Expression of synaptotagmin 1 in the taste buds of rat gustatory papillae*

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Summary. Synapses between taste receptor cells and primary sensory afferent fibers transmit the output signal from taste buds to the central nervous system. The synaptic vesicle cycle at the synapses involves vesicle docking, priming, fusion, endocytosis, and recycling. Many kinds of synaptic vesicle proteins participate in synaptic vesicle cycles. One of these, synaptotagmin 1, binds Ca²⁺ phospholipids with high affinity and plays a role in Ca²⁺ regulated neurotransmitter release in the central and peripheral nervous systems. However, the expression patterns of synaptotagmin 1 in rat taste tissues have not been determined. We therefore examined the expression patterns of synaptotagmin 1 and several cell specific markers of type II and III cells in rat taste buds. RT-PCR assay showed that synaptotagmin 1 mRNA was expressed in circumvallate papillae. In fungiform, foliate, and circumvallate papillae, the antibody against synaptotagmin 1 yielded the labeling of a subset of taste bud cells and intra- and subgennal nerve processes. Double labeled experiments showed that synaptotagmin 1 positive cells co-expressed type III cell markers, PGP 9.5, and NCAM. Intragenimal nerve processes positive for synaptotagmin 1 co-expressed PGP 9.5. Conversely, all synaptotagmin 1 expressing cells did not co-express type II cell markers, PLCβ2, or gustducin. These results show that synaptotagmin 1 may play some regulatory roles in vesicle membrane fusion events with the plasma membrane at the synapses of type III cells in rat taste buds.

Introduction

In mammals, taste buds comprise a heterogeneous population of 50–80 taste cells specialized for the detection of aqueous chemical stimuli. Taste cells also can be classified on the basis of their morphological and immunocytochemical characteristics. Dark cells or type I cells possess an electron-dense cytoplasm with several 100–400 nm electron-dense granules in their apical processes and abundant chromatin and are slender in shape (Murray, 1973). Type I cells are thought to have supporting and secretory functions. Type II cells have an electronlucent cytoplasm and large circular or ovoid nuclei without apparent synaptic vesicles (Kinnaman et al., 1985). Gustducin, phospholipase Cβ2 (PLCβ2), and type III IP₃ receptor (IP₃R3), the downstream components of the taste transduction cascade, are immunocytochemical markers of type II cells (Clapp et al., 2001, 2004; Yang et al., 2000b). Type III cells contain both small, clear, synaptic type vesicles and dense-core granules and make synaptic contacts with gustatory nerves (Murray et al., 1969; Takeda and Hoshino 1975). In type III cells, three immunocytochemical markers, serotonin (5-HT), ubiquitin carboxyl terminal hydrolase (PGP 9.5), and the neural cell adhesion molecule (NCAM), have been reported (Takeda et al., 1992; Nelson and Finger, 1993; Kim and Roper, 1995; Kanazawa and Yoshie, 1996; Yee et al., 2001). Approximately 50% of taste bud cells can be classified as

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type I cells, 15–30% as type II cells, and 10% as type III cells (Murray, 1973).

Synaptic vesicle exocytosis requires three neuronal SNAREs: vesicle-associated membrane protein (VAMP2, also called synaptobrevin), syntaxin 1, and the 25-kDa synaptosomal-associated protein (SNAP-25) (Li and Chin, 2003). Synaptobrevin-2 and SNAP-25 are expressed in type III cells of rat taste buds (Yang et al., 2000a, 2004). In addition to these SNAREs, synaptic vesicle exocytosis uses a set of unique components—such as synaptogamin, complexin, Munc13, and RIM—to meet the special needs of fast Ca\(^{2+}\)-triggered neurotransmitter releases in the central and nervous systems. Among these molecules, synaptogamin 1 is the best-characterized candidate Ca\(^{2+}\) sensor in triggering neurotransmitter releases (Li and Chin, 2003). Ca\(^{2+}\)-binding to synaptogamin reacts with the SNAREs and triggers the exocytosis of synaptic vesicles. Synaptogamin 1 is an integral membrane glycoprotein of neuronal synaptic vesicles and secretory granules of neuroendocrine cells that is widely expressed in the central and peripheral nervous systems. Synaptogamin 1 has a variable N-terminal domain that is exposed to the lumen of the vesicle and a conserved cytoplasmic tail that contains two Ca\(^{2+}\)-binding C2-domains. At least eight different isoforms of synaptogamin (synaptogamin 1–8) are expressed in the brain, four of which (synaptogamin 4, 5, 7 and 8) are also expressed in non-neuronal tissues (Ullrich et al., 1994; Li et al., 1995). The deletion of synaptogamin 1 results in severely impaired Ca\(^{2+}\)-triggered neurotransmitter releases (Geppert et al., 1994). Thus, we became interested in synaptogamin 1 as a candidate for the Ca\(^{2+}\) sensor in triggering neurotransmitter releases in taste buds. Although the expression patterns of neuronal SNAREs, SNAP-25 and synaptobrevin-2 have been revealed, no expression patterns for synaptogamin 1 have been reported.

In the present study, we used a reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry to examine the expression of synaptogamin 1 in rat gustatory tissues. We further performed double immunolabelings to compare the expression patterns of synaptogamin 1 relative to gustducin, PLC\(\beta\)2, PGP 9.5, or NCAM.
**Materials and Methods**

Methods used in this study were approved by the Institutional Animal Care and Use Committee at Kyushu Dental College.

**RT-PCR**

Adult male Sprague-Dawley (SD) rats were killed *via* overdose with sodium pentobarbital by an intraperitoneal injection. The lingual tissues containing circumvallate papillae, lingual tissues without taste papillae, and whole brain were removed, and total RNAs were isolated using RNeasy Protect Mini (QIAGEN, Japan) as described by the manufacturer’s instruction and incubated with DNase I to remove any contaminating genomic DNA. First-strand cDNA syntheses were performed by the reverse transcription of 1 μg of total RNA using a Omniscript™ Reverse Transcriptase (QIAGEN) with oligo dT primer. Primer sequences for each PCR were:

-synaptotagmin 1: 5’-ATGGCTGTGATGACTTTGATC GCT-3’(forward) and 5’-GAAGACTTTGCGATGCGTGTT-3’ (reverse); product size, 456bp.

-GAPDH: 5’-TGAAGGTGGTGTGAAACGGA-3’(forward) and 5’-GTACATCCGGTACTCCAGGT-3’ (reverse); product size, 984bp. PCR amplifications were performed under the following conditions: 94°C for 30 sec, 57°C for 1 min, and 72°C for 1 min for a total of 40 cycles for synaptotagmin 1. The reverse transcriptase step was omitted in the controls to confirm the removal of all genomic DNA. Amplification products were analyzed on 2% agarose gels and visualized with ethidium bromide. All PCR products were gel-purified, cloned into pGEM-T easy cloning vector (Promega Madison, WI, USA), and transformed into XL-1 BLUE MRF+ competent cells. Plasmid DNAs were purified using QIAGEN Plasmid Midi Kits (QIAGEN) and sequenced with Thermo Sequenase II dye terminator cycle sequencing kits (Amersham, Pharmacia Biotech Inc., Piscataway, PH, USA). The sequence reactions were analyzed by an ABI373S DNA sequencer (PerkinElmer, USA).

**Immunohistochemistry**

Rats were anesthetized deeply with intraperitoneal injection of chloral hydrate (350 mg/kg) and transcardially perfused with a fixative containing 0.5% picric acid and 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3) for
Fig. 3. Double-label immunohistochemical images of synaptotagmin 1 and gustducin or PLCβ2 in rat circumvallate taste buds. Immunofluorescence of synaptotagmin 1 (red, a), gustducin (green, b) and their overlay (c) in a transverse section. Immunofluorescence of synaptotagmin 1 (red, d), PLCβ2 (green, e) and their overlay (f) in a transverse section. All cells positive for synaptotagmin 1 did not co-express gustducin and PLCβ2. Scale bars: 20 μm (a–f).

15 min. The circumvallate, foliate, and fungiform papillae were removed and rinsed overnight in a phosphate buffer containing 30% sucrose. Tissues were then embedded in Tissue-Tek and snap-frozen in a dry ice-isopentane mixture. Sections were cut at 8 μm in a cryostat, thaw-mounted onto MAS-coated glass slides (Matsunami, Japan), and air-dried for 30 min. After a brief wash in phosphate-buffered saline (PBS), the section were treated with a 10 mM citrate buffer (pH 6.0) for 10 min in an autoclave at 121°C. After a 5 min wash in PBS, the sections were placed in a humidified chamber and preincubated with a solution of 0.15% normal goat serum (Vector Laboratories Inc., Burlingame, CA, USA) in PBS for 40 min.

Immunohistochemical staining was performed using the indirect immunofluorescence method with antibodies to synaptotagmin 1 (1:50; BD Transduction Laboratories, USA) as the primary antibody and Alexa 546-conjugated goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR, USA) as the secondary antibody, and then observed under a fluorescence microscope. The specificity of the synaptotagmin 1 immunoreactivity was determined by substitution of the buffer for the primary antibody. Double-labeled experiments for synaptotagmin 1 and gustducin, PLCβ2, PGP 9.5, or NCAM involved incubations with the anti-synaptotagmin 1 mouse monoclonal antibody and anti-gustducin rabbit polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, UA, USA), anti-PLCβ2 rabbit polyclonal antibody (1:1,500; Santa Cruz Biotechnology), anti-PGP 9.5 rabbit polyclonal antibody (1:1,600; Ultra Clone Ltd., Isle of Wight, UK), or anti-NCAM rabbit polyclonal antibody (1:200; Chemicon International, Temecula, CA, USA) as primary antibodies and subsequent incubation with Alexa 488-conjugated goat anti-rabbit IgG (1:200) and Alexa 546-conjugated goat anti-mouse IgG (1:200) as secondary antibodies.

The sections were then washed in PBS and cover slipped with Vectashield (Vector Laboratories). All images were obtained by changing the filter cube without altering the portion of the section and focus with a cooled CCD camera (Olympus, Tokyo). Images were overlaid with Adobe Photoshop (Adobe systems; USA). Quantitative analyses were performed from randomly selected fields of the transverse sections. The numbers of cells showing immunoreactivity for synaptotagmin 1, PGP 9.5, and NCAM were counted.

Results

RT-PCR analysis

To examine the presence of mRNAs for synaptotagmin 1, we used RT-PCR in rat circumvallate papillae. In the RT-PCR for synaptotagmin 1, RNAs from the whole brain and circumvallate papillae yielded amplification products of the expected size and sequence. RT-PCR analyses showed that mRNAs for synaptotagmin 1 were expressed in the circumvallate papillae (Fig. 1). No fragment was detected when the reverse transcriptase was omitted from the reverse transcription mixture, indicating the absence of contamination by genomic DNA.
**Immunohistochemistry**

In cryosections of fungiform, foliate, and circumvallate papillae, the monoclonal antibody against synaptotagmin 1 gave labeling of a subset of taste cells and nerve processes of all taste buds examined (Fig. 2). Positive signals for synaptotagmin 1 were detected in intra- and subgenmal nerve processes and 10% of the cells within taste buds in all gustatory papillae. In contrast, no positive signals for synaptotagmin 1 were detected in the nerve processes in the connective tissues below the taste bud-bearing epithelium. In the fungiform papillae, synaptotagmin 1 immunopositive nerves occasionally appeared in the epithelium outside the taste bud (Fig. 2b).

In double immunolabeling of synaptotagmin 1 and gustducin or PLCβ2, synaptotagmin 1 expressing taste bud cells did not co-express gustducin or PLCβ2 (Fig. 3). In double immunolabeling of synaptotagmin 1 and PGP 9.5 or NCAM, approximately 90.9% (20/22) of PGP 9.5 expressing cells showed synaptotagmin 1 immunoreactivity (Fig. 4a–c, arrowheads). However, not all PGP 9.5-positive cells showed synaptotagmin 1 immunoreactivity (Fig. 4b, c, arrows). Approximately 87.0% (20/23) of NCAM expressing cells showed synaptotagmin 1 immunoreactivity (Fig. 4d–f, arrowheads), but a subset of NCAM-positive cells did not co-express synaptotagmin 1 (Fig. 4e, f, arrows). These expression patterns are illustrated graphically in Figure 5.
Discussion

In the present study, we found that synaptotagmin 1 mRNA was expressed in circumvallate papillae by using RT-PCR. Positive signals for synaptotagmin 1 were detected in a subset of taste bud cells in all gustatory papillae examined by using immunohistochemistry. All synaptotagmin 1 expressing cells co-expressed PGP 9.5 and NCAM. It is known that synaptotagmin exhibits Ca\textsuperscript{2+}-dependent interactions with a variety of other molecules, including the SNARE complex (Li and Chin, 2003). One of the SNAREs, synaptobrevin-2, is co-localized with serotonin, PGP 9.5, as is another of the SNAREs, SNAP-25, in a subset of type III cells (Yang et al., 2000a; Yang et al., 2004). In addition, serotonin localizes in a subset of NCAM expressing taste cells (Yee et al., 2001). Taken together, these facts show that synaptotagmin 1 may regulate the release of serotonin from type III cells as the Ca\textsuperscript{2+} sensors with the members of SNAREs, SNAP-25 and synaptobrevin-2.

Positive signals for synaptotagmin 1 were detected in intra- and subgennal nerve processes. Synaptobrevin-2 is also expressed in both intra- and subgennal nerve processes in which numerous vesicles are present (Yang et al., 2004). By using colloidal gold immunoelectron microscopy with a synaptobrevin-2 antibody, the vesicles display synaptobrevin-2 immunoreactivities labeling with colloidal gold particles in some nerve processes. In addition, SNAP-25 is co-expressed with synaptobrevin-2 in nerve processes (Yang et al., 2000a). These results suggest that synaptotagmin 1 may play some regulatory roles in vesicle membrane fusion events with synaptobrevin-2 and SNAP-25 in intra- and subgennal nerve processes and taste bud cells. In the fungiform papillae, synaptotagmin 1 immunopositive nerves occasionally appeared in the epithelium outside the taste bud. These nerves may derive from the lingual branch of the trigeminal nerves that promote somatosensory functions.

Synapses of synaptotagmin 1 knockout mice lack the fast-component of Ca\textsuperscript{2+} dependent neurotransmitter release but exhibit no changes in the slow. Ca\textsuperscript{2+} independent component of synaptic vesicle exocytosis (Geppert et al., 1994). It should be interesting to analyze the roles of synaptotagmin 1 in taste transduction by use of synaptotagmin 1 knockout mice. A number of possible neurotransmitter candidates have been found in taste buds. These include enkephalin (Yoshie et al., 1993), γ-aminobutyric acid (GABA) (Obata et al., 1997) and glutamate (Astbäck et al., 1995). A comparison of the expression and localization patterns between synaptotagmin 1 and these neurotransmitter candidates in taste buds should also prove interesting. In the present study, we clearly showed that synaptotagmin 1 and PGP 9.5 or NCAM were co-expressed in the rat taste buds. Since PGP 9.5 and NCAM are known to be possible markers of type III cells (Takeda et al., 1992; Nelson and Finger, 1993; Kanazawa and Yoshih, 1996; Yee et al., 2001), the co-expression of synaptotagmin 1 and these proteins suggests that synaptotagmin 1 can be used as an additional marker for type III cells in rat taste buds.

References


