J. Membrane Biol. 161, 287–296 (1998)

The Journal of
Membrane
Biology

Transient Swelling of Salivary Acinus Induced by Acetylcholine Stimulation: Water Secretion Pathway in Rat Submandibular Gland

T. Nakahari, Y. Imai

Department of Physiology, Osaka Medical College, 2-7 Daigaku-cho Takatsuki 569-8686, Japan

Received: 3 February 1997/Revised: 29 October 1997

Abstract. The volume changes of isolated acini and acinar cells from rat submandibular glands were measured from digitized images recorded upon stimulation of acetylcholine (ACh) or reduction of the perfusate osmolarity and water secretion pathway in salivary gland was studied. When acinus is exposed to a hyposmotic solution, water flows into the acinar cells and into the lumen via acinar epithelia. If the water enters the lumen chiefly via the cells, the swelling of the lumen would follow the same time course as the cell swelling or slower. The results show that reduction of the perfusate osmolarity evoked a transient increase followed by a gradual increase in the volume of unstimulated acinus, while it evoked only a gradual increase in the volumes of unstimulated acinar cells. Thus, the time course of the acinar swelling is faster than that of the acinar cell swelling. Reduction of the perfusate osmolarity also evoked a transient swelling in ACh stimulated acini. When acinus is stimulated by ACh, water also flows into the lumen via acinar epithelia according to the osmotic gradient which was generated by the active electrolyte transport of acinar cells. If the water enters the lumen chiefly from the cells, there would be no overall change in acinar volume. The results show that stimulation of ACh (5 μM) evoked a transient increase followed by a gradual decrease in the volume of the acinus, while it evoked only a decrease in the volume of acinar cells. Video-enhanced optical microscopy exhibited that ACh stimulation caused transient swelling of the luminal space, prior to causing the volume of acinar cells to decrease and the transient swelling of the lumen followed the same time course as that of acinus. Thus, the transient acinar swelling is explained by the transient swelling of luminar volume. These results suggest that water is probably drawn into the lumen from interstitial space directly in the salivary acinus.

Key words: Paracellular pathway — Osmotic flow — Salivary secretion — Submandibular gland — Cell volume

Introduction

The salivary acinar cells generate a primary fluid that is similar in composition to plasma (Martinez, Holzgreve & Frick, 1996; Young & Schögel, 1996), and the composition of this primary fluid is modified as it flows through the duct system of the gland. The water flow generated in the acinus is widely believed to be an osmotic flow driven by active transport of electrolytes, principally Na^+ and Cl^-, into the acinar lumen. However, both the route and the driving force for water transport remain uncertain. The route could be transcellular, which would require water flow to be generated across both the basolateral and luminal membranes, or it could be paracellular which would require a driving force for water flow at the tight junction, or it could be a combination of the two.

There is some evidence to suggest that the acinar cells form a leaky epithelium (Burgen, 1957; Burgen & Emmelin, 1961; Martin & Burgen, 1962). There is also good evidence for some degree of paracellular permeability to extracellular makers such as horseradish peroxidase (Garrett, 1981; Garrett, Klinger & Parsons, 1982; Takai et al., 1995) and lucifer yellow or fluorescent dextrans (Segawa et al., 1991; Segawa, 1994). The magnitude of the paracellular permeability to water, however, is unknown. Furthermore, attempts to detect paracellular water flow have not proved conclusive. Nonelectrolytes smaller than mannitol show evidence of

Correspondence to: T. Nakahari

solvent drag which suggests that the effective pore radius of the water secretion pathway is about 0.4 nm (Case et al., 1985; Howorth, Case & Steward, 1987). However, this pathway could be either transcellular or paracellular since molecules of this size (0.4 nm) are not confined to the extracelullar space.

Conversely, it is not clear whether the water permeability of the transcellular pathway would be sufficient to generate the observed salivary secretory rates in response to a small osmotic gradient. Measurements of the water permeability of the basolateral membrane suggest that the osmotic gradient required there would be of the order of 10–40 mosmol l^{-1} (Steward et al., 1990). In view of the much smaller area of the luminal membrane (Poulsen & Bundgaard, 1994), an even larger gradient might be required at that membrane.

The recent development of video-optical microscopy allowed us to continuously observe the morphological changes in living cells. We have also reported the shrinkage of salivary acinar cells induced by acetylcholine (ACh), using video-enhanced optical microscopy (Nakahari, Murakami & Kataoka, 1989, Nakahari et al., 1990, 1991). Similar cell shrinkages were reported in other exocrine glands or epithelial cells (Foskett & Melvin, 1989; Suzuki et al., 1991; Park et al., 1994; Nakahari & Marunaka, 1996, 1997). These results demonstrated that cell volume change is an indicator of water and ion transport of cell, and also suggest that cell volume changes are different between preparations, such as whole gland, acinar preparation, and single cell preparation. The acinus consisted of acinar cells and the lumen. Therefore, the volume changes of the acinus may reflect the volume changes not only of acinar cells but also of the luminal space. If luminal fluid is supplied via a transcellular route, the volume change of acinar cells may precede that of the acinus. In the present study we measured volume changes of both an acinus and acinar cells in the acinus upon ACh stimulation or reduction of the perfusate osmolarity and the osmotic water permeability of acinus was compared with that of acinar cells.

Materials and Methods

The control solution (solution A) contained (in mM): NaCl 121, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 1.5, $NaHCO_3$ 25, NaHEPES 5, HHEPES 5, glucose 5, pH 7.4. In the hypotonic solutions the concentration of NaCl was reduced to 91 mM (solution B). The osmolarities of solutions A and B were 287 mosmol l^{-1} and 235 mosmol l^{-1}.

Cell Preparation and Volume Measurements

Adult male Wistar rats (Slc:Wistar/ST, Japan SLC Hamamatsu, Japan) weighing 200–250 g were purchased and fed a standard pellet diet and water. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal 60–70 mg/kg). The submandibular gland was removed from the animals for cell isolation. The submandibular glands were washed with solution A, which was cooled (4°C) and aerated with a gas mixture (95% O_2 and 5% CO_2). Collagenase (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in solution A containing 1% bovine serum albumin (BSA) and the concentration of collagenase used was 0.1%. The glands were minced with fine forceps and incubated for 15 min in collagenase solution (37°C). The digested tissue was resuspended in solution A (4°C) containing 1% BSA and the suspension was filtered through a nylon mesh of 75 μm squares to remove undigested tissues. After filtering, the suspension was centrifuged at 300 rpm for 2 min, washed three times with centrifugation between each wash and suspended in solution A containing 1% BSA. The suspension was stored at 4°C and used for experiments within 3 hr. Isolated acini were placed on a coverslip that was precoated with neutralized Cell-Tak (Becton Dickinson Labware, Bedford, MA) to attach cells firmly to the coverslip. The coverslip with cells was set on the perfusion chamber as described elsewhere (Takemura et al., 1991). The perfusion chamber was set on the stage of a differential interference contrast (DIC) microscope (BX50Wi, Olympus, Tokyo, Japan) connected to a video-enhanced contrast (VEC) system (ARGUS-10, Hamamatsu Photonics, Hamamatsu, Japan), and images were recorded continuously using a video recorder. The volume of the perfusion chamber was about 50 μl, and the rate of perfusion was 300 μl/min. Experiments were performed at 37°C and the ACh concentration used for stimulation was 5 μM.

For cell volume measurements, an acinus was selected (Fig. 1). To estimate cell volume, the areas of acinar cells in an acinus and the acinus were measured on the video image. An average value from 6 images measured in the first 2 min was used as the control (A_0). The relative volume of the acinus or the acinar cell was estimated as $V/V_0 = (A/A_0)^{1.5}$, where V is the volume, A is the area, and subscript 0 indicates the control value. Thus, the values of relative cell volume (V/V_0) are normalized to the control value. Volume estimation of acini or acinar cells was based on the assumption that the volume changed the same amount in all three dimensions. The method of cell volume measurement has been described in details previously (Foskett & Melvin, 1989; Nakahari et al., 1989, 1990, 1991; Suzuki et al., 1991).

Briefly, the acinus on the video image was digitized on the monitor screen and its area was measured using the ARGUS-10 system. Acinar cells which existed in the acinus on the video image were also digitized and their areas were measured. The sampling interval for volume measurement was 1–2 sec. The values of V/V_0 from 4–8 experiments were expressed as means ± SE. The statistical significances of differences between mean values were assessed using paired and unpaired Student's t tests as appropriate.

Results

Effects of Hyptonic Stress

In the unstimulated acinus, the perfusate osmolarity was suddenly reduced to 235 mosmol l^{-1} by switching from solution A to solution B. On reduction of the perfusate osmolarity (solution B), the acinus exhibited a transient increase followed by a gradual increase in its volume, while the acinar cells only exhibited a gradual increase in their volume (Fig. 1). The V/V_0 values of the acinus at 2 sec, 3 sec and 30 sec after reduction of the perfusate osmolarity were 1.036 ± 0.007, 1.020 ± 0.005 and 1.101 ± 0.007 respectively (data were obtained from 8 experi-

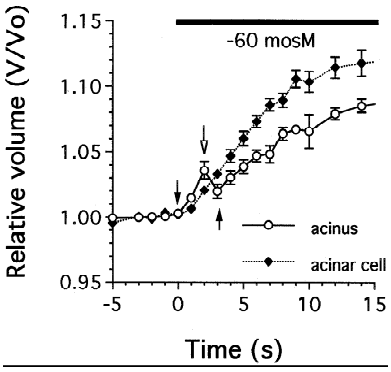

Fig. 1. Volume changes of an unstimulated acinus and acinar cells on reduction of the perfusate osmolarity. The perfusate osmolarity was reduced by switching to solution B which contained 30 mM less NaCl than solution A. The acinus exhibits transient swelling followed by gradual swelling and the volume of the acinus reaches a plateau within 30 sec after the start of hypotonic perfusion. The acinar cells show only swelling. The volume of the acinus at the plateau is smaller than that of the acinar cells. Values show means ± SE of 8 experiments. The value indicated by open arrow is significantly different from those by closed arrows ($P < 0.001$).

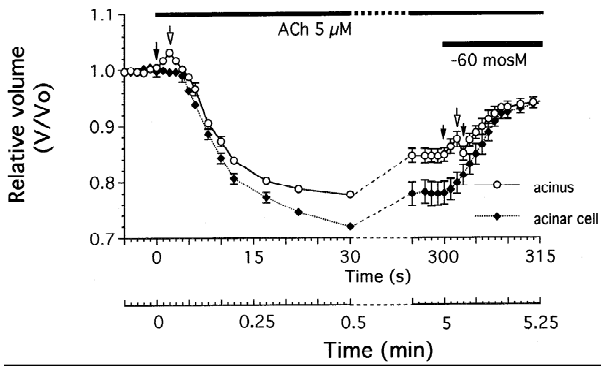

Fig. 2. Volume changes of the acinus and acinar cells caused by ACh stimulation (5 μM). The volume of the acinus increases transiently followed by a gradual decrease, while the volume of acinar cells only decreases gradually. The volumes of the acinus and acinar cells at 30 sec after the start of ACh stimulation are about 0.78 and 0.72. Both the acinus and acinar cells exhibit a decrease in volume in the first 30 sec, followed by a gradual increase in volume. The volume changes of acinar cells are greater than that of the acinus. The perfusate osmolarity was reduced by switching to solution B which contained 30 mM less NaCl than solution A at 5 min after the start of ACh stimulation. The acinus shows transient swelling followed by gradual swelling and the volume of the acinus reaches a plateau within 30 sec after the start of hypotonic perfusion. The acinar cells show only gradual swelling. The volumes of the acinus and acinar cells at the plateau are similar, although the volume of the acinus before the start of hypotonic perfusion is larger than that of the acinar cells. Values show means ± SE of 4 experiments. The values indicated by open arrow is significantly different from those by closed arrows ($P < 0.01$).

ments). The value of V/V_0 at 2 sec was significantly different from those at 0 sec and 3 sec ($P < 0.001$). The acinar cells exhibited only a gradual increase in their volume and V/V_0 value of acinar cell was 1.134 ± 0.007 at 30 sec after switching of the perfusate from solution A to solution B. Upon reduction of the perfusate osmolarity, volume changes were smaller for the acinus (about 10%) than for the acinar cells (about 13%).

After their volume reached a peak value at 30 sec, both acinar cells and acinus exhibited decreases in their volume (V/V_0 of the acinar cells was 1.084 ± 0.013 at 5 min after reduction of the perfusate osmolarity). The volume of the acinar cells, however, does not recover to that before application of hypotonic stress. Thus, the acinar cells exhibited regulatory volume decrease (RVD) (Foskett et al., 1994), but RVD was incomplete form in the acinus preparation.

Effects of ACh

The volume changes of acinus and acinar cells are shown in Fig. 2 (data were obtained from 4 experiments). Upon ACh stimulation (5 μM) the volume of the acinus increased transiently ($V/V_0 = 1.031 \pm 0.001$ at 3 sec after the start of stimulation), then it decreased gradually and reached a plateau ($V/V_0 = 0.777 \pm 0.002$ at 30 sec after the start of stimulation). The value of V/V_0 at 3 sec was significantly different from that at 0 sec ($P < 0.01$). Thus, stimulation by 5 μM ACh evoked a transient increase followed by a gradual decrease in the volume of the isolated submandibular acinus. The volume change of the acinar cells was quite different from that of the acinus. ACh stimulation did not evoke the transient increase in the volume of the acinar cells ($V/V_0 = 0.990 \pm 0.001$ at 3 sec after the start of stimulation) but rather evoked a gradual decrease in the volume which reached a plateau ($V/V_0 = 0.720 \pm 0.051$ at 30 sec after the start of stimulation). The time course of the volume decrease is similar between the acinus and the acinar cells, although the values of the acinar volume and acinar cell volume at 30 sec after the start of stimulation were different (Fig. 2). The values of the acinar volume and acinar cell volume decreased to a plateau and then they increased gradually during continuous ACh stimulation (Fig. 2). The values of relative volume (V/V_0) of the acinus at 30 sec and 5 min after the start of ACh stimulation were 0.777 ± 0.002 and 0.848 ± 0.012, and those of the acinar cells at 30 sec and 5 min were 0.720 ± 0.005 and 0.780 ± 0.020, respectively. Thus, V/V_0 values of the acinus at 30 sec and 5 min after the start of stimulation were about 5% greater than those of the acinar cells.

Effect of Hypotonic Stress with ACh

At 5 min after the start of ACh stimulation, the perfusate osmolarity was suddenly reduced to 235 mosmol l^{-1} by

switching to solution B which contained 30 mM less NaCl than solution A. On reduction of the perfusate osmolarity (solution B), the acinus exhibited a transient increase followed by a gradual increase in its volume (V/V_0 of acinus at 302 sec was significantly different from those at 300 sec and 303 sec), while the acinar cells only exhibited a gradual increase in their volume (Fig. 2). At 30 sec after reduction of the perfusate osmolarity, the V/V_0 of both the acinus and acinar cells reached a plateau. On reduction of the perfusate osmolarity, the V/V_0 of the acinus increased from 0.848 ± 0.012 to 0.956 ± 0.010 within 30 sec and that of the acinar cell increased from 0.780 ± 0.020 to 0.957 ± 0.004. Thus, on reducing the perfusate osmolarity, the volume increase of the acinus was about 11%, while the volume increase of the acinar cells was about 18%. The volume changes were smaller for the acinus than for the acinar cells. ACh-stimulated acinus did not exhibit the RVD, as previously reported (Larcombe-McDouall et al., 1991; Foskett et al., 1994).

Figure 3*A–C* shows video images of an acinus recorded using the DIC optics and VEC system. Figure 3*D–F* show outline traces of Fig. 3*A–C*. Figure 3*A* and *D* show an unstimulated acinus. In this optical section which was obtained at a focal plane just below the coverslip, two acinar cells were detected, but we could detect another 3 cells by changing the focal plane of the DIC microscope. Acinar cells and the intercellular canaliculi (IC) which is a part of the luminal space, are clearly shown in Fig. 3. The surface of basolateral and luminal membranes are smooth. Figure 3*B* and *E* show the acinus at 3 sec after the start of ACh stimulation (5 μM). These figures shows the swelling of IC and swelling of the acinus (the outline of unstimulated acinus (Fig. 3*A*) was depicted by dotted line in Fig. 3*E*). Figure 3*C* and *F* shows the acinus at 11 sec after the start of ACh stimulation. These figures clearly show shrinkage of the acinus and acinar cells (the outline of unstimulated acinus (Fig. 3*A*) was depicted by dotted line). However, the IC still appears to swell. The luminal surfaces of acinar cells are irregular, and vacuoles, which are connected to the IC, exist in the acinar cells.

Figure 4*A–D* shows changes in the IC of acinus on video images upon stimulation of ACh (5 μM). Figure 4*E–H* shows outline traces of Fig. 4*A–D*. An unstimulated acinus was shown in Fig. 4*A* and *E*, in which acinar cells and the intercellular canaliculi between acinar cells were detected. The surfaces of basolateral and luminal membranes are smooth. Figure 4*B* and *F* show the acinus at 1 sec after the start of ACh stimulation. These figures show the swelling of the IC. ACh stimulation caused the rapid swelling of IC followed by a burstlike fluid secretion to the opening of acinus (* in Fig. 4*E–H* shows the opening of acinus which normally connects to the duct lumen). Figure 4*C* and *G* show the acinus at 3 sec after the start of ACh stimulation. The volume of IC in Fig. 4*C* decreased and the width of IC was about half of that in Fig. 4*B*. Figure 4*D* and *H* shows 3 min after the start of ACh stimulation. The luminal surfaces of acinar cells are irregular, and small vacuoles, which are connected to the luminal space, exist in the luminal surface of acinar cells. These figures also show that ACh stimulates exocytotic activity of acinar cells. The volume of IC was maintained well.

We can also observe fluid flow in the IC by detecting movements of cellular fragments such as small particles. The direction of fluid flow was from the peripheral end of IC to the opening of acinus (* of Fig. 4) and it took about 1 sec from the end of IC to the opening of acinus. Furthermore, upon stimulation of ACh (in another experiments), the swelling of IC first occurred in the peripheral end of acinus, and then was followed by the IC of its central part where fluid entered from several peripheral ICs. Finally fluid secreted leaked out of acinus as shown in Fig. 4. These observations indicate that fluid flows from the peripheral end of IC where the tight junction exists to the opening of acinus (duct lumen), even in the isolated acinus.

The width of IC in Fig. 4 were measured and plotted in Fig. 5. The measured point of intercellular canaliculi was marked in Fig. 4 (dotted line). The width of IC was around 0.3 μm in unstimulated acinus of Fig. 4. ACh stimulation caused the rapid increase (3.1 μm at 1 sec after start of stimulation) followed by a sharp decrease (1.5 μm at 3 sec) in the width of IC. Then the width of IC increased gradually (about 2.6 μm at 10 sec), then it decreased gradually and reached a plateau value (about 2 μm). This figure clearly shows volume changes of IC evoked by ACh stimulation.

Thus, the transient acinar swelling induced by ACh is explained by swelling of IC (lumen). This result suggests that the difference of volume changes between acinus and acinar cells is explained by volume changes in IC. In unstimulated acini, transient swelling of IC, during transient swelling of the acinus caused by reduction of perfusate osmolarity, occurred. However, changes in width of IC was not so obvious compared with those induced by ACh stimulation. In a case, the width of IC (0.6 μm) increased to 1.3 μm at 2 sec after reduction of the perfusate osmolarity. However, at 30 sec after reduction of the perfusate osmolarity, the width of IC reduced to 0.3 μm. The similar results were also obtained in ACh stimulated acini upon application of hyposmotic stress. In a case, the width of IC was 3.1 μm at 5 min after the start of stimulation, and then perfusate osmolarity was reduced. The width of IC increased to 3.6 μm at 2 sec after reduction of the perfusate osmolarity and it reduced to 2.6 μm at 30 sec after reduction of the perfusation osmolarity. The swelling of the acinar cells caused by the hypotonic solution appears to reduce the width of IC.

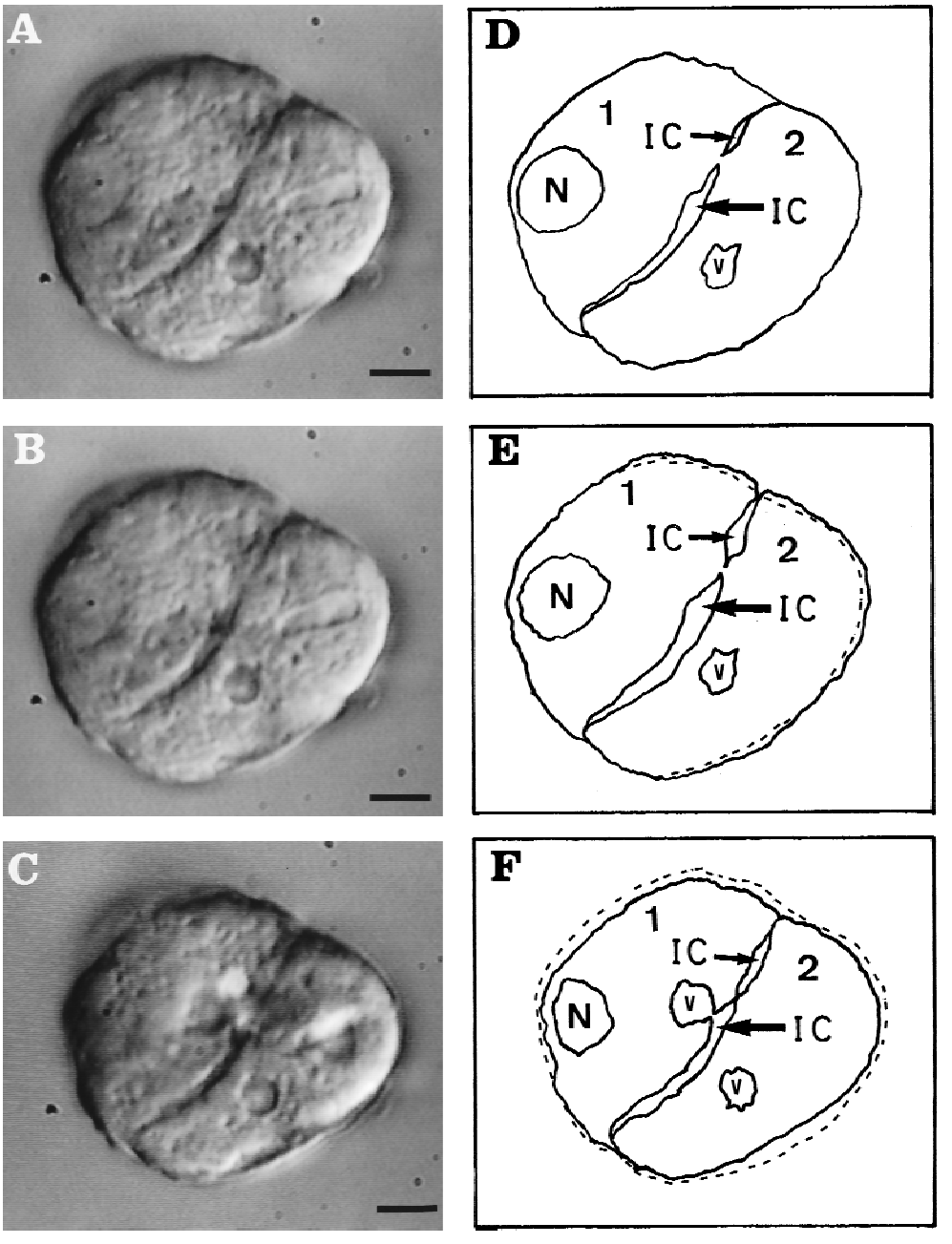

Fig. 3. Photographs of DIC image recorded by VEC system. The acinus was first perfused with solution A in the perfusion chamber set on the stage of the DIC microscope, then stimulated with 5 μM ACh. (*A*) Unstimulated acinus. The acinar cells are clearly visible and the luminal space can be observed between acinar cells. (*B*) Acinus at 3 sec after the start of ACh stimulation. This photograph shows the swelling of the acinus and the expansion of the luminal space, but the volume of the acinar cells has changed very little. (*C*) Acinus at 11 sec after the start of ACh stimulation. This photograph shows the shrinkage of both the acinus and the acinar cells. The luminal space remains expanded. The surfaces of the luminal membrane became irregular and vacuoles appear in acinar cells. The scale bar shows 8 μm. (*D–F*) Outline traces of Fig. 3*A–C*. IC is the intercellular canaliculi, V is the vacuoles and N is the nucleus. 1 and 2 indicate acinar cells. The dotted lines in Fig. 3*E* and *F* show the outlines of unstimulated acinus (Fig. 3*A* and *D*). Figure 3*E* and *F* clearly shows the acinar swelling and shrinkage respectively.

Discussion

Cholinergic stimulation causes the acinar cell volume to decrease in the salivary gland (Burgen & Emmelin, 1961; Nauntofte & Poulsen, 1986; Foskett & Melvin, 1989; Foskett, 1991; Nakahari et al., 1989, 1990, 1991; Larcombe-McDouall, Nakahari & Steward, 1991; Larcombe-McDouall, Seo & Stewart, 1994). This agonist-induced cell shrinkage occurs under isotonic conditions and is explained by KCl release from the acinar cells on

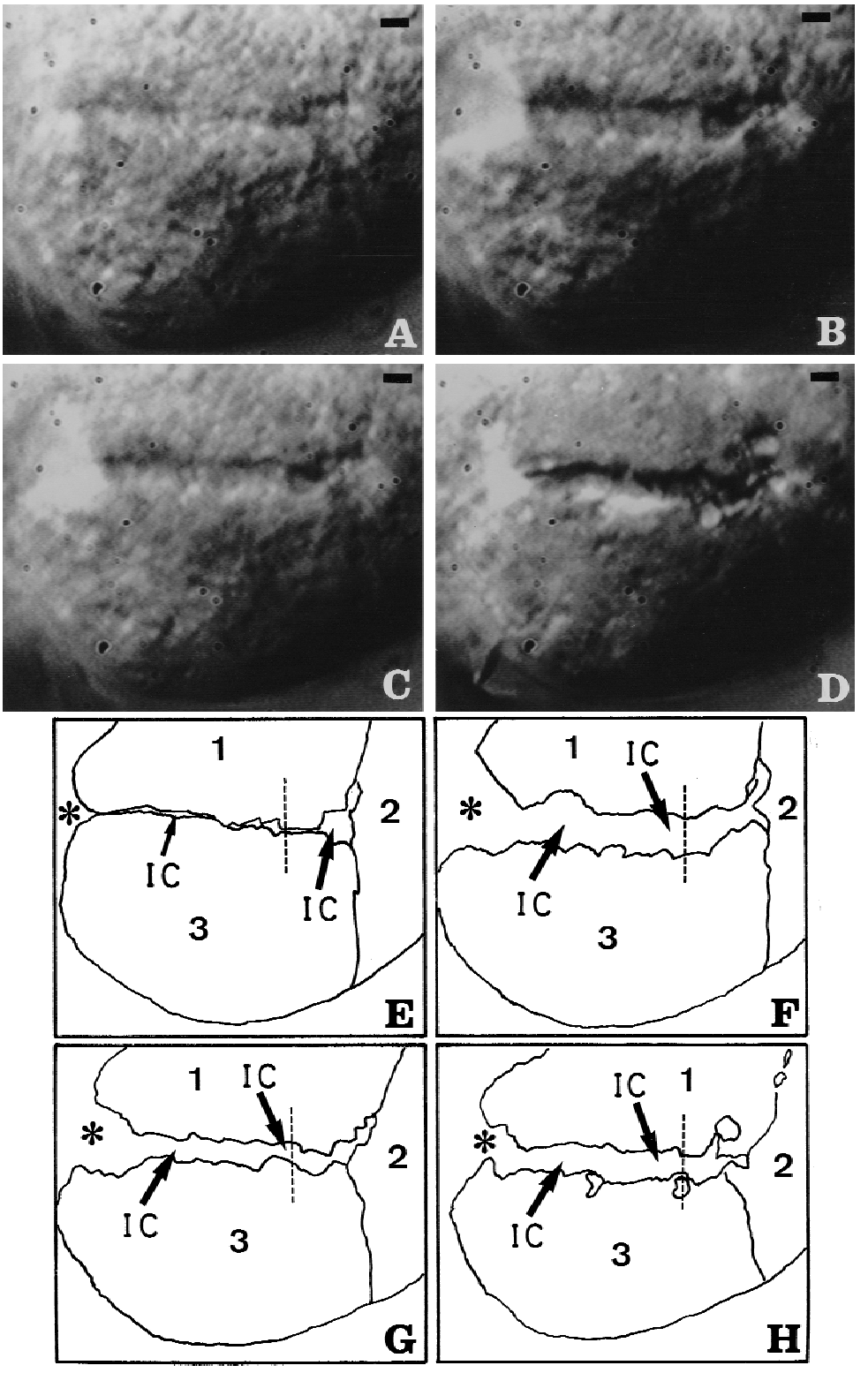

Fig. 4. Changes in the intercellular canaliculi (IC) detected by VEC system upon stimulation of ACh (5 μM). (*A*) Unstimulated acinus. The acinar cells are clearly visible and the IC can be observed between acinar cells. (*B*) Acinus at 1 sec after the start of ACh stimulation. This photograph shows the swelling of IC. In the video images fluid flow was observed from the peripheral end of IC to the opening of acinus (* in Fig. 4*E–H*). (*C*) Acinus at 3 sec after the start of ACh stimulation. IC was less swelled compared with IC at 1 sec after ACh stimulation (Fig. 4*F*). (*D*) Acinus at 3 min after the start of ACh stimulation. The surfaces of the luminal membrane became irregular and vacuoles appear in the luminal surface of acinar cells which connected to IC. This photograph shows the shrinkage of both the acinus and the acinar cells. The scale bar shows 2 μm. (*E–H*) Outline traces of Fig. 4*A–D*. IC is the intercellular canaliculi, * is the opening of acinus, which normally connects to the duct lumen. 1–3 indicate acinar cells. At the dotted lines width of IC was measured and the results are shown in Fig. 5.

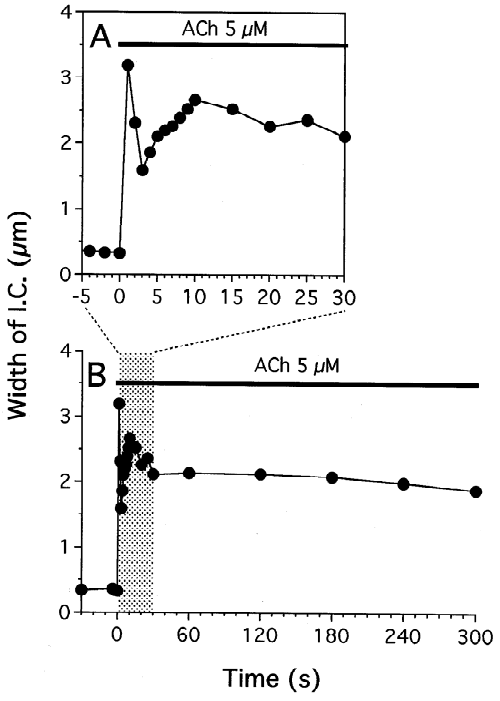

Fig. 5. Changes in width of IC upon stimulation of ACh (5 μM). The width of IC was measured continuously on the video images of Fig. 4. The width of IC expanded within 1 sec after ACh stimulation and decreased sharply (2 sec), then increased gradually and reached a peak value and decreased gradually to a plateau value. This figure shows a typical change in IC with ACh stimulation. ACh stimulation evoked swelling of the IC.

cholinergic stimulation (Suzuki et al., 1991). Similar isotonic cell shrinkages have been reported for sweat glands (Suzuki et al., 1991) and fetal lung epithelial cells (Marunaka, Doi & Nakahari, 1995; Nakahari & Marunaka, 1996, 1997). Shrinkage induced by cholinergic stimulation has been reported for isolated acinar cells, acini, and whole glands and detected using morphometric, impedance, and NMR methods and tissue weight measurement. But the extent of shrinkage observed was different between cells and whole glands or among the methods to measure the degree of shrinkage, as previously reported that the acinar cell volume measured using a morphometric method and an impedance method decreased to about 70–75% of the prestimulation volume (Foskett & Melvin, 1989; Nakahari et al., 1989, 1990, 1991), while the water content of the whole gland decreased to 80–85% of the prestimulation water content (Nakahari et al., 1990; Larcombe-Mcdouall et al., 1994). An explanation is that spaces other than the intracellular space exist in the whole gland, such as duct spaces and luminal spaces. The results of the present study also demonstrated differences in volume changes of an acinus and the acinar cells of the acinus on ACh stimulation or reduction of perfusate osmolarity. The acinus, which was surrounded by myoepithelial cells and connective tissue, consisted of acinar cells, which adhered to each other by junctional complexes, and three compartments, the intracellular space, the luminal space and the intercellular space. The volume of the acinus was affected by these three compartments.

Upon reduction of the perfusate osmolarity, the volume increase of the acinus at 30 sec after reduction of the perfusate osmolarity was also smaller than that of the acinar cell. The reduction of the intercellular fluid osmolarity caused the cell swelling in unstimulated acinus and it may cause the luminal space to decrease by the compression, because the transepithelial osmolarity gradient is reduced immediately as shown in the transient fluid shift experiments (Nakahari, Yoshida & Imai, 1996) and consequently it is difficult to maintain the open luminal space against cell swelling. In ACh stimulated acinus, the luminal space was still maintained due to the fluid secretion even during hyposmotic stress, but the volume of luminal space decreased compared with that before hyposmotic stress. These volume changes in luminal space may cause the swelling of acinus following the initial transient swelling to be slower compared with the swelling of acinar cells. On ACh stimulation, the volume decrease of the acinus at 30 sec after the start of ACh stimulation was smaller than that of the acinar cell. ACh stimulation caused the fluid secretion to increase continuously, and the continuous fluid flow into the lumen keep the luminal volume increase (as shown in Fig. 5). This may cause the shrinkage of acinus to be slower compared with the shrinkage of acinar cells. Thus, the differences in volume changes between the acinus and acinar cells can be explained by luminal volume changes.

ACh causes rapid loss of electrolytes from the acinar cells which increases the osmolarity of lumen and this causes a flow of water into the lumen not from the cells (because cell volume showed only small shrinkage) but rather from the interstitium. If the acinar cells lost KCl during the initial stage of ACh stimulation, these cells should have lost cell volume, because Cl^- secretion across apical membrane should be accompanied by K^+ release across basolateral membranes. However the cells lost only a small volume (about 1%) during the initial stage of ACh stimulation. This suggests that electrolyte loss is regained by activation of the ion transporters, such as Na/K/2Cl cotransport and Na/H and Cl/HCO_3 coupled exchanger, at a rate equivalent to the secretion of Cl^- across the luminal membrane (Cook et al., 1994).

The most probable explanation for transient swelling of the acinus upon reduction of intercellular fluid osmolarity is that the osmotic driving force for fluid shift between the interstitium and the lumen increases and causes a fluid shift from the interstitium to the lumen either via the cells or via the paracellular pathway. The

fluid shift into the lumen causes the expansion of the luminal space which causes the luminal hydrostatic pressure to increase, then fluid of the lumen leaked into the duct lumen (outside of acinus) and the volume of lumen decreases again. These decreases in luminal space caused the decrease of acinar volume. If the water entered lumen via the cells, the luminal swelling would follow the same time course as the cell swelling or slower. The result shows that the time course of the transient acinar swelling is faster than that of cell swelling.

The transient swelling of the acinus on ACh stimulation is explained as follows: electrolyte secretion into the lumen is activated by ACh stimulation and causes the luminal osmolarity to increase, and the osmolarity gradients generated between the lumen and the interstitium drive fluid flow into the lumen. The fluid influx into the lumen may cause increases in the luminal volume which causes hydrostatic pressure of the lumen to increase, and then fluid of the lumen leaks out of the acinus and the luminal space decreases again. These decreases in luminal space caused the decrease of acinar volume. If the water entered the lumen chiefly from the cells, there would be no overall change in acinar volume. The fact is that the acinar volume increases transiently prior to causing the acinar cell volume to decrease. As previously reported, the reduction of the perfusate osmolarity during ACh stimulation caused the transient increases in fluid secretion in the perfused rat submandibular gland by increasing the driving force for fluid secretion between interstitium and lumen (Nakahari et al., 1997; Yoshida, Nakahari & Imai, 1997). Thus, the transient acinar swelling of ACh-stimulated acinus upon reduction of the interstitial osmolarity is explained by the transient increases in the fluid flow. These observations indicate that water is probably drawn into lumen from interstitium directly in the salivary acinus.

The question in here is the site of the fluid secretion pathway. One is the paracellular pathway which is composed of the tight junctions and the other is the opening of the acinus (artificial pathway) where it connects to the salivary ducts. As mentioned in the Results, however, we observed that the fluid flowed from the end of acinus to the opening of the acinus during ACh stimulation and that the lumen was expanded from the acinar end to its opening during the first second of ACh stimulation. Unfortunately, we did not observe the fluid flow directly in unstimulated acinus upon application of hyposmotic stress, although fluid flow from the end to the opening was still observed in ACh-stimulated acinus and fluid shift was also detected in unstimulated glands upon application of hyposmotic stress (Nakahari et al., 1996). Segawa et al. (1991) reported that lucifer yellow which is an extracellular marker did not appear in the lumen in unstimulated isolated acinus using the laser confocal microscopy. We also observed the fluorescence of lucifer yellow in the isolated acinus using confocal laser microscopy upon application of hyposmotic solution, and we did not detect the fluorescence in the lumen of isolated acinus (Nakahari *unpublished observation*). Thus, the lumen of the isolated acinus is not likely to connect the interstitium directly via the opening of the acinus. If the opening of the acinus is not closed, the diffusion of lucifer yellow there may be slow. When applying hyposmotic perfusate, ions of lumen diffuse outside of the acinus at the opening of acinus, while water flow into the lumen via the opening of acinus is negligibly small without barriers for ions, and ion movements reduce the volume changes of lumen. Furthermore, the hydrostatic pressure differences between the lumen and the outside of acinus are negligible. These suggest that the amount of fluid entered via the opening of acinus is small. From these observations we think that the artificial pathway does not contribute to the acinar swelling largely upon application of hyposmotic solution. However, we did not completely reject the possibility that this artificial pathway contributed to the transient acinar swelling in unstimulated acinus. On the other hand, the perfusate may easily access the intercellular space near the tight junctions. Therefore, water may be drawn into the lumen via the tight junctions upon application of hyposmotic perfusate. Thus, our data suggest that the noncellular pathway contributes fluid flow into the lumen largely, but we did not identify the site of the water flow in the acinus exactly.

Many reports on paracellular fluid transport pathways in the salivary gland have been published. Electron microscopy showed that extracellular markers such as horseradish peroxidase and lead ion appeared in the lumen during secretory stimulations (Garret, 1981; Simson & Bank, 1984; Takai et al., 1995). Laser confocal microscopy has also shown that lucifer yellow or fluorescent dextran appeared in the lumen during secretory stimulations. Martin and Burgen (1962) reported that polar nonelectrolytes with radii smaller than 1.2 nm appeared in the saliva during adrenaline stimulation. Furthermore, nonelectrolytes smaller than mannitol show evidence of solvent drag which suggests that the effective radius of water secretion pathway is about 0.4–0.45 nm (Case et al., 1985; Howorth et al., 1987). We reported a similar result, that the effective radius for nonelectrolyte passage was about 0.4 nm in a study on transepithelial fluid shift generated by osmolarity gradients between the interstitium and lumen (Nakahari et al., 1996).

In our previous study the osmotic water permeability of salivary epithelium has been reported to be 13–15 $\mu l\ min^{-1}\ (mosmol\ l^{-1})^{-1}\ g^{-1}$ (Nakahari et al., 1997; Yoshida et al., 1997). This indicates that if the osmolarity of the lumen is 10 mosmol l^{-1} higher than that of the intersti-

tium, steady-state flow is maintained. The permeabilities of the basolateral membranes of the acinar cells in rat and rabbit submandibular glands have been estimated in NMR studies of diffusive water exchange between intra- and extracellular fluid (Steward et al., 1990, 1992). In those studies, the osmotic water permeabilities of the basolateral membranes were estimated to be 3–18 μl $(\text{mosmol l}^{-1})^{-1}\ \text{min}^{-1}\ \text{g}^{-1}$. Although the upper value is comparable to the transepithelial one (Nakahari et al., 1997; Yoshida et al., 1997), water flow via the transcellular route also requires a sufficient water permeability of the luminal membrane. In view of the much smaller area (one-twelfth) of the luminal membrane (Poulsen & Bundgaard, 1994), it would have to have a much higher water permeability per unit area of membrane than the basolateral membrane.

Although the data presented in this paper suggest that the noncellular pathway makes a major contribution to fluid secretion, we cannot exclude the possibility that the transcellular pathway also has a significant role. A water channel protein, aquaporin 5 (AQP 5), has recently been identified in salivary and lacrimal glands (Raina et al., 1995) and it is present in the luminal membrane of acinar cells (He et al., 1997). AQP5 may contribute the water secretion in the salivary gland.

This work is partly supported by a grant to T.N. from Jinsenkai, Osaka Medical College.

References

Burgen, A.S.V. 1957. The secretion of nonelectrolytes in the parotid saliva. *J. Cell. Comp. Physiol.* **48:**113–138

Burgen, A.S.V., Emmelin, N.G. 1961. Theories of secretion. *In:* H. Barcroft, H. Davson and W.D.M. Paton, editors. pp. 195–228. Physiology of the Salivary Glands. Arnold Publishers, London

Case, R.M., Cook, D.I., Hunter, M., Steward, M.C., Young, J.A. 1985. Transepithelial transport of nonelectrolyte in the rabbit mandibular salivary gland. *J. Membrane Biol.* **84:**239–248

Cook, D.I., van Lennep, E.W., Roberts, M.L., Young, J.A. 1994. Secretion by the major salivary glands. *In:* L.R. Johnson editor. Physiology of the Gastrointestinal Tract, 3rd ed. pp. 1061–1117. Raven Press, New York

Foskett, J.K. 1991. $[Ca^{2+}]_i$ modulation of Cl^- content controls cell volume in single salivary acinar cells during fluid secretion. *Am. J. Physiol.* **259:**C998–C1004

Foskett, J.K., Melvin, J.E. 1989. Activation of salivary secretion: coupling of cell volume and $[Ca^{2+}]_i$ in single cells. *Science* **244:**1582–1585

Foskett, J.K., Wong, M.M.Y., Sue-A-Quan, G., Robertson, M.A. 1994. Isosmotic modulation of cell volume and intracellular ion activities during stimulation of single exocrine acinar cells. *J. Exp. Zool.* **268:**104–110

Garrett, J.R. 1981. Permeability of salivary glands to macromolecules. *Adv. Physiol. Sciences* **28:**109–117

Garrett, J.R., Klinger, A.H., Parsons, P.A. 1982. Permeability of canine submandibular glands to blood-borne horseradish peroxidase during parasympathetic secretion. *Quart. J. Exp. Physiol.* **67:**31–39

He, X., Tse, C.-M., Donowitz, M., Alper, S.L., Gabriel, S.E., Baum, B.J. 1997. Polarized distribution of key membrane transport proteins in the rat submandibular gland. Pfluegers Arch. **433:**260–268

Howorth, A.J., Case, R.M., Steward, M.C. 1987. Effects of acetylcholine and forskolin on the nonelectrolyte permeability of the perfused rabbit mandibular gland. Pfluegers Arch. **408:**209–214

Larcombe-McDouall, J.B., Nakahari, T., Steward, M.C. 1991. Cell volume changes in perfused salivary glands measured by H-1 NMR relaxometry. *In:* Y. Seo, M. Murakami, O. Ichikawa, editors. Relaxation Mechanism of Intra- and Extracellular Sodium (International Workshop on MRI&S, Okazaki, Japan, 1991), pp. 97–100. National Institute for Physiological Sciences (Kenbun Printing, Ltd.) Okazaki, Japan

Larcombe-McDouall, J.B., Seo, Y., Steward, M.C. 1994. Continuous measurement of cell volume changes in perfused rat submandibular glands by proton NMR. *Magnetic Resonance in Medicine* **31:**131–138

Martin, K., Burgen, A.S.V. 1962. Changes in the permeability of the salivary gland caused by sympathetic stimulation and by catecholamines. *J. Gen. Physiol.* **46:**225–243

Martinez, J.R., Holzgreve, H., Frick, A. 1966. Micropuncture study of submaxillary glands of adult rats. Pfluegers Arch. **290:**124–133

Marunaka, Y., Doi, Y., Nakahari, T. 1995. Bumetanide and bicarbonate increase short-circuit current in fetal lung epithelium. *Gen. Pharm.* **26:**1513–1517

Nakahari, T., Marunaka, Y. 1996. Regulation of cell volume by β_2-adrenergic stimulation in rat fetal distal lung epithelial cells. *J. Membrane Biol.* **151:**91–100

Nakahari, T., Marunaka, Y. 1997. β-agonist-induced activation of Na^+ absorption and KCl release in rat fetal distal lung epithelium: a study of cell volume regulation. *Exp. Physiol.* **82:**521–536

Nakahari, T., Murakami, M., Kataoka, T. 1989. Shrinkage of rat mandibular acinar cell with acetylcholine detected by video-enhanced contrast in microscopy. *Jpn. J. Physiol.* **39:**609–615

Nakahari, T., Murakami, M., Sasaki, Y., Kataoka, T., Imai, Y., Shiba, Y., Kanno, Y. 1991. Dose effects of acetylcholine on the cell volume of rat mandibular salivary acini. *Jpn. J. Physiol.* **41:**153–168

Nakahari, T., Yoshida, H., Imai, Y., Murakami, M., Seo, Y. 1992. Osmotically inactive space during hyperosmotic stress in the perfused submandibular gland of the rat. *Jpn. J. Physiol.* **42:**977–985

Nakahari, T., Murakami, M., Yoshida, H., Miyamoto, M., Sohma, Y., Imai, Y. 1990. Decrease in rat submandibular acinar cell volume during ACh stimulation. *Am. J. Physiol.* **258:**G878–G886

Nakahari, T., Steward, M.C., Yoshida, H., Imai, Y. 1997. Osmotic flow transients during ACh stimulation in the perfused rat submandibular gland. *Exp. Physiol.* **82:**55–70

Nakahari, T., Yoshida, H., Imai, Y. 1996. Transepithelial fluid shift generated by osmolarity gradients in unstimulated perfused rat submandibular glands. *Exp. Physiol.* **81:**767–779

Nauntofte, B., Poulsen, J.H. 1986. Effects of Ca^{2+} and frosemide on Cl^- transport and O_2 uptake in rat parotid acini. *Am. J. Physiol.* **251:**C175–C185

Park, K-P., Beck, J.S., Douglas, I.J., Brown, P.D. 1994. Ca^{2+}-activated K^+ channels are involved in regulatory volume decrease in acinar cells isolated from the rat lacrimal gland. *J. Membrane Biol.* **141:**193–201

Poulsen, J.H., Bundgaard, M. 1994. Quantitative estimation of the area of luminal and basolateral membranes of rat parotid acinar cells: some physiological applications. *Pfluegers Arch.* **429:**240–244

Raina, S., Preston, G.M., Guggino, W.B., Agre, P. 1995. Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal, and respiratory tissues. *J. Biol. Chem.* **270:**1908–1912

Segawa, A. 1994. Tight junctional permeability in living cells: dynamic changes directly visualized by confocal laser microscopy. *J. Electron Microscopy* **43:**290–298

Segawa, A., Terakawa, S., Yamashina, S., Hopkins, C.R. 1991. Exocytosis in living salivary glands: Direct visualization by video-enhanced microscopy and confocal laser microscopy. *Eur. J. Cell Biol.* **54:**322–330

Simson, J.A.V., Bank, H.L. 1984. Freeze-fracture and lead ion tracer evidence for a paracellular fluid secretion pathway in rat parotid glands. *Anat. Rec.* **208:**69–80

Steward, M.C., Larcombe-McDouall, J.B., Nakahari, T., Case, R.M. 1992. Salt-water coupling in salivary secretion. *In:* M. Murakami, Y. Seo, T. Ishikawa, editors. Salivary Secretion: Control and Mechanisms, pp. 77–80. National Institute for Physiological Sciences, Okazaki, Japan

Steward, M.C., Seo, Y., Rawlings, J.M., Case, R.M. 1990. Water permeability of acinar cell membranes in the isolated perfused rabbit mandibular salivary gland. *J. Physiol.* **431:**571–583

Suzuki, Y., Ohtsuyama, M., Samman, G., Sato, F., Sato, K. 1991. Ionic basis of methacholine-induced shrinkage of dissociated eccrine clear cells. *J. Membrane Biol.* **123:**33–41

Takai, N., Uchihashi, K., Miyao, H., Murakami, H., Yoshida, Y. 1995. Chorda-evoked opening of tight junctions in rat submandibular salivary acini demonstrated by microperoxidase. *Arch. of Oral Biol.* **40:**1077–1080

Takemura, T., Sato, F., Saga, K., Suzuki, Y., Sato, K. 1991. Intracellular ion concentrations and cell volume during cholinergic stimulation of eccrine secretory coil cells. *J. Membrane Biol.* **119:**211–219

Yoshida, H., Nakahari, T., Imai, Y. 1997. A method to estimate the transient interstitial osmolarity change upon application of osmotic stress in perfused rat submandibular gland. *Jpn. J. Physiol.* **47:**311–316

Young, J.A., Schögel, E. 1996. Micropuncture investigation of sodium and potassium excretion in rat submaxillary saliva. *Pfluegers Arch.* **291:**85–98