Immunolocalization of SNARE proteins in both type II and type III cells of rat taste buds*

Katsura Ueda, Yasuo Ichimori, Hiroyuki Okada, Shiho Honma, and Satoshi Wakisaka

Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan

Summary. Double immunohistochemistry of soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins [synaptosomal-associated protein of 25kDa (SNAP-25), syntaxin and vesicle-associated protein-2 (VAMP-2)], and specific cell markers of taste buds cells [α -gustducin and phospholipase $C\beta 2$ (PLC $\beta 2$) for type II cells; neural cell adhesion molecule (NCAM) for type III cells] was applied to gustatory epithelia of the rat circumvallate papillae. All three SNARE proteins were present in some elongated taste buds cells as well as intra-, peri- and subgemmal nerve fibers. Double immunohistochemistry revealed that nearly all α -gustducin and PLC $\beta 2$ immunoreactive cells expressed SNAP-25, syntaxin, and VAMP-2. A majority of NCAM immunoreactive cells showed immunoreactivity for these SNARE proteins. These results indicate that these synapse-associated proteins (SNAP-25, syntaxin and VAMP-2) are present in both type II cells and type III cells. Moreover, more than 50% of intragemmal cells containing SNARE proteins showed immunoreactivities for α -gustducin,

PLC $\beta 2$, and NCAM, suggesting the possible presence of transitional cells having histochemical properties of both type II and type III cells.

Introduction

Mammalian taste buds are localized mostly in lingual and palatal epithelia, and a few taste buds are present at the laryngeal surface of the epiglottis. In the lingual epithelium of the rat, taste buds are localized in three lingual papillae — fungiform, foliate and circumvallate papillae — and taste buds are also present in the nasoincisor papilla and soft palate. Taste buds contain proliferative basal cells (progenitor cells) as well as 50–80 elongated epithelial cells (taste cells). Traditionally, taste cells are classified into two types of cells — dark and light. Dark cells or type I cells have an electron-dense cytoplasm, and are thought to be supporting cells. Light cells are characterized by the presence of an electron-lucent cytoplasm and are further divided into two types in regard to the presence of synapses with nerve terminals of the gustatory nerve fibers. Light cells without apparent synapse contact are called type II cells, and those with a synapse contact with a gustatory nerve are termed type III cells (Murray *et al.*, 1969; Takeda and Hoshino, 1975).

Tastants may depolarize taste cells via direct opening membrane channels or via second-messenger systems, and the depolarization of taste cells induces neurotransmitter release by a mechanism probably similar to that occurring at presynaptic nerve terminals. It is known that many synapse-associated proteins are involved in synaptic vesicle cycles; namely docking, priming, fusion, exocytosis, and recycling during neurotransmitter release. One of the key events in neurotransmitter release from synaptic terminals is the formation of the soluble *N*-ethylmaleimide-sensitive fusion protein attachment

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Address for correspondence: Prof. Satoshi Wakisaka, Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry, 1-8, Yamadaoka, Suita, Osaka, 565-0871 Japan
Tel: +81-6-6879-2871, Fax: +81-6-6879-2875
E-mail: wakisaka@dent.osaka-u.ac.jp

protein receptor (SNARE) complex. The SNARE complex consists of three main proteins: the synaptosomal-associated protein of 25kDa (SNAP-25), syntaxin, and the vesicle-associated membrane protein (VAMP, also called synaptobrevin). Previous immunohistochemical studies showed the presence of SNAP-25 (Pumplin and Getschman, 2000; Yang *et al.*, 2000a, 2004; Yee *et al.*, 2001), syntaxin (Aguado *et al.*, 1999; Pumplin and Getschman, 2000), and VAMP (Pumplin and Getschman, 2000; Yang *et al.*, 2004) in a subset of taste bud cells and nerve fibers as well as other synapse-related proteins such as synapsin (Finger *et al.*, 1990), synaptophysin (Pumplin and Getschman, 2000) and synaptotagmin 1 (Kohno *et al.*, 2005). As type III cells make synaptic contact with gustatory nerve terminals and contain many synaptic vesicles (Murray *et al.*, 1969; Takeda and Hoshino, 1975), it is reasonable that type III cells contain these synapse-related proteins; indeed, both VAMP and SNAP-25 are localized in the type III cells.

Recently, much evidence has been accumulated in support of the idea that type II cells, which do not have apparent synaptic contacts with gustatory nerve terminals, act as taste receptor cells. Immunohistochemical studies demonstrated that type II cells contain α -gustducin, a taste-specific G protein, phospholipase C β 2 (PLC β 2), and type III inositol 1, 4, 5-triphosphate receptor (IP3R3), all the downstream components of the taste transduction cascade (Boughter *et al.*, 1997; Cho *et al.*, 1998; Yang *et al.*, 2000b; Miyoshi *et al.*, 2001; Clapp *et al.*, 2001, 2004), and that type III cells express immunoreactivity for the neural cell adhesion molecule (NCAM) (Nelson and Finger, 1993; Smith *et al.*, 1993). Recently, the presence of SNARE proteins in the type II cell has been reported. Yang *et al.* (2004) demonstrated the presence of VAMP-2 in type II cells (IP3R3 immunoreactive cells) as well as type III cells. There is argument over the presence of SNAP-25 in the type II cells; Yang *et al.* (2000a) reported that SNAP-25 is present in type III cells only, while Oike *et al.* (2006) reported that SNAP-25 is also detected in type II cells (PLC β 2-immunoreactive cells). In the present study, therefore, we re-examined the relation between SNARE proteins (SNAP-25, syntaxin and VAMP-2) and specific markers of type II cells (α -gustducin and PLC β 2) and type III cells (NCAM) in the taste buds of circumvallate papillae of the rat.

Materials and Methods

Animals and tissue preparation

Male Sprague-Dawley rats, weighing 200–250g, were

used in the present study. All animal experiments were reviewed and approved by Intramural Animal Use and Care Committee at Osaka University Graduate School of Dentistry prior to the experiments (Project # 03-002).

The animals were deeply anesthetized with chloral hydrate (600mg/kg, i.p.), and transcardially perfused with a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in a 0.1M phosphate buffer (PB; pH 7.2) followed by 0.02M phosphate-buffered saline (PBS; pH 7.4). Circumvallate papillae were carefully removed and further fixed in 4% paraformaldehyde in 0.1M PB for an additional 2–3 days. Specimens were dehydrated through an ascending series of ethanol, embedded in paraffin, and cut at a thickness of 4–5 μ m.

Paraffin sections were deparaffinized with xylene, rehydrated through a descending series of ethanol, and rinsed with PBS. Sections were treated with 0.1M sodium citrate buffer (pH 6.0) for 5 min in a microwave for antigen retrieval and then processed for the indirect immunofluorescence method. Sections were incubated either with monoclonal anti-SNAP-25 (1:500; Synaptic System, Göttingen, Germany), or with monoclonal anti-syntaxin (1:500; SIGMA, St. Louis, MO), or with monoclonal anti-VAMP-2 (1:2000; Synaptic System) for 16–18 hours at room temperature. All monoclonal antibodies were raised in a mouse. Sections were then incubated with either Alexa488- or Cy3-conjugated anti-mouse IgG preabsorbed with rat IgG (1:100; Vector, Burlingame, CA) for 90 min. They were coverslipped with Vectashield (Vector) and observed with a fluorescein microscope (Axioskop 2 plus, Carl Zeiss, Germany) with an appropriate exciting filter. Fluorescent images were captured with a CCD camera (AxioCam, Carl Zeiss).

For the double labeling experiment, the sections were first incubated either with monoclonal anti-SNAP-25 (1:500), or monoclonal anti-syntaxin (1:500), or with monoclonal anti-VAMP-2 (1:500) for 16–18 h, and then either with polyclonal anti- α -gustducin (1:1000; Santa Cruz Biotechnologies, Santa Cruz, CA), polyclonal anti-PLC β 2 (1:500; Santa Cruz Biotechnologies), or with polyclonal anti-NCAM (1:1000; Chemicon International, Temecula, CA) for 16–18 h each at room temperature. All polyclonal antibodies were raised in a rabbit. Following several rinses with PBS, sections were then incubated with a mixture of Alexa 488-conjugated anti-rabbit IgG (1:500; Molecular Probe, OR) and Cy3-conjugated anti-mouse IgG preabsorbed with rat IgG (1:100; Molecular Probe) for 90 min at room temperature. The immunostained sections were mounted onto gelatin-subbed glass slides, cover-slipped with VectaShield, and observed with a fluorescein microscope (Axioskop 2 plus) or confocal laser scanning microscope (LSM510, Carl Zeiss). All

Fig. 1. SNAP-25 (A), syntaxin (B) and VAMP-2 (C) in the circumvallate papilla of the adult rat. Some elongated taste cells (arrows) show an immunoreactivity for SNAP-25 (A), syntaxin (B), and VAMP-2 (C). SNAP-25 and VAMP-2 are also detected in intra-, peri- and subgemmal nerve fibers. Scale bar = 100 μ m

fluorescent images were transferred to Adobe Photoshop (Ver. 5.0).

For quantification, 15 randomly selected taste bud profiles from circumvallate papillae having a taste pore were used. The numbers of cells showing SNAP-25, syntaxin, VAMP-2, α -gustducin, PLC β 2, and NACM immunoreactivity were counted. Only those cells having nuclear profiles were counted.

Results

SNARE proteins in circumvallate papillae

SNAP-25, syntaxin, and VAMP-2 immunoreactivities were detected in a subset of elongated taste bud cells (Fig. 1A, B, C). Immunoreactions were restricted within the cytoplasm, and their nuclear profiles were devoid of immunoreactions. In addition to taste buds cells, intragemmal and perigemmal nerve fibers as well as subgemmal nerve fibers in the lamina propria also expressed immunoreaction for SNAP-25 and VAMP-2 (Fig. 1A, C)

Relation between SNARE proteins and type II cells

In the cross sections of circumvallate papillae, taste buds cells were clearly recognized by round profiles. All SNARE proteins were localized in the cytoplasm in the subsets of the taste cells (Fig. 2A, D, G, Fig. 3A, D, G). Double labeling showed a majority of α -gustducin and PLC β 2 immunoreactive cells to have an immunoreaction for SNARE proteins, while some cells having immunoreactivity for SNAP-25, syntaxin and VAMP-2 lacked any immunoreaction for α -gustducin and PLC β 2 (Fig. 2C, F, I). Quantitative analysis showed that all α -gustducin immunoreactive cells displayed an immunoreaction for SNAP-25 (98/98), syntaxin (283/283), and VAMP-2 (204/204). Similarly, all PLC β 2 immunoreactive cells exhibited SNAP-25 (128/128) and syntaxin (256/256), and almost all PLC β 2 immunoreactive cells showed VAMP-2 immunoreactivity (97.1%; 165/170). In contrast, nearly 70% of SNAP-25 immunoreactive cells showed α -gustducin (68.5%; 361/527) and PLC β 2 (75.8%; 336/443). Approximately 57.9% (165/285) and 55.4% (256/462) of syntaxin immunoreactive cells had α -gustducin and PLC β 2, respectively. Similarly, approximately 64.6% (204/316) and 68.9% (283/411) of VAMP-2 immunoreactive cells showed an immunoreactivity for α -gustducin and PLC β 2, respectively.

Fig. 2. Double labeling of SNARE proteins (SNAP-25, syntaxin, VAMP-2) and the type II marker (PLC β 2 and α -gustducin) in the rat circumvallate papilla. **A–C:** SNAP-25 (**A**), PLC β 2 (**B**) and their merged image (**C**). All PLC β 2 immunoreactive cells exhibit SNAP-25 immunoreactivity, while some SNAP-25 immunoreactive cells do not have an immunoreaction for PLC β 2 (arrows). **D–F:** Syntaxin (**D**), α -gustducin (**E**) and their merged image (**F**). All α -gustducin immunoreactive cells exhibit syntaxin immunoreactivity, while some syntaxin immunoreactive cells lack any immunoreactivity for α -gustducin (arrows). **G–I:** VAMP-2 (**G**), PLC β 2 (**H**) and their merged image (**I**). All PLC β 2 immunoreactive cells exhibit VAMP-2 immunoreactivity, while some VAMP-2 immunoreactive cells are devoid of any immunoreaction for PLC β 2 (arrows). Scale bar = 50 μ m

Fig. 3. Double labeling of SNARE proteins (SNAP-25, syntaxin, VAMP-2) and the type III marker (NCAM) in the rat circumvallate papilla. Nearly all NCAM immunoreactive cells (**B**, **E**, **H**) display an immunoreactivity for SNAP-25 (**A**), syntaxin, (**D**) and VAMP-2 (**G**), while some cells showing immunoreactivity for SNARE proteins lack this in NCAM (arrows). Scale bar = 50 μ m

SNARE proteins and type III cells

NCAM immunoreactivity was detected in the cytoplasm of intragemmal cells and was devoid of nuclei. The immunoreactions in individual cells varied among individual cells; immunoreactions were restricted to the cell surface in some cells, and they were diffusely observed in the entire cytoplasm in other cells (Fig. 3B, E, H). In addition to intragemmal cells, a part of intragemmal and perigemmal nerve fibers also showed immunoreactivity. Double labeling showed a majority of NCAM immunoreactive cells displayed immunoreactivity for SNAP-25 (95.0%; 134/141, Fig. 3A–C), syntaxin (97.7%; 336/344, Fig. 3D–F), and VAMP-2 (87.8%; 361/411, Fig. 3G–I). Not all cells having SNARE protein immunoreactivity showed NCAM immunoreaction; approximately 49.4% (134/271) SNAP-25-, 82.0% (128/156) of syntaxin- and 59.4% (98/165) of VAMP-2-immunoreactive cells showed an immunoreaction for NCAM.

Discussion

The present study showed that a subset of elongated intragemmal cells as well as intra-, peri- and subgemmal nerve fibers showed immunoreactivities for SNAP-25, syntaxin, and VAMP-2, in accordance with previous studies (Aguado *et al.*, 1999, Pumplin and Getschman, 2000; Yang *et al.*, 2000a, 2004; Yee *et al.*, 2001). Moreover, a double labeling experiment revealed that nearly all α -gustducin- or PLC β 2- or NCAM-immunoreactive elongated taste cells exhibited an immunoreactivity for SNAP-25, syntaxin, and VAMP-2.

Both intragemmal and perigemmal nerve fibers as well as some subgemmal nerve fibers showed immunoreactivities for SNAP-25 and VAMP-2, although these proteins are closely associated with the presynaptic plasma membrane. Aguado *et al.* (1999) reported that syntaxin 1 was present within intragemmal and perigemmal nerve fibers. We recently reported that mRNA and proteins of SNAP-25, syntaxin, and VAMP-2 are present in the trigeminal ganglion neurons, and that ligation of the inferior alveolar nerve causes the accumulation of these proteins at the site proximal to the ligation (Honma *et al.*, 2005). The presence of syntaxin-1 in the dorsal root ganglion neurons also has been reported (Aguado *et al.*, 1999). Moreover, synaptic vesicles were also present in the nerve terminals of the gustatory nerve which makes synaptic contact with type III cells of the guinea pig (Yoshie *et al.*, 1990). Taking all these finding together, we speculated that these three SNARE

proteins in intra-, peri- and subgemmal nerve fibers of the circumvallate papillae were synthesized and transported from petrosal ganglion neurons. Further analysis is required whether petrosal ganglion neurons express these SNARE proteins.

SNAP-25 is a neuron-specific protein associated with the presynaptic membrane. A previous study by Yang *et al.* (2000a) showed that more than 90% (80/87) of taste cells with synapses displayed an immunoreactivity for SNAP-25 using an immunoelectron microscopic technique, indicating that SNAP-25 is present in type III cells. However, Oike *et al.* (2006) reported that SNAP-25 is coexpressed in group IIA phospholipase A2 that is co-localized with PLC β 2, suggesting the possible co-localization of SNAP-25 with PLC β 2. Our present results showing that nearly all SNAP-25 cells are immunoreactive for α -gustducin or PLC β 2 are consistent with those from Oike *et al.* (2006). One of the possible explanations for the discrepancy on the immunohistochemical localization of SNAP-25 in taste buds cells is due to the difference of antibodies. A biochemical study reported the presence of two isoforms of SNAP-25, SNAP-25A, and SNAP-25B, which differ in 9 amino acids, including 6 amino acids in the N-terminal SNARE motif and 3 amino acids in the linker region, (Bark and Wilson, 1994). Yang *et al.* (2000a) used a monoclonal antibody, while Oike *et al.* (2006) examined with a polyclonal antibody. We used different clones of a monoclonal antibody. Different antibodies that may recognize the different epitopes of different isoforms of SNAP-25 resulted in the different staining patterns.

Recent studies showed that IP3R3 is a taste-signaling molecule related to the transduction of bitter, sweet and umami tastes (Perez *et al.*, 2002; Zhang *et al.*, 2003), and that IP3R3 is localized in type II cells (Miyoshi *et al.* 2001; Clapp *et al.*, 2001). Yang *et al.* (2004) reported that approximately 73% of VAMP-2 immunoreactive cells also exhibit IP3R3 immunoreactivity, suggesting the presence of VAMP-2 in type II cells. Our present results on the colocalization of VAMP-2 with α -gustducin and PLC β 2 further support the idea that VAMP-2 is also present in type II cells.

The colocalization among SNAP-25, syntaxin, and VAMP-2 was not examined in the present study. The present quantification that nearly all SNAP-25, syntaxin, and VAMP-2 immunoreactive cells showed α -gustducin and PLC β 2 indicates that these three SNARE proteins were colocalized. This speculation is supported by another group showing the partial colocalization of SNAP-25 and VAMP-2 (Yang *et al.*, 2004).

The functional significance of the presence of SNAP-25, syntaxin, and VAMP-2 in type II cells remains

to be elucidated. One possible explanation is that the immunoreaction for SNARE proteins we observed in type II cells is related to the subsurface cisternae known to exist in type II cells, and can be considered to have an important role in neurotransmitter release. IP3R3-immunoreactive cells were reported to be associated with subsurface cisternae in an ultrastructural study (Clapp *et al.*, 2004), and approximately three fourths of VAMP-2 immunoreactive cells express IP3R3 immunoreactivity (Yang *et al.*, 2004). It is therefore possible that VAMP-2—and also SNAP-25 and syntaxin—in the type II cells are also related to the subsurface cisternae and take part in taste transmission.

The similarity between type II and type III cells regarding the expression of SNARE proteins provides a cue for understanding the cell-lineage of taste bud cells. Most taste bud cells have a life span of 9–10 days (Beidler and Smallman, 1965; Farbman, 1980), indicating that rapid cell differentiation occurs in the taste buds. It has been now accepted that round cells at the basal portion of taste buds, i.e. basal cells (type IV cells), are progenitor cells that differentiate into other types of taste bud cells. There are three theories concerning the cell-lineage of taste buds. The first is that all types of taste buds cells have the same progenitor cells (one-cell line theory). The second is a "multi-cell line theory": each type of spindle-shaped cell (type I, type II and type III cells) has its own progenitor cells—there are at least three types of progenitor cells that differentiate into type I, I and III cells, respectively. The third is a mixture of the one-cell line theory and multi-cell line theory; namely there are two types of progenitor cells: those differentiated into dark cells (type I cells) and light cells (type II and III cells)—both type II cells and type III cells—are in the same cell lineage. If type III cells differentiate into type II cells, the histochemical properties of immature type II cells have a similarity to that of (older) type III cells, or there are transitional cells having similar histochemical properties of type II cells and type III cells. One example of the possible presence of transitional cells is cells expressing protein gene product 9.5 (PGP 9.5). Yee *et al.* (2001) reported that there are two subpopulations of PGP 9.5-immunoreactive cells: a majority of PGP 9.5-immunoreactive cells are type III cells that do not express any immunoreactivity for serotonin, and very few PGP 9.5-immunoreactive cells belong to type II cells that do not show immunoreactions for α -gustducin. The presence of SNARE proteins in both type II and type III cells is also explained by the presence of transitional cells between type II and type III cells.

Despite the fact that nearly all SNAP-25-, syntaxin- and VAMP-2-immunoreactive taste cells express α -

gustducin or PLC β 2 immunoreactivity, a few NCAM immunoreactive cells lack any immunoreaction for SNAP-25, syntaxin, or VAMP-2. There are two possible explanations: one is that, although VAMP, syntaxin and/or SNAP-25 are present in the type III cells, the antibodies used in the present study could not recognize the epitope of antigen. Similar to the presence of the isoform of SNAP-25, among the several isoforms of syntaxin and VAMP-2, syntaxin-1A, 1B, 2A and 2B, and VAMP-2A and VAMP-2B, are major isoforms in neuronal tissues. The other explanation is that other proteins participate in synapse transmission. This speculation is supported by the fact that not all synapses use SNAP-25 (Duc and Catscias, 1995).

It is now accepted that α -gustducin and PLC β 2 are present only in type II cells and that NCAM is present only in type III cells. In the present quantitative analysis, nearly all intragemmal cells displaying an immunoreactivity for SNARE proteins have immunoreactions for α -gustducin or PLC β 2, and a majority of SNARE protein immunoreactive cells showed immunoreaction for NCAM. In addition, nearly 70% and 60% of VAMP-2 immunoreactive cells displayed PLC β 2 and NCAM immunoreactivity, respectively, suggesting that at least 30% of VAMP-2 immunoreactive cells displayed an immunoreactivity for both PLC β 2 and NCAM. Similar results by Miura *et al.* (2005) report that approximately 20% of NCAM immunopositive cells express mRNA for α -gustducin in adult mice. The possible presence of taste cells expressing both α -gustducin/PLC β 2, markers of type II cells, and NCAM, a marker for type III cells, further supports the idea of the presence of transitional cells between type II and type III cells, although the definition of type II and type III was originally characterized by the ultrastructural profiles of rabbit foliate papillae (Murray *et al.*, 1969).

In conclusion, the present study revealed that SNAP-25, syntaxin, and VAMP-2 are present in both type II and type III cells as well as intragemmal and perigemmal nerve fibers of circumvallate papillae of the adult rat. The role of these proteins in type II cells that have no synaptic contacts remains to be elucidated. In addition, the partial colocalization of α -gustducin/PLC β 2 and NCAM suggests the possible presence of transitional cells having histochemical properties of both type II and type III cells.

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