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Immunohistochemical observation of actin filaments in epithelial cells encircling the taste pore cavity of rat fungiform papillae

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SUMMARY

Epithelial cells are connected to each other around taste pores in rat fungiform papillae. Cytoskeletal components are responsible for the maintenance of intracellular adhesion, and we investigated the identification and localization of actin filaments around taste pores. On the basis of observations made by immunohistochemical transmission electron microscopy comparing with confocal laser scanning microscopy using actin-lectin double staining, actin filaments were found to be localized, encircling the squeezed taste pore cavity, in epithelial cells a few micrometers below the papilla surface. In addition, these observations suggest that the organization of actin filaments around taste pores might be involved in the constriction of taste pores.

INTRODUCTION

The taste pore of fungiform papillae is a pit in the papillary epithelium (Parks and Whitehead, 1998), filled with glycoproteins (Brouwer and Wiersma, 1978; Witt and Miller, 1992; Menco and Hellekant, 1993). In the taste pore cavity, taste receptor cells protrude microvilli containing receptor molecules to establish contact with external taste substances through glycoproteins (Farbman 1965; Hoon *et al.*, 1999). The taste pore and glycoproteins are essential for receiving the external taste stimuli via the microvilli of taste receptor cells (Graziadei, 1969; Mistretta, 1989; Farbman 1965; Hoon *et al.*, 1999). However, the structural components responsible for the maintenance of taste pores remain unknown.

Epithelial cells around taste pores (taste pore cells) are connected to each other via tight junctions (Akisaka and Oda, 1978; Jahnke and Baur, 1979). The cytoarchitecture through tight junctions is maintained with cytoskeletal components such as actin filaments (Mitic and Anderson, 1998; Denker and Nigam, 1998). Confocal laser scanning microscopy on staining of fungiform papillae with rhodamine phalloidin reveals positive reactions, such as ring shape fluorescence, due to actin filaments encircling taste pores (Ohishi *et al.*, 1999); but for the identification and

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localization of actin filaments in epithelial cells around the taste pores, transmission electron microscopy with immunohistochemical techniques is a potentially powerful tool. In addition, elucidating the positional relationship of glycoproteins in the taste pore cavity with ring shape fluorescence should improve our understanding of their localization.

In the present study, we investigated the identification and localization of actin filaments in taste pore cells, using immunohistochemical transmission electron microscopy comparing with confocal laser microscopy.

MATERIALS AND METHODS

Animals

Male Wistar rats (250-350 g) were used throughout the study. The animals were anesthetized with pentobarbital sodium (Nembutal, 65 mg/kg, i.p.). For experiments, the lateral portion of the anterior one-third of the tongue, where numerous fungiform papillae are distributed, was used.

Immunohistochemistry for actin filaments in epithelial cells around taste pore of rat fungiform papillae

Five rats were anesthetized with sodium pentobarbital and perfusion-fixed with 4% paraformaldehyde/1% glutaraldehyde in 0.067 M phosphate buffer, pH 7.4. The tongue was removed and cut into small pieces containing a fungiform papilla. After a wash in phosphate buffer, the samples were dehydrated in graded N,N-dimethylformamide and embedded in glycol methacrylate at –20°C (Uchida, 1985). Ultrathin longitudinal sections (about 85 nm) of taste buds were mounted on nickel grids for electron microscopic immunohistochemistry. They were processed for immunogold-silver staining (Uchida *et al.* 1991). The sections were first immersed for 5 min in a blocking solution consisting of 0.2% casein, 0.2% ovalbumin, 0.1% Tween-20, and 0.1% Triton X-100 dissolved in Tris-buffered saline (TBS; 20 mM Tris-HCl, 150 mM NaCl, pH 8.2). They were then incubated for 15 min with anti F-actin primary antibodies (Transformation Research, Inc., MA. USA) diluted 1:200 with blocking solution. After rinsing excess antibodies with TBS, the sections were immersed in colloidal gold particles (average diameter, 3 nm) conjugated with goat anti-rabbit Ig G antibodies diluted 1:400 with blocking solution for 30 min. They were washed with TBS followed by distilled water and air-dried. Silver intensification (Uchida *et al.* 1991) was carried out for 7 min. The sections were stained with uranyl acetate followed by lead citrate and examined with a transmission electron microscope (Hitachi H-300, Hitachi, Tokyo, Japan). All the processes described above were carried out at room temperature. Immunohistochemical specificity was checked by the absorption test as the negative control.

Confocal horizontal sectioning of fungiform papilla stained with rhodamine phalloidin

Four rats were anesthetized with sodium pentobarbital. Tongues were amputated, cut into small pieces with a fungiform papilla and washed twice containing phosphate-buffered saline (PBS, pH 7.4). The samples were fixed in 4% formalin solution for 15 min and dehydrated with acetone at –20°C for 5 min. After drying at room temperature, the samples were incubated with 70 ng/ml rhodamine-phalloidin for 20 min (Molecular Probes, USA). Thereafter, these non-sectioned samples were observed with confocal horizontal sectioning from the surface of papillae under a confocal laser scanning microscope (Carl Zeiss LSM410, Carl Zeiss, Germany) at an excitation wave length of 543 nm and an emission wave length of 570 nm.

Longitudinal frozen sections of fungiform papillae for actin staining

Tongues removed from the four anesthetized rats were embedded in O.C.T. compound (Miles, USA) and frozen in liquid nitrogen. The tongues were longitudinally sectioned to 5-µm in thickness at –20°C, mounted on silanized glass slides, and stained with rhodamine-phalloidin for 20 min as described above. Longitudinal sections of the fungiform papillae were observed under a confocal laser scanning microscope at an excitation wave length of 543 nm and an emission wave length of 570 nm.

Lectin histochemistry

The small pieces of rat tongues with a fungiform papilla and longitudinal frozen sections of fungi-

form papillae stained with rhodamine-phalloidin described above were used for actin-lectin double staining. Fluorescein-isothiocyanate conjugated *Ulex europeus* agglutinin-I (FITC conjugated UEA-I) was obtained from E-Y Laboratories (San Meteo, Calif., USA) and diluted to a concentration of 20 µg/ml in PBS. After being rinsed with PBS, samples were incubated with a diluted solution of FITC conjugated UEA-I (20 µg/ml) for 30 min at room temperature in a moist chamber. These samples were examined under a confocal laser scanning microscope, at an excitation wave length of 488 nm and an emission wave length of 510 nm.

Scanning electron microscopy of fungiform papillae

Four rats were anesthetized with sodium pentobarbital. The small pieces of rat tongues with a fungiform papilla were fixed with 10% formalin solution for 6 hrs and dehydrated through a graded series of alcohols. After being dried with a critical point dryer, the samples were coated with gold in vacuo. The samples were observed with a scanning electron microscope (JEOL JMS-6300, JEOL, Tokyo, Japan).

RESULTS

Immunocytochemistry of actin filaments in epithelial cells around taste pore

The cylindrical pore cavity is 2-5 µm in diameter and about 10 µm deep from the keratinized free surface of the papilla forming a narrow region in an outer part of the pore cavity (Fig. 1A). At ultrastructural resolution, silver grains representing immunoreactivity of actin filaments were found in epithelial cells constructing the taste pore cavity (taste pore cells) (Fig. 1B). Intense immunoreactivity to anti-F actin was observed in the restricted region adjacent to the taste pore cavity in taste pore cells, which were the second or third epithelial layer from the papilla surface. The cavity was filled with an amorphous material (Fig. 1A and B). A dense substance was seen in the upper taste pore cavity (Fig. 1B).

Double histochemical staining: rhodamine-phalloidin/FITC conjugated UEA-Iin longitudinal sections

In the longitudinal sections of the fungiform papilla from frozen samples, strong positive reactions with rhodamine-phalloidin were detected in two positions around the upper taste pore cavity: a

Fig. 1 - Electron micrographs of the area around the taste pore showing immunolocalization of actin filaments in longitudinal sections of the fungiform papilla. **A:** Epithelial cells (E) construct the cylindrical pore cavity (PC), making a narrow region (arrows) under the pore surface. Secretory dense substance (DS) is noted beyond the narrow squeezed cavity. MV: microvilli. Bar=5 µm. **B:** Higher magnification view of the epithelial cells around the taste pore in Fig. 1A. Intense immunoreactivity of actin filaments in epithelial cells, just adjacent to the pore cavity, is observed about 2-4 µm under the pore surface (arrows). Bar=1 µm.

Fig. 2 - Confocal laser micrographs of the longitudinal frozen sections of the fungiform papilla stained with rhodamine-phalloidin and FITC conjugated UEA-I. **A:** Strong reactions with rhodamine-phalloidin (shown as red) are identified near the top of the taste pore cavity (arrow) and in the apical portion of the taste cells in the taste bud. The taste bud is shown by white broken lines. Binding to UEA-I (shown as green) is observed near the surface of the taste pore cavity. Bar=20 μm. **B:** Higher magnification view of Fig. 2A. Strong reactions of actin filaments (shown as red spots indicated by arrows) are observed around the taste pore cavity a few micrometers below the papilla surface. Binding to UEA-I (shown as green) is observed in the upper taste pore cavity. Other strong reactions with rhodamine-phalloidin are observed in the apical portion of the taste cells (star). Bar=5 μm.

few micrometers below the papilla surface (Fig. 2A and B). Positive reactions with UEA-I were observed at the site of reaction with rhodamine-phalloidin (Fig. 2B), suggesting that the dense amorphous substances seen under a transmission electron microscope (Fig. 1) are glycoproteins. The positional relationships between the fluorescent products of the two reactions is consistent with the findings of immunohistochemical transmission electron microscopy. Another strong reaction with rhodamine-phalloidin was observed in the apical region of the taste bud (Fig. 2A and B). The distance between these two positivities was about 7 μm (Fig. 2B). This is consistent with the interval between grains in epithelial cells a few micrometers below the surface and in microvilli in the apical portion of the taste cells observed in the TEM image of Fig. 1A.

Scanning electron microscopy and double histochemical staining (rhodamine-phalloidin/FITC conjugated UEA-I) in horizontal cross sections

The central portion of the taste pore in fungiform papillae strongly binds to UEA-I in horizontal cross sections under a confocal laser microscope (Fig. 3A). Encircling this UEA-I binding, positive staining with rhodamine-phalloidin was observed as a ring shape in the same sample (Fig. 3A). The inner diameter of the rings was 3.1±0.8 μm (mean±SE, n=40). One, sometimes two, taste pores were observed in the central portion of the fungiform papilla surface by scanning electron microscopy (Fig. 3B). The diameter of the pore observed from right overhead was 3.0±0.5 μm (mean±SE, n=40) under the scanning electron microscope. These observations indicate that a ring of fluorescence due to actin filaments in the epithelial cells encircles taste pores.

DISCUSSION

In the present study, by comparing the staining with anti-actin antibody and with rhodamine-phalloidin, we clarified the identification and the localization of actin filaments in epithelial cells constructing the taste pore (taste pore cells). Actin filaments in the taste pore cells were present a few

Fig. 3 - Confocal laser micrograph of the taste pore in the fungiform papilla after staining with rhodamine-phalloidin and FITC conjugated UEA-I, and scanning electron micrograph of the dorsal surface of the rat tongue. **A:** Reaction with rhodamine-phalloidin (shown as red) is observed as a ring in the horizontal cross sections of the fungiform papilla near the surface under a confocal laser microscope. Other marginal lines of epithelial cells are faintly stained. In the center of the ring, another reaction with UEA-I (shown as green) is observed in the horizontal cross sections of the fungiform papilla near the surface. Bar=5 µm. **B:** A taste pore in the central portion of the fungiform papilla as observed under a scanning electron microscope. The pore is encircled by epithelial cells (taste pore cells). Bar=10 µm.

micrometers below the surface of papillae and encircled taste pores.

Glycoproteins occupy the taste pore cavity and cover the receptor microvilli of taste receptor cells (Brouwer and Wiersma, 1978; Witt and Miller, 1992; Menco and Hellekant, 1993). The UEA-I binding glycoproteins account for some of the electron-dense materials seen in the taste pore cavity. These substances were present in the upper portion of the cavity, adjacent to a ring of fluorescence obtained with rhodamine-phalloidin and immunohistochemical reactions with anti-actin antibody, indicating that ring fluorescence is due to actin filaments.

In addition, we obtained clear fluorescence in the apical region of the taste receptor cells about 10 µm below the surface of fungiform papillae. Intense immunoreactivity of actin filaments is found over receptor microvilli of taste cells in the apical portion of the taste bud (Jahnke and Baur, 1979; Takeda *et al.*, 1989) and some sensory organs (Flock and Cheung, 1977; Balboni and Vannelli, 1982). These observations indicate that the fluorescence obtained with rhodamine-phalloidin in the fungiform papillae under the confocal laser microscope is due to actin filaments.

The maintenance of taste pores requires the presence of nerve terminals, and denervation results in a loss of taste pores of taste buds (Oakley *et al.*, 1993). Since nerve fibers extend close to the taste pores (Azzali *et al.*, 1996; Kanazawa and Yoshie, 1996; Müller, 1996; Müller and Jastrow, 1998), the presence of nerve terminals around taste pores might contribute to the maintenance of taste pores through actin filament organization. Further study is required to clarify how the neurotrophic action is involved in the maintenance of actin filament organization.

Taste stimulation modulates taste pore size (Graziadei, 1969; Henkin and Bradley, 1969; Ciges *et al.*, 1976). It is possible that the taste pores are contractile. The special localization of actin filaments in taste pore cells is likely related to a contractile function of the pores, since actin filaments are assumed to contribute to contractile function in many types of cells (Aderem, 1992). The upper portion of the pore cavity filled with UEA-I reactive glycoproteins is rather narrow, and actin filaments are localized here. Treatment of fungiform papillae with cytochalasin D enlarges the diameter of the ring of actin filaments

around taste pores (Ohishi *et al.*, 1999). Thus, it is likely that actin filaments are involved in the constriction of taste pores. The contractile function of unfixed taste pores has yet to be demonstrated, but high resolutive observation of unfixed taste pores should provide an insight into the contractile function of taste pores.

In conclusion, actin filaments are identified in taste pore cells as a ring around the squeezed taste pore cavity. The localization of actin filaments encircling taste pores might be related to the constriction of taste pores.

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