ORIGINAL

Opened and Closed States of Tight Junctions by Secretory Stimuli in Rat Submandibular Acini

Yuichi Sakaguchi, Noriyasu Takai and Yo Yoshida

Department of Physiology, Osaka Dental University

(Chief : Prof. Yo Yoshida)

8-1, Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan

[Received on July 26, 1999 ; Accepted on November 25, 1999]

Key words : submandibular gland / tight junction / secretion / microperoxidase

Abstract : It is widely accepted that the salivary glands secrete primary saliva by osmosis at the acini, and that some of the salivary fluid flows through the tight junctions (paracellular secretory pathway). The aim of this study was to examine the changes in permeability of the acinar tight junction in rat submandibular glands *in vivo* by electrical stimulation of parasympathetic and sympathetic secretory nerves. The permeability changes of the tight junctions after the stimulation were also examined. The permeability of the tight junction from the interstitium to the lumen was examined by close-arterial infusion with a small ultra-structural tracer, microperoxidase (MW=1,630 Da), and this tracer was observed by electron microscopy. In the resting gland, the tracer was observed in the interstitial and intercellular spaces, but not within the lumen (closed tight junction). Intraductal injection of hypertonic sucrose (570—998 mOsm) caused a subsequent elevation of the luminal pressure, indicating that osmotic fluid had passed into the lumen. However, the tight junction in the gland remained in the closed state. In the chorda stimulated gland and the stimulated gland of the superior cervical ganglion (stimulated for 2 min), the tracer entered the lumen through all the tight junctions, that is, they became permeable to the tracer (the tight junction switched to an opened state). However, the opened tight junction reverted to the closed state after 15—30 min. These findings suggest that submandibular acinar tight junctions switch rapidly to an opened state by nerve stimulation and close again within 15—30 min after the stimulation, and that the paracellular secretory pathway may be involved in the salivary fluid secretion from the acini.

抄録 : 唾液腺の腺房部細胞で生成される原唾液の水はポンプやチャンネルによって作られた浸透圧によって基底側間質から腺腔に流れ込むことによって供給されるが、その際の分泌経路は腺房部細胞内を通るルート（経細胞分泌経路）と細胞間隙を通るルート（傍細胞分泌経路）の2つが想定されている。本研究は、傍細胞分泌経路におけるtight junction (TJ) の基底側間質から腺腔への物質透過バリアーとしての機能について、ラット顎下腺を用いて検討した。TJのバリアー機能はmicroperoxidase (50 μ l/min で顔面動脈の顎下腺枝から投与) をトレーサーとして用いて電顕的に調べた。安静時には、TJはトレーサーの基底側間質から腺腔への透過を阻止しており、腺腔にはトレーサーはみられなかった。また、安静時の腺腔に高張ショ糖液を導管内から逆行性に注入すると、腺腔への水の流入を生じたが、この際にもやはりTJは、トレーサーの腺腔への透過を阻止していた。鼓索神経または上顎神経節のいずれの電気刺激(2分間)でも、トレーサーは細胞間隙からTJを越えて腺腔にみられるようになった。すなわち、副交感性および交感性分泌刺激によって、TJはトレーサーを透過させるようになった。さらに、刺激停止15分後のTJも透過性を示していたが、30分後のTJは安静時と同様に非透過性であった。以上の実験結果から、顎下腺腺房部細胞のTJは、安静時には閉じているが、副交感性および交感性分泌刺激によってこれが速やかに開いて傍細胞分泌経路が通じるようになることが明らかとなった。そして、分泌刺激によって開いたTJは、刺激停止後にはすぐには閉じるわけではなく、再び閉じるのは15—30分後であった。

Introduction

In all secretory epithelia, including the salivary gland, fluid transport is thought to be driven osmotically in response to the transepithelial gradient of electrolytes, principally Na^+ and Cl^- . This gradient is generated by the active transport system localized in the luminal or basolateral membranes of the epithelial cells. In the salivary gland, it is now accepted that primary salivary fluid is osmotically drawn into the acinar lumen through the basolateral and luminal membranes (transcellular secretory pathway) or through the intercellular junctional complexes (paracellular secretory pathway), or a combination of the two routes^{1,2}. In the paracellular secretory pathway, the tight junction (*zonula occludens*) is the sole barrier function between the basolateral and luminal compartments, and selectively controls the passive diffusion of electrolytes and small water-soluble solutes. There is considerable evidence in different epithelial tissues that the tight junction, once regarded as rather static, is a dynamic structure that readily adapts to a variety of developmental, physiological and pathological conditions^{3,4}. Although our previous study showed that electrical stimulation of the chorda (parasympathetic stimulation) caused an increase in the permeability of the acinar tight junctions of the rat submandibular gland⁵, there is no information on the states of tight junctions after the stimulation.

As there is no direct method of determining the fluid flow through the tight junction in salivary acini *in vivo*, we used an indirect approach employing an ultrastructural tracer to histochemically clarify the route and the states of the tight junction by electron microscopy. Horseradish peroxidase has been used as a tracer in this type of experiment in salivary glands⁵⁻⁷, but more recently, this enzyme has been found to have a toxic effect on the cell membrane and tight junction⁸. Microperoxidase is considered to be a more suitable tracer than horseradish peroxidase, as it is very small (1,630 Da) and does not exhibit any toxic effects^{9,10}. Additionally, the molecular size of this tracer is small enough (0.2 nm)¹¹ to pass through the secretory water pathway in the acini. Studies to

determine solvent drag have shown that an effective pore radius for the water pathway is 0.4–0.45 nm^{12,13}. These studies suggest that the passage for microperoxidase in the salivary gland is *via* a water transport pathway. Therefore, we tested the permeability of the tight junction for water and small molecules from the interstitium to the lumen using microperoxidase as a tracer. In the present study, we assessed the effect of parasympathetic or sympathetic stimulation on the permeability of the acinar tight junctions, and the states of tight junctions after the stimulation.

Materials and Methods

1. Measurements of luminal pressure and stimulation of the gland

Male Wistar rats (200–250 g) were fasted for 12 hrs before the experiments, but were given free access to water, and were subsequently anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Additional doses of the anesthetic were given through a femoral venous cannula when required. A tracheal cannula was inserted, and the body temperature was maintained at 37°C with the aid of a heating pad.

A 30-mm length of polyethylene tubing (I. D.=0.28 mm, O. D.=0.61 mm : Intramedic PE-10, Becton & Dickinson, USA) was inserted into the oral opening of the submandibular duct and fixed in place with an adhesive.

For the secretory stimulation of parasympathetic and sympathetic nerves, the chorda on the main submandibular duct, and the superior cervical ganglion were electrically stimulated continuously for 2 min with 3.0–5.0 V pulses at 20 Hz of 5.0 ms duration using a bipolar electrode. The luminal pressure was measured 2 min after the stimulation by a pressure transducer connected to the tubing.

In order to produce an osmotic driving force for water influx to the acinar lumen, the glands were retrogradely injected with 30 μl of 570, 713 and 998 mOsm sucrose solution as uniformly as possible using a microsyringe attached between the tubing, with a pressure transducer connected between the syringe

Fig. 1 Diagram of equipment used to measure the luminal pressure in the submandibular gland. Three way-cocks are for removal of air bubbles, pressure adjustment to zero, and calibration.

and the tubing (Fig. 1). The control gland was injected with 285 mOsm of isotonic sucrose. The luminal pressure was measured 2 min after the intraductal injection. Previously, we reported that an intraductal injection of 30 μ l of 0.9 % saline caused no functional or histological damage to the rat submandibular gland¹⁴, and that 30 μ l of solution can easily reach the acinar lumen¹⁵.

The data were presented as means $\pm$ SE, and Student's t-test was used for statistical analysis; a *p*-value less than 0.05 was considered significant.

2. Histochemical detection of microperoxidase

Three or 5 heparinized (500 IU/kg) rats were used in each series. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the facial artery was retrogradely cannulated with the cannula tip positioned within 1 mm of the glandular branch junction. Microperoxidase (MP-9, Sigma, USA) was dissolved in 0.1 M phosphate-buffered saline (pH 7.4) at a concentration of 100 mg/ml, and infused through the arterial cannula using a syringe pump (Harvard, USA) at 50 μ l/min for 2 min, as described by Takai *et al.*¹⁶ In the nerve stimulated or the gland undergoing intraductal injection, the tracer infusion was simultaneous with the stimulation or intraductal injection. In some cases, the tracer was infused 15 or 30 min after the stimulation.

The rat was then perfused through the aorta with ice-cold normal saline containing heparin (0.5 IU/ml), followed by 500 ml of ice-cold fixative containing 2.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The glands were then dissected and postfixated in the same fixative for 12–24 hrs at 4°C. For the demonstration of microperoxidase, the tissues were sliced into 75 μ m sections on a cryostat and incubated in a modified medium used by Simionescu *et al.*¹⁷ containing 3,3'-diaminobenzidine tetrahydrochloride (12.5 mg), imidazole (0.1 M), and H₂O₂ (0.5%, 0.1 ml) in 25 ml of 0.1 M tris buffer (pH 8.6) at room temperature for 20–30 min. After dehydration through a graded ethanol series, the sections were embedded in Quetol 635/ERL 4206 resin (Taab Lab, England), and then examined with a transmission electron microscope.

Results

1. Luminal pressure

In the resting gland, no spontaneous increase in intraductal pressure was found. Electrical stimulation of the chorda and superior cervical ganglion increased the luminal pressure to 85.6 ± 13.2 and 52.5 ± 20.4 mmHg, respectively (Fig. 2).

Although the intraductal injection of isotonic sucrose caused slight (less than 5 mmHg) or no subsequent rise in intraductal pressure, injection of the

Fig. 2 Luminal pressure changes caused by parasympathetic or sympathetic stimulation, and by intraductal injection of isotonic or hypertonic sucrose.

* Significantly different ($p < 0.05$) from control (injection of isotonic sucrose). The data are presented as Mean + SE. The numbers in parentheses are numbers of samples.

Chorda : Electrical stimulation of chorda

SCG : Electrical stimulation of superior cervical ganglion

hypertonic sucrose solution caused a sustained elevation of pressure in an osmolarity-dependent manner (Fig. 2). The typical pattern of pressure change under each condition is shown in Figure 3.

2. Tracer localization

When the microperoxidase was administered through the glandular artery to the intact resting gland, the reaction products of the tracer were consistently observed in the interstitial and intercellular spaces, but not seen within the lumen in the tight junction region (Fig. 4-A). This indicated that the tight junction is impermeable to microperoxidase, and thus, in the resting gland, is closed to the tracer. Additionally, tight junctions of the resting gland, which was injected intraductally with hypertonic sucrose (998 mOsm), were closed (Fig. 4-B).

In the stimulated gland of the chorda, the reaction products were present in most of the intercellular spaces in acinar cells, including the lumen (Fig. 4-C). A similar distribution of the reaction product was seen when the superior cervical ganglion was stimulat-

Fig. 3 The typical pattern of luminal pressure changes caused by parasympathetic or sympathetic stimulation, and by intraductal injection of isotonic or hypertonic sucrose.

A. Electrical stimulation of chorda, B. Electrical stimulation of superior cervical ganglion, C. Intraductal injection of isotonic sucrose, D. Intraductal injection of 570 mOsm sucrose, E. Intraductal injection of 713 mOsm sucrose, F. Intraductal injection of 998 mOsm sucrose.

Initial steep rise in pressure in (C-F) was produced artificially by intraductal injection of solutions.

Horizontal bar : electrical stimulation for chorda in A or superior cervical ganglion in B.

▲ : Injection.

Fig. 4 Electron micrographs of adjacent acinar cells in the submandibular gland infused with microperoxidase. In the resting gland (A), the reaction product of the tracer is seen in the space between acinar cells, but not within the lumen (*) in the tight junction region (▲). Similar localization of the reaction product was observed in a gland intraductally injected with hypertonic sucrose (B). In the gland stimulated for 2 min by chorda (C) or by superior cervical ganglion (D), the reaction product is seen in the space between acinar cells, extending all the way into the lumen. In the gland 15 min after the stimulation of chorda (E), or superior cervical ganglion (F) similar localization of the reaction product was observed (Bar=1 μ m).

Table 1 The states of the acinar tight junctions demonstrated by microperoxidase

Experimental condition	State of tight junctions	
	Resting	Closed
Intraductal inject	Intraductal injection of	
	Isotonic (285 mOsm) sucrose	Closed
Tracer Infusion (2 min) ↑ Fix	570 mOsm sucrose	Closed
	713 mOsm sucrose	Closed
	998 mOsm sucrose	Closed
Stim (2 min)	Parasymp stim	Opened
Tracer infusion (2 min) ↑ Fix	Symp stim	Opened
Stim (2 min)	15 min after	
15 or 30 min resting	parasymp stim	Opened
Tracer infusion (2 min) ↑ Fix	symp stim	Opened
	30 min after	
	parasymp stim	Closed
	symp stim	Closed

Parasymp stim : Electrical stimulation of chorda for 2 min.

Symp stim : Electrical stimulation of superior cervical ganglion for 2 min.

ed (Fig. 4-D). These findings indicated that tight junctions are permeable to the tracer, and thus caused them to open under parasympathetic or sympathetic secretory stimulation. The tight junctions remained open 15 min later (Fig. 4-E). After stimulation of the superior cervical ganglion, the tight junctions also remained open (Fig. 4-F), and 30 min later, they had closed. The states of the acinar tight junctions using the tracer are summarized in Table 1.

Discussion

The present study revealed that secretory stimulation caused the opening of acinar tight junctions in the submandibular gland, and that the opened tight junctions did not immediately close again after the stimulation stopped.

The barrier function of the closed tight junctions of the salivary acini in the resting gland prevents the movement of salivary components into the interstitial fluid and, conversely, movement of interstitial components into the luminal saliva. There is considerable histological evidence describing the impermeability in the tight junctions of the salivary gland to extracel-

lular markers such as lead ions¹⁸⁾ and horseradish peroxidase⁵⁻⁷⁾. Our histochemical results using microperoxidase confirmed that the resting acinar tight junction is impermeable to the tracer. In the resting gland injected intraductally with hypertonic sucrose, a sustained elevation of luminal pressure was observed. The luminal membrane of the acinar cells would be exposed by hypertonic sucrose. However, in this gland, the tight junctions remained closed. Thus, luminal pressure elevation was caused by transcellular osmotic water flowing into the lumen, due to the presence of a hypertonic solution without tight junctions open. These findings showed that acinar tight junctions are closed in the resting gland.

Stimulation of different autonomic divisions in the rat submandibular gland produces markedly different responses. Parasympathetic stimulation causes continuous and marked secretion with a relatively low concentration of protein. On the other hand, sympathetic stimulation causes only small transient secretion with a high concentration of protein. In either case, stimulation mobilizes the fluid in the acinus, and results in secretion of water by osmosis across the acini^{1,2)}. A confocal laser microscopic study demon-

strated that both Lucifer Yellow and dextrans entered the lumen through acinar tight junctions after carbachol stimulation¹⁹). In addition, our previous transmission electron microscopic study using microperoxidase showed that electrical stimulation of the chorda caused an increase in the permeability of the tight junctions to the tracer²⁰). In the present study, we showed that electrical stimulation of the superior cervical ganglion also caused an increase in the tight junction permeability to microperoxidase. Thus, these findings clearly show that submandibular acinar tight junctions switch rapidly to an opened state by the stimulation of parasympathetic or sympathetic nerves, and that primary salivary fluid is drawn into the lumen through the paracellular pathway.

We also found that the opened tight junction, as a result of the parasympathetic or sympathetic stimulation, remained open even after secretory stimulation was stopped, and the tight junction finally closed 15–30 min later. The frequency of electrical stimulation may affect the duration of opening of tight junctions. Electrical stimulation of the chorda at low frequency induces the release of acetylcholine alone from the nerve terminals, while one or more peptides, for example, substance P, in addition to acetylcholine, are released in response to high-frequency stimulation^{5,21}). It is reported that substance P has an effect of opening of the tight junction⁵). Additionally, the extension of the stimulation period may extend the period of the opening of the tight junctions after the stimulation is stopped. When the tight junctions of an unstimulated gland were in the opened state after the stimulation had been stopped, no salivary secretion occurred because there was no driving force. The tracer entered the lumen by simple diffusion through the opened tight junction. The rapid fall of luminal pressure after nerve stimulation can be explained by water efflux from the lumen to the interstitium through the opened tight junction.

The cellular mechanisms involved in parasympathetic and sympathetic regulation in the opened and closed states of the tight junctions in rat submandibular gland are unknown. However, several possible explanations can be considered. It is well known that stimulation in rat submandibular acinar cells is a

multi-stage process including signal transduction by cyclic AMP, phospholipase C, IP_3 , protein kinase C, or activation of the Ca^{2+} signaling system²²). For example, Ca^{2+} and cyclic AMP interaction with cytoskeletal elements may be involved in the opened and closed states of tight junctions²³). Cyclic AMP increases paracellular permeability in the proximal tubules of the rabbit kidney²⁴), and removal of Ca^{2+} causes disorganization of tight junction elements in various epithelial tissues, including exocrine glands²⁵). Presumably, any or all of these processes could affect junctional permeability, as observed in several other types of epithelial tissue^{3,4}). It was also reported that shrinkage during secretion occurs as a result of cholinergic stimulation of isolated acinar cells in the rat²⁶). This shrinkage has also been observed *in vivo* in the rat submandibular gland perfused with acetylcholine²⁷). It is possible that acinar cell shrinkage results in some form of mechanical disruption of the tight junction, and may result in its opening.

The paracellular secretory pathway for primary salivary fluid is unlikely the only secretory pathway, because we showed that transcellular osmotic fluid could flow into the lumen due to the luminal presence of hyperosmolarity without the tight junction being open. Judging from this observation and the results from nerve-stimulated glands, it is suggested that both the transcellular and paracellular secretory routes for the luminal fluid are present in the rat submandibular acini. The present study, however, was unable to identify the magnitude for each secretory route. A water channel protein, aquaporin 5, has recently been identified in salivary and lacrimal glands²⁸) and the luminal membrane of acinar cells²⁹). This water channel protein may contribute to water secretion in the transcellular water transport system in the salivary acini. However, it remains uncertain whether the fluid primarily flows through the transcellular pathway across the basolateral and luminal membranes, or through the paracellular pathway through the tight junction between the acinar cells. To address this question, further investigation is necessary.

Acknowledgement

This study was supported by the Proposal-Based New Industry Creative Type Technology R&D Promotion Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan. We would like to express our gratitude to Drs. K. Uchihashi and K. Higuchi for their technical help. We also thank Ms. S. Yamaoka for her editorial work and typing the manuscript.

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