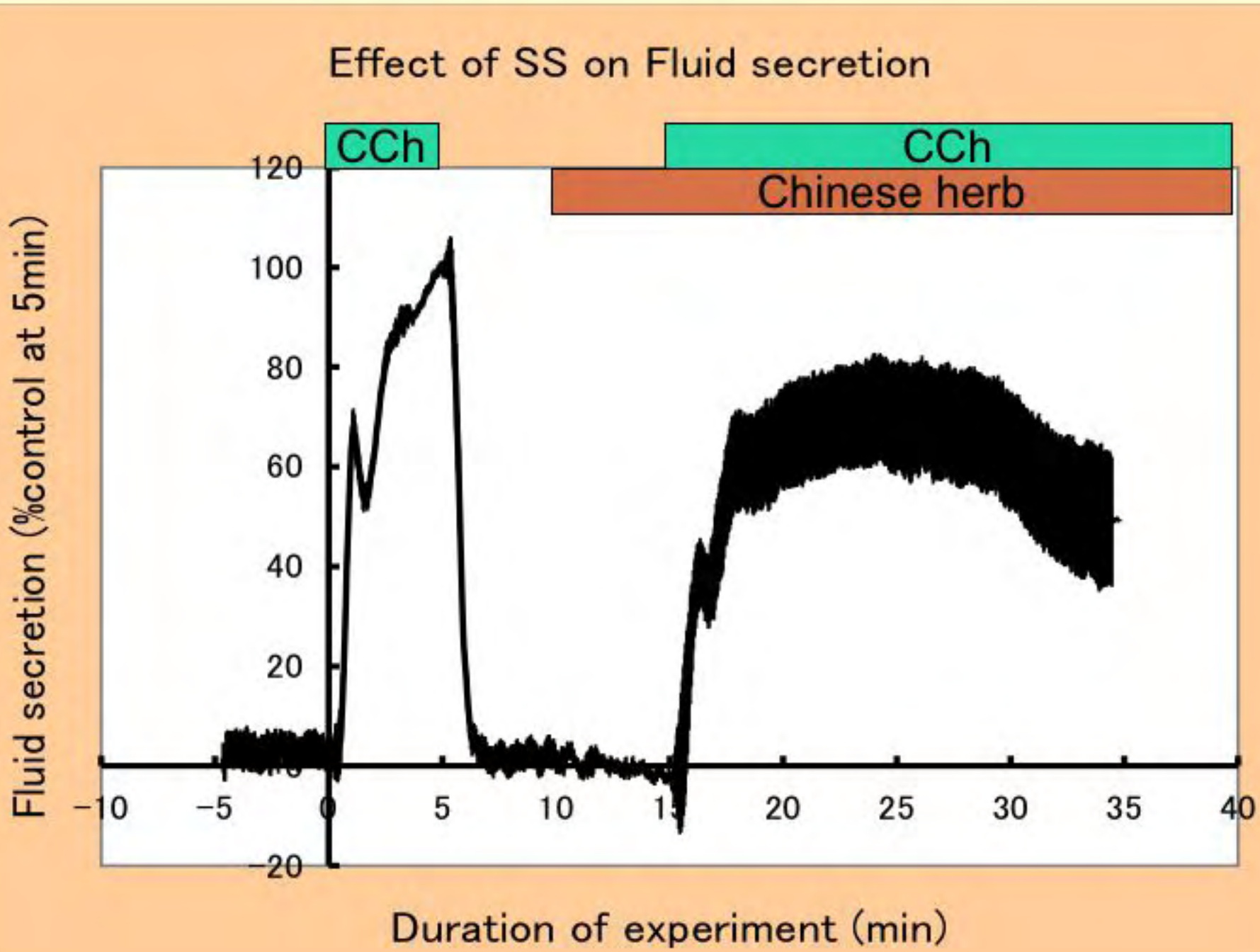
However, it is likely that the saliva glucose measurement before meal is useful for health care, as a result of error grid analysis suggested.

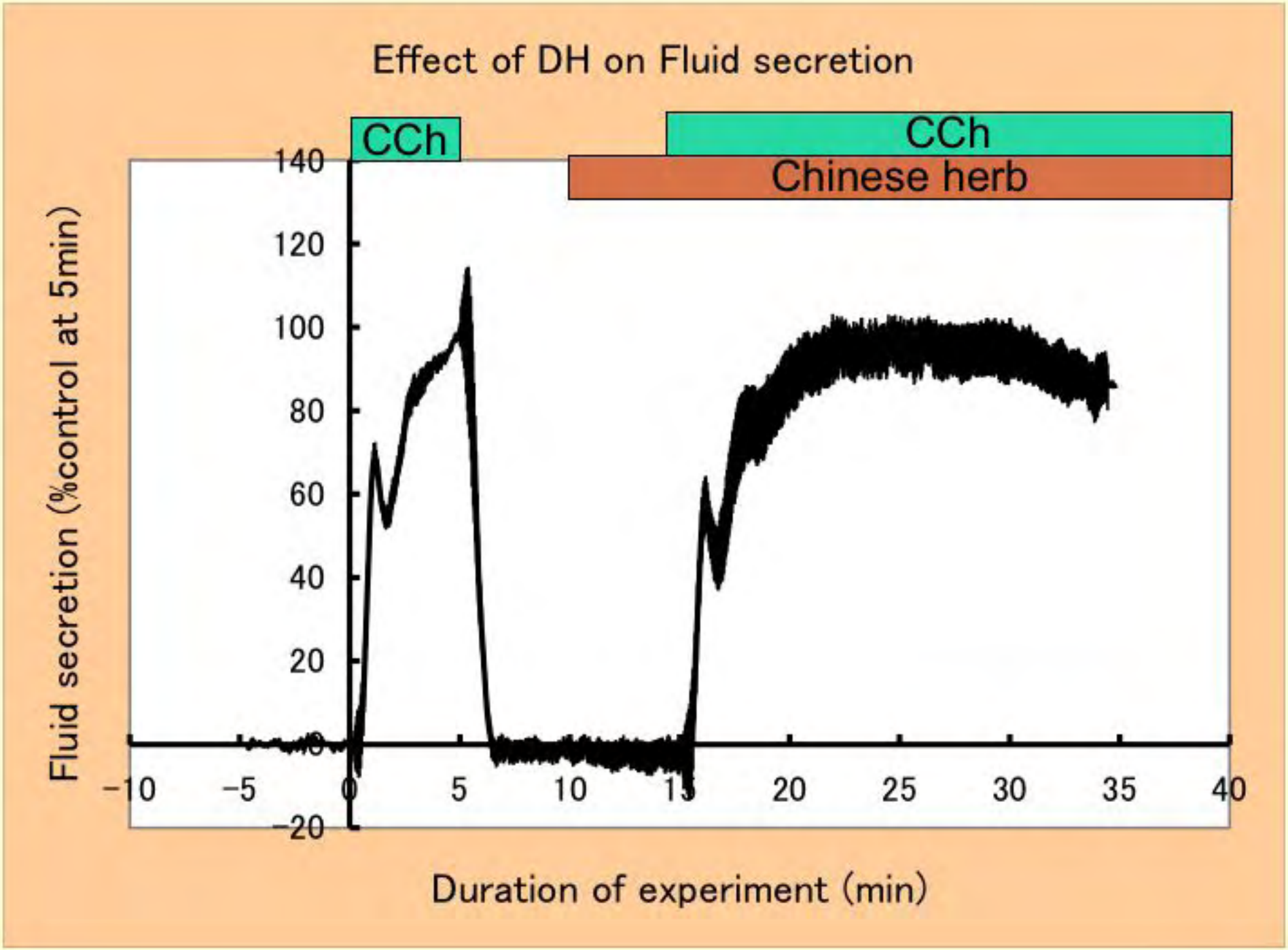
By modification of the saliva sampling method, the saliva glucose measurement will be able to replace the SMBG in the future.

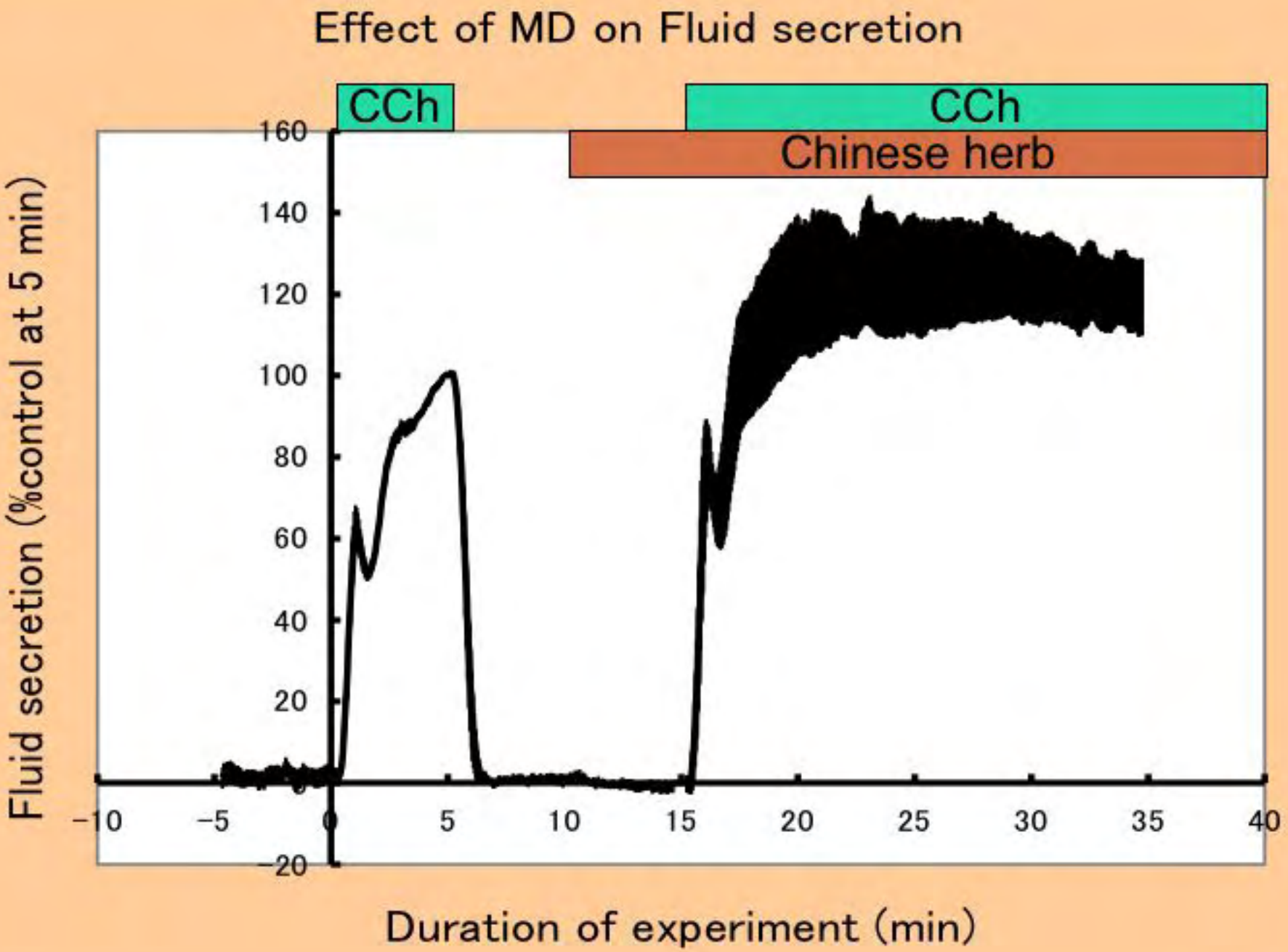

Effect of XS on Fluid secretion
CCh
CCh
Chinese herb
120
100
80
60
40
20
−20
−10
−5
0
5
10
15
20
25
30
35
40
Fluid secretion (%control at 5min)
Duration of experiment (min)

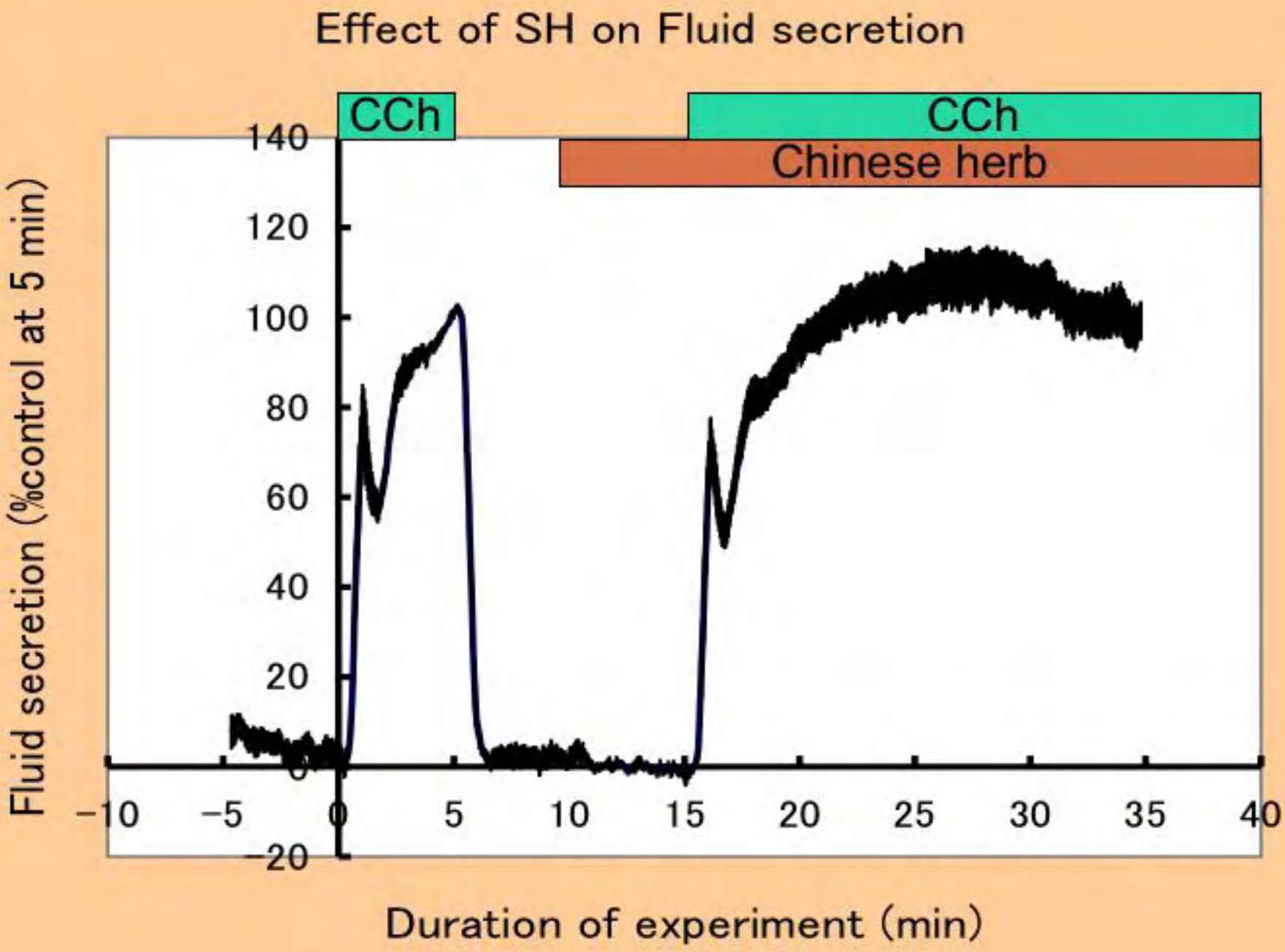

Effect of YZ on Fluid Secretion
CCh
CCh
Chinese herb
Fluid Secretion (%control at 5 min)
160
140
120
100
80
60
40
20
0
-20
-40
-10
-5
0
5
10
15
20
25
30
35
40
Duration of Experiment (min)

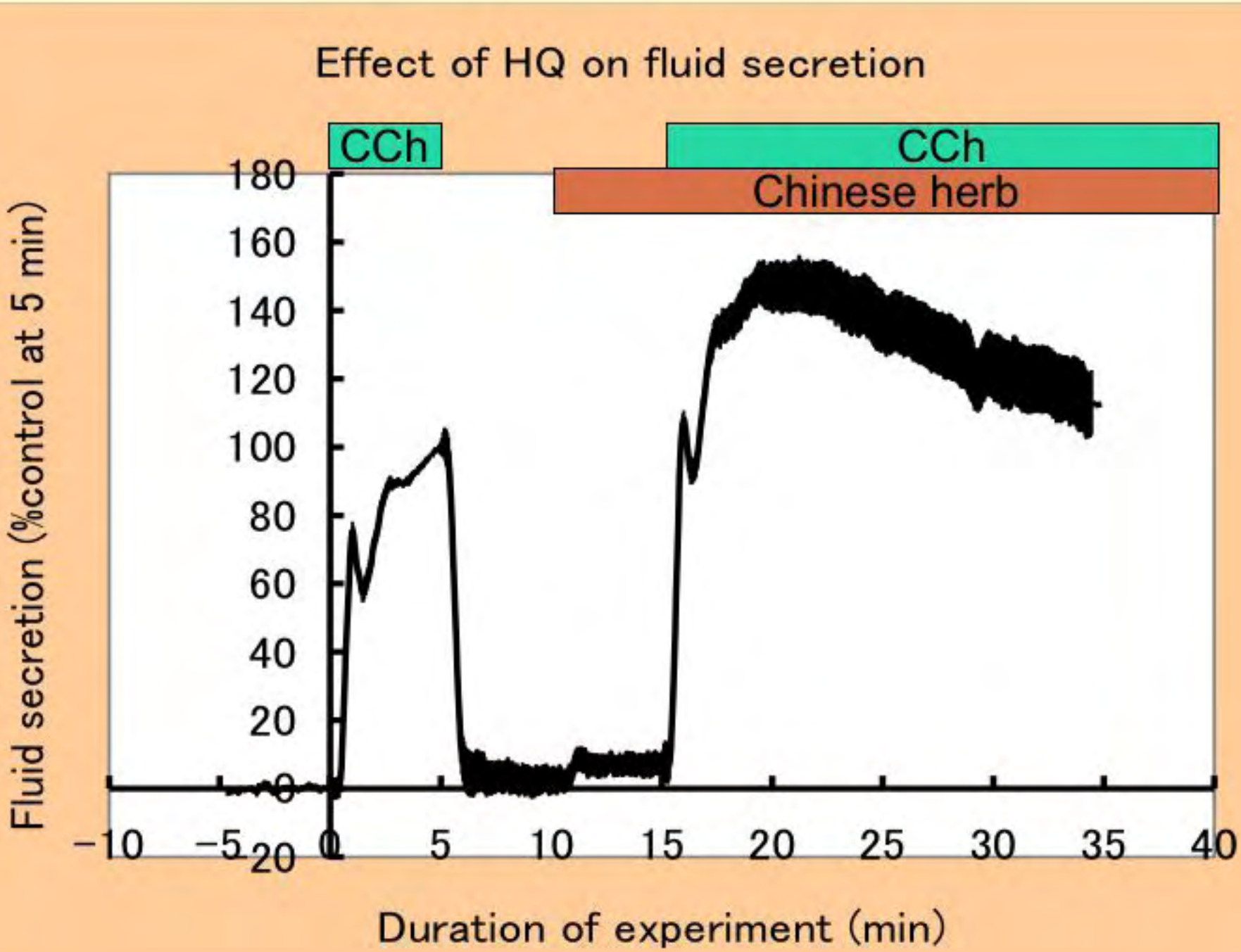

Effect of THF on Fluid secretion
CCh
CCh
Chinese herb
160
140
120
100
80
60
40
20
0
-20
-40
-10
-5
0
5
10
15
20
25
30
35
40
Fluid secretion (%control at 5 min)
Duration of experiment (min)

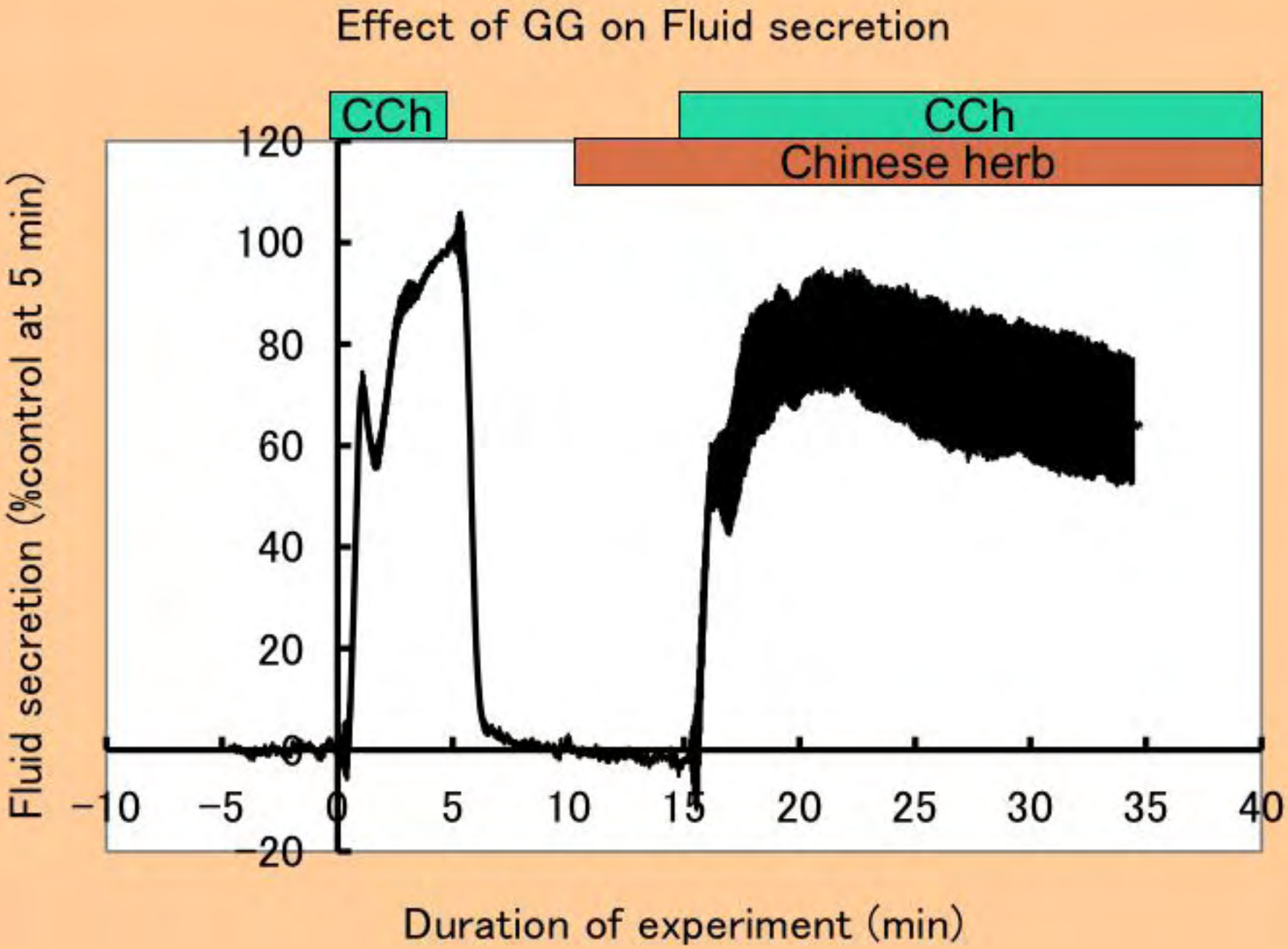

Effect of SS on Fluid secretion
CCh
CCh
Chinese herb
120
100
80
60
40
20
−20
−10
−5
0
5
10
15
20
25
30
35
40
Fluid secretion (%control at 5min)
Duration of experiment (min)


Effect of DH on Fluid secretion
CCh
CCh
Chinese herb
Fluid secretion (%control at 5min)
140
120
100
80
60
40
20
0
-20
-10
-5
0
5
10
15
20
25
30
35
40
Duration of experiment (min)


Effect of MD on Fluid secretion
CCh
CCh
Chinese herb
160
140
120
100
80
60
40
20
−20
−10
−5
0
5
10
15
20
25
30
35
40
Fluid secretion (%control at 5 min)
Duration of experiment (min)

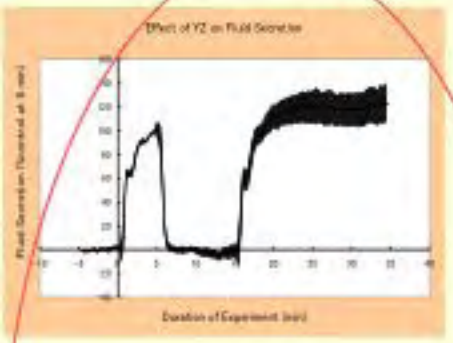

Effect of SH on Fluid secretion
CCh
CCh
Chinese herb
Fluid secretion (%control at 5 min)
140
120
100
80
60
40
20
0
-20
-10
-5
0
5
10
15
20
25
30
35
40
Duration of experiment (min)

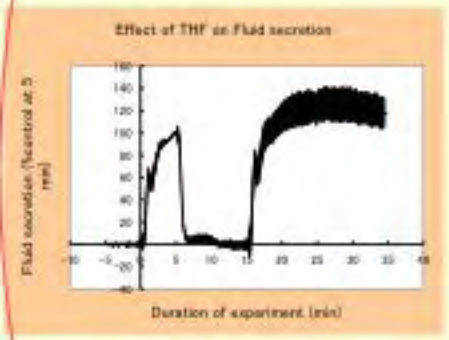

Effect of HQ on fluid secretion
CCh
CCh
Chinese herb
Fluid secretion (%control at 5 min)
180
160
140
120
100
80
60
40
20
0
-20
-10
-5
0
5
10
15
20
25
30
35
40
Duration of experiment (min)

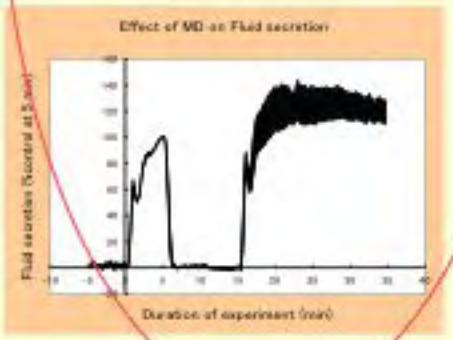

Effect of GG on Fluid secretion
CCh
CCh
Chinese herb
120
100
80
60
40
20
0
-20
-10
-5
0
5
10
15
20
25
30
35
40
Fluid secretion (%control at 5 min)
Duration of experiment (min)

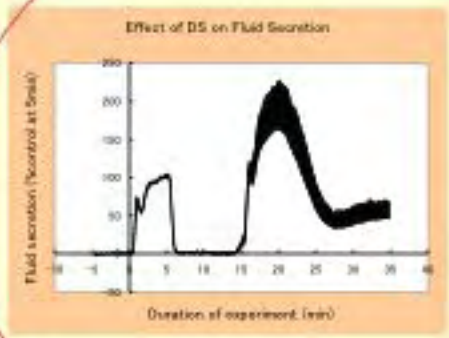
Blood-activating Herb

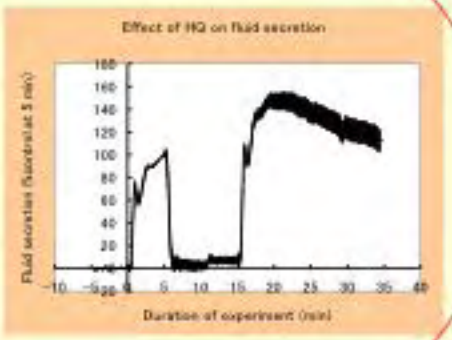
Qi-enhancing Herb



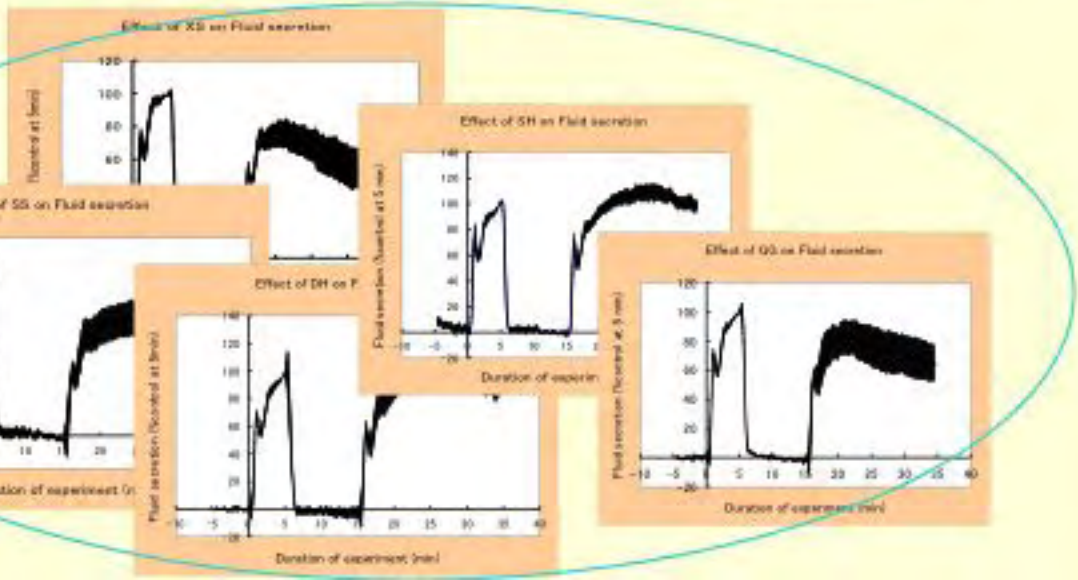


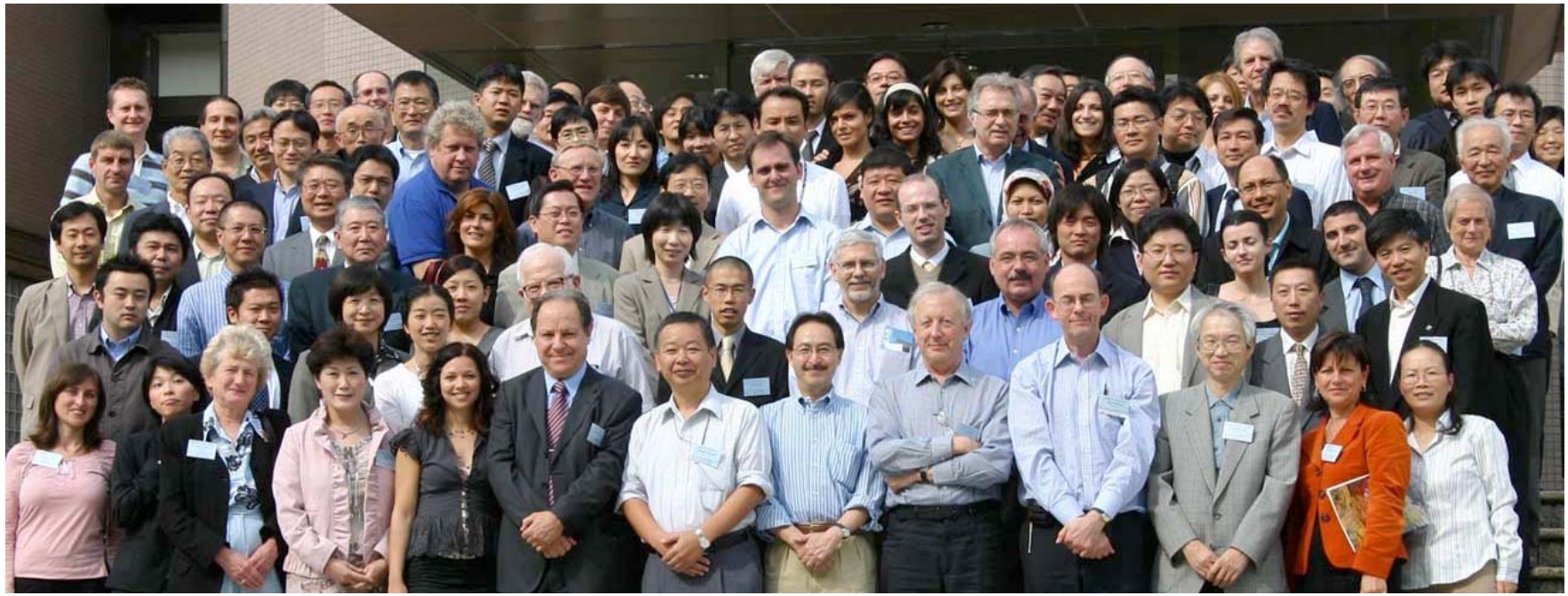






Yin-nourishing Herbs





Change of Claudin Expression in Primary Cultured Parotid Acinar Cells

Junko Fujita-Yoshigaki, Bing Qi, Hiroshi Sugiya

Department of Physiology, Nihon University School of Dentistry at Matsudo

Tight junctions are essential structures for epithelial cells to keep their polarity. We have previously established a system for primary culture of parotid acinar cells that retained abilities to generate new secretory granules and to secrete proteins signal-dependently (1). Because cell polarity and cell-cell adhesion are prerequisites for epithelial tissues, we investigated structures of tight junctions in the culture.

We found that the cells formed two types of cellular organization: monolayers and semispherical clumps. Tight junctions were observed at the apical part of lateral membrane between cells in the monolayers and cells at the surface of the clumps. The cells inside the clumps surrounding lumens also had tight junctions. The cells inside the clumps kept secretory granules better than the other two types of cell, suggesting that they retained the original character as acinar cells. Although claudin-4 was not detected in the cells just after the isolation from the glands, it began to be expressed and its expression level increased during culture. Immunofluorescence microscopy showed that claudin-4 was expressed in the monolayers and at the surface of the clumps, but not inside the clumps. Only claudin-3, which is expressed in the original acinar cells just after the isolation and in the intact gland, was detected inside the clumps. These results suggest that the difference in claudin expression is related to the three-dimensional structures of cell cultures and the functions of acinar cells.

To identify the intracellular signaling to induce claudin-4 expression, we examined the effects of Src kinase inhibitors on the primary culture. PP1 analog (4-amino-1-tert-butyl-3-(1'-napthyl) pyrazolo [3,4-d] pyrimidine) and PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine), and PP3 (4-amino-7-phenylpyrazol [3,4-d] pyrimidine), negative control of PP2, were added to

the medium during the culture. As a result, PP1 and PP2 suppressed the claudin-4 expression, whereas PP3 had no effect. PP1 and PP2 also suppressed the reduction of amylase activity, in turn, the relative amylase activity in the lysate of the cells cultured in the presence of PP1 was 3-fold higher than that of control after 3 days in culture.. There was a negative correlation between the expression of claudin-4 and amylase. There is a possibility that inhibition of cellular signaling to induce claudin-4 expression lead to prolongation of lifespan of the culture.

1. Fujita-Yoshigaki, J. *et al. Tissue Cell Res.* **320:** 455-464 (2005)