

Expression of Gustducin Overlaps with That of Type III IP3 Receptor in Taste Buds of the Rat Soft Palate

Hirohito Miura¹, Ayumi Nakayama¹, Yoichiro Shindo², Yuko Kusakabe³, Hiroshi Tomonari¹ and Shuitsu Harada¹

¹Oral Physiology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima-shi, Kagoshima 890-8544, Japan, ²Fundamental Research Laboratory, Asahi Breweries Ltd., 1-1-21 Midori, Moriya-shi, Ibaraki 302-0106, Japan and ³Gustatory Biology Laboratory, National Food Research Institute, 2-1-12 Kannondai, Tsukuba-shi, Ibaraki 305-8642, Japan

Correspondence to be sent to: Hirohito Miura, Oral Physiology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima-shi, Kagoshima 890-8544, Japan. e-mail: hmiura@denta.hal.kagoshima-u.ac.jp

Abstract

Type III IP3 receptor (IP3R3) is one of the common critical calcium-signaling molecules for sweet, umami, and bitter signal transduction in taste cells, and the total IP3R3-expressing cell population represents all cells mediating these taste modalities in the taste buds. Although gustducin, a taste cell-specific G-protein, is also involved in sweet, umami, and bitter signal transduction, the expression of gustducin is restricted to different subsets of IP3R3-expressing cells by location in the tongue. Based on the expression patterns of gustducin and taste receptors in the tongue, the function of gustducin has been implicated primarily in bitter taste in the circumvallate (CV) papillae and in sweet taste in the fungiform (FF) papillae. However, in the soft palate (SP), the expression pattern of gustducin remains unclear and little is known about its function. In the present paper, the expression patterns of gustducin and IP3R3 in taste buds of the SP and tongue papillae in the rat were examined by double-color whole-mount immunohistochemistry. Gustducin was expressed in almost all (96.7%) IP3R3-expressing cells in taste buds of the SP, whereas gustducin-positive cells were 42.4% and 60.1% of IP3R3-expressing cells in FF and CV, respectively. Our data suggest that gustducin is involved in signal transduction of all the tastes of sweet, umami, and bitter in the SP, in contrast to its limited function in the tongue.

Key words: taste bud, Trpm5, T1r 3, T2rs, whole-mount immunohistochemistry

Introduction

In mammals, taste buds are distributed in the epithelium of the 3 types of tongue papillae (fungiform [FF], foliate [FL], and circumvallate [CV]) and the soft palate (SP) and larynx. During development, the time course for the maturation of taste buds varied among loci in the oral cavity. In newborn rats and hamsters, about half of the taste buds on the SP had taste pores, indicating that SP taste buds are morphologically differentiated immediately after birth (Belecky and Smith 1990; Harada et al. 2000). In contrast, there is no discernible taste bud structure in the CV and FL of the tongue at birth. Taste pores occurred in about 14% of FF taste buds at birth, but the number of taste buds bearing taste pores in the FF was only about one-fifth of that on the SP. Therefore, taste buds on the SP are considered to have an important role in the detection of nutrients in neonatal rodents. In adult rats

and hamsters, although the greater superficial petrosal nerve (GSP) innervating the SP shows pronounced electrophysiological responses to all basic tastes, sweet, umami, bitter, salty, and sour, electrophysiological recordings to sweet stimuli from the GSP in rats and hamsters were reported to be of a larger magnitude than those from the chorda tympani (CT) or glossopharyngeal nerves (GL) innervating taste buds on the tongue (Nejad 1986; Harada and Smith 1992; Harada et al. 1997). Behavioral experiments of taste nerve–transected rats further suggested the functional importance of SP taste buds in adult rats and hamsters for the detection of sweet and umami substances (Krimm et al. 1987; Harada 1992; Sako et al. 2000).

Contemporary research has significantly advanced our understanding of the molecular mechanisms of taste reception.

IP3R3, PLC β 2, and Trpm5, coexpressed in the taste cells in rodents and humans, were shown to be common essential components for sensing sweet, umami, and bitter tastes (Clapp et al. 2001; Max et al. 2001; Miyoshi et al. 2001; Perez et al. 2002; Zhang et al. 2003). In rats and mice, the taste cell populations expressing these molecules are reported to include T1r and T2r taste receptor-expressing cells; however, the sweet taste receptor (T1r2/3)- and the bitter taste receptor (T2rs)-expressing cell populations are segregated (Adler et al. 2000; Matsunami et al. 2000; Kim et al. 2003; Mueller et al. 2005). Gustducin, a taste cell-specific G-protein involved in sweet and bitter sensation (Wong et al. 1996), is coexpressed dominantly in the CV with bitter taste receptors, but not with the sweet taste receptor, in rats and mice (Adler et al. 2000). Recently, it was reported in mice that gustducin is coexpressed with the sweet taste receptor in the FF but not in the CV (Kim et al. 2003), indicating a regional difference of signal transduction molecules concerning sweet and bitter taste stimuli on the tongue. Gustducin is assumed to be involved primarily in bitter taste in the CV and in sweet taste in the FF. In the SP, it was recently reported that most sweet taste receptor (T1r2/3)-expressing cells and a half of umami taste receptor (T1r1/3)-expressing cells were gustducin positive in mice, suggesting the function of gustducin in the SP (Stone et al. 2007). However, in spite of the functional importance of SP taste buds, the information regarding the molecular characteristics of the taste buds in the SP is still limited in comparison to that in the tongue.

In the present study, we first confirmed the expression of taste receptors and taste transduction molecules in the rat SP by *in situ* hybridization. Coexpression patterns of gustducin and IP3R3 were then compared precisely in individual taste buds among the SP, FF and CV, using double-color whole-mount immunohistochemistry. The results demonstrated that almost all (96.7%) IP3R3-expressing cells expressed gustducin in the SP, in contrast to only 42.4% in the CV and 60.1% in the FF, raising the possibility that gustducin is involved in the SP in signal transduction of sweet, umami, and bitter tastes in rats.

Materials and methods

Experimental animals and tissue preparation for whole-mount immunohistochemistry

Experimental animals used in the present investigation were 10-week-old male rats (Sprague Dawley) purchased from KYUDO Laboratory Animal Center (Kumamoto, Japan). According to the guidelines of our institute for the care and use of experimental animals, rats were killed by injection of an excessive dose of sodium pentobarbital (250 mg/kg, intraperitoneally; Nembutal; Abbott Laboratories, Abbott Park, IL). The tongue was excised and placed into Ringer's solution (150 mM NaCl, 4.7 mM KCl, 3.3 mM CaCl₂, 0.1 mM MgCl₂, 2 mM HEPES, and 7.8 mM glucose).

The SP was rinsed with Ringer's solution. Ringer's solution containing 2.5 mg/ml Collagenase type IV (Worthington Biochemical, Lakewood, NJ) and 2 mg/ml Elastase (Worthington Biochemical) was injected beneath the epithelial layers of the tongue and SP. After incubation for 15 min at room temperature, the epithelium of the tongue and SP were peeled off for the following immunohistochemical experiments.

Double-color whole-mount immunohistochemistry

The epithelia of the tongue and SP were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature. After washing with TBST (Tris-buffered saline [TBS] containing 0.05% Tween 20), tissues were immersed in methanol at -20 °C for 20 min. After washing with TBST, tissues were placed in TBSTB (TBS containing 2% Blocking reagent [Roche Diagnostics GmbH, Mannheim, Germany]) for 30 min at room temperature to block non-specific staining. Tissues were incubated for 6 h at room temperature and for 18 h at 4 °C with primary antibodies (anti-gustducin antibody derived from rabbit, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, and anti-IP3R3 mouse monoclonal antibody, 1:100; BD Bioscience, San Jose, CA) in TBSTB. After washing with TBST, tissues were incubated overnight at 4 °C with secondary antibodies (Alexa 568-conjugated anti-rabbit IgG and Alexa 488-conjugated anti-mouse IgG, 1:200; Invitrogen, Carlsbad, CA) in TBSTB. After washing with TBST, the immunofluorescence of labeled taste cells was photographed with a 100 \times /1.4 numerical aperture lens on a Leica TCD4D confocal laser-scanning microscope (CLSM). A series of optical sections was acquired at approximately 1–2.5 μ m intervals from the bottom to the apical portion of the taste buds. Three-dimensional projection images at a particular single angle were reconstructed from Z stacks using the Zeiss LSM 5 Image Browser. Immunohistochemistry was performed by 2 combinations of antibody as a negative control: 1) anti-IP3R3 mouse monoclonal antibody and Alexa 568-conjugated anti-rabbit IgG, 2) anti-gustducin rabbit polyclonal antibody and Alexa 488-conjugated anti-mouse IgG. No specific signal was observed by both antibody combinations.

Cell quantification and statistical analysis

Immunoreactivity for gustducin and IP3R3 was observed throughout the cytoplasm of labeled cells. Gustducin-and/or IP3R3-expressing cells in each taste bud were quantified by confirming the cell shape and nuclear profile in the original Z-series sections in serial order. Results were presented as means \pm SEMs. The number of each gene-expressing cells and the ratio of gustducin and IP3R3 double-positive cells in IP3R3-expressing cells were compared between the 3 regions (SP, FF, and CV) by one-way analysis of variance (ANOVA). Post hoc comparisons between regions were made using Scheffe's test for multiple comparisons (StatView, version 5.0.1, SAS Institute, Cary, NC). Three

rats were used for statistical analysis. In the SP, 52 taste buds located in the Geschmacksstreifen were examined. In the FF, 43 taste buds located within 5 mm from the tip of the tongue were examined. In the CV, 69 taste buds were examined.

Western blot analysis

The epithelia within approximately 10 mm from the tip of the tongue and the epithelia of CV region were peeled off in the same way as in whole-mount immunohistochemistry and used as tongue sample (T). The epithelium of the entire SP region was used as SP sample. The peeled epithelium was homogenized in ice-cold lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris [pH 7.5], 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 10 IU/ml aprotinin, and 10 µg/ml leupeptin). After centrifugation at $14\,000 \times g$, the supernatant was mixed with sodium dodecyl sulfate loading buffer containing β-mercaptoethanol, boiled for 5 min, and placed on ice for 5 min. Proteins were separated on a SuperSep HG 5–20% gradient gel (Wako, Osaka, Japan) and transferred to a Hybond-P membrane (GE Healthcare, Amersham Place, UK) by electroblotting. The membrane was incubated for 1 h at room temperature with 5% bovine serum albumin in TBST and then incubated at 4 °C overnight with primary antibodies (anti-gustducin antibody derived from rabbit, 1:1000; Santa Cruz Biotechnology or anti-IP3R3 mouse monoclonal antibody, 1:1000; BD Bioscience) in TBST. After washing with TBST, the membrane was incubated for 1 h at room temperature with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, 1:2000; GE Healthcare). Signal was detected with Lumigen PS-3 detection reagent (GE Healthcare) and digitally scanned by LAS 1000 (Fujifilm, Tokyo, Japan).

In situ hybridization

The SP and tongues were excised, placed in embedding compound (OCT; Sakura Finetech, Los Angeles, CA), and were rapidly frozen in a liquid nitrogen bath. Tissues were sectioned at 5 µm. Antisense cRNA probes were transcribed in vitro with digoxigenin-UTP using an RNA transcription kit (Roche Diagnostics GmbH) from a linearized plasmid containing one of the following cDNA inserts: T1r1, T1r2, T1r3, T2r5, T2r8, T2r18, T2r19 (Kim et al. 2003), gustducin (1123–1623: Genbank accession no. X65747), and Trpm5 (1–3777: Genbank accession no. NM020277). A mixture of 4 T2r cRNA probes was used to detect T2r expression, whereas other cRNA probes were used independently. The sections were hybridized with cRNA probes in hybridization buffer (50% formamide, 5× saline sodium citrate [SSC], 5× Denhardt's solution, 500 µg/ml salmon sperm DNA, and 250 µg/ml tRNA) overnight at 65 °C and washed with 0.2× SSC at 65 °C for 90 min. Following the blocking with TBSTB, sections were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics

GmbH, 1:400). After washing with TBST, the color reaction was performed with 1.5 mg/ml nitoroblu tetrazolium and 0.75 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 50 mM MgCl₂). In situ hybridization with sense probes was performed as a negative control. No specific signal was found with any of the sense probes.

Results

Expression of taste reception-related genes in the rat SP

In order to confirm that the mRNA for molecules involved in taste reception in taste buds is shared in SP and tongue, the expression of taste receptors T1r and T2r and the intracellular signal transduction molecules gustducin and Trpm5 was examined in the SP by in situ hybridization (Figure 1). The expression of T1r and T2r receptors was detected in the SP, whereas the signal intensity for T2r receptors was weaker than that of the T1r receptors. Both gustducin and Trpm5 were expressed strongly and showed similar expression patterns with each other in taste buds on the SP. The number of gustducin- and Trpm5-expressing cells was greater than that of each taste receptor-expressing cells, raising the possibility that gustducin is expressed in almost all the Trpm5-expressing cells, which includes the sweet, umami, and bitter responsive taste cells in SP taste buds.

Whole-mount immunohistochemistry of gustducin and IP3R3 in the rat taste buds

To analyze gustducin expression in SP taste buds, whole-mount immunostaining of the epithelium was used, which allowed us to compare precisely the number of each gene-expressing cell in individual taste buds in the SP and tongue by eliminating the difficulties in the reconstruction of serial sections into whole taste buds (Bigiani et al. 2002). IP3R3 is previously reported to be coexpressed with Trpm5 and PLCβ2 in taste cells responsive to sweet, umami, and bitter stimuli in rats and mice (Clapp et al. 2001; Miyoshi et al. 2001; Perez et al. 2002; Zhang et al. 2003). Double-color whole-mount immunohistochemistry of IP3R3 and gustducin was performed with an anti-IP3R3 antibody suitable for whole-mount immunohistochemistry of the epithelium of the SP, FF, and CV.

The specificity of antibodies was examined by western blot analyses (Figure 2). Anti-gustducin and anti-IP3R3 antibodies detected protein bands of the expected size, approximately 45 kDa (Ozden et al. 2006) and 300 kDa (Jayaraman and Marks 1997), respectively, in the epithelial lysate of the SP and tongue. A series of optical sections of whole-mount immunohistochemistry was obtained using CLSM. Figure 3 showed one of the optical sections of taste buds in each region, and Figure 4 showed 3-dimensional

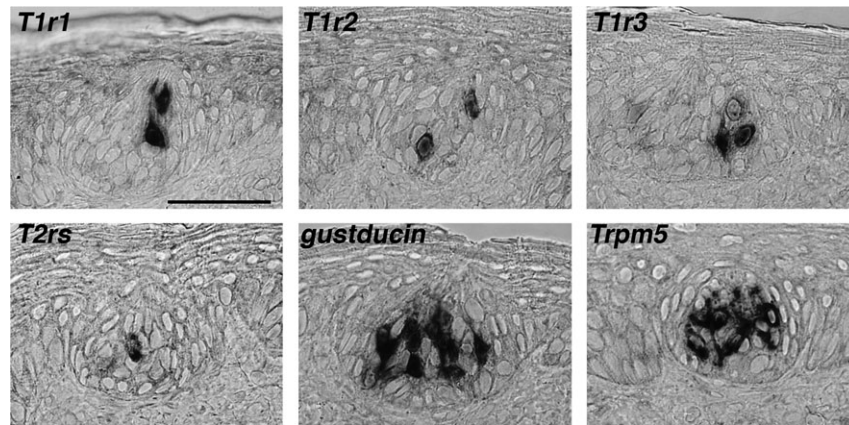


Figure 1 Expression of T1r1, T1r2, T1r3, T2rs, gustducin, and Trpm5 in rat soft palate. In situ hybridization was used for detecting the expression. T2r was detected by mixed probe of T2r5, T2r8, T2r18, and T2r19. The scale bar indicates 50 μm .

projection images reconstructed from Z stacks. Nuclear profiles are clearly observed in each optical section (Figure 3), and immunoreactivity for gustducin and IP3R3 was noted throughout the cytoplasm of labeled cells (Figure 4), allowing us to precisely analyze the immunoreactive cells by confirming nuclear profile and cell shape. All the gustducin-expressing cells were included in IP3R3-expressing cells. Three-dimensional images of whole taste buds showed obvious differences in the coexpression pattern of gustducin and IP3R3 between the SP and tongue. Gustducin and IP3R3 were coexpressed exclusively in the SP, whereas the IP3R3-expressing cells without gustducin were evident in the FF and CV (Figure 4). The number of gustducin- and IP3R3-expressing cells was counted for 43–69 taste buds in each epithelial region bearing taste buds (Table 1). The number of gustducin-expressing cells in individual taste buds varied significantly in the SP, FF, and CV (ANOVA; $F_{(2,161)} = 82.671$; $P < 0.0001$). As shown in Figure 5, rat SP taste buds had more gustducin-expressing cells (14.8 ± 0.7) than the other 2 regions (Scheffe's test; $P < 0.0001$). The number of gustducin-expressing cells also showed significant difference between FF (5.3 ± 0.8) and CV (7.8 ± 0.4) (Scheffe's test; $P < 0.005$). The number of IP3R3-expressing cells in individual taste buds showed less difference than that of gustducin-expressing cells (ANOVA; $F_{(2,161)} = 4.306$; $P < 0.05$), whereas significant difference was noted only between SP (15.3 ± 0.8) and FF (12.6 ± 0.5) (Scheffe's test; $P < 0.05$). The ratio of gustducin and IP3R3 double-positive cells in IP3R3-expressing cells in individual taste buds varied significantly in the SP, FF, and CV, reflecting the difference in the number of gustducin-expressing cells in individual taste buds (ANOVA; $F_{(2,161)} = 300.031$; $P < 0.0001$, Scheffe's test; $P < 0.0001$) (Figure 5).

Discussion

The GSP innervating taste buds on the SP shows pronounced responses to all basic tastes, with a relatively larger

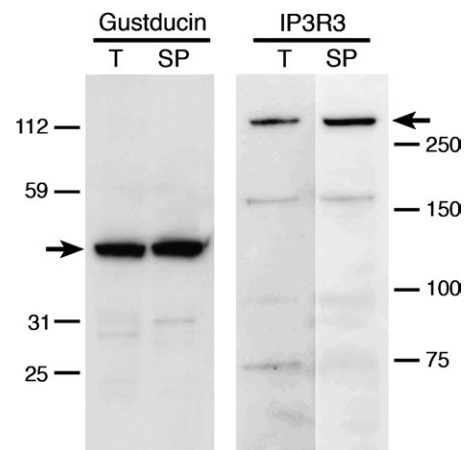


Figure 2 Confirmation of antibody specificity by western blot analyses. Molecular weight (in thousands) is indicated on the left for gustducin and on the right for IP3R3. Arrows indicate the positions of expected sizes (~45 and 300 kDa for gustducin and IP3R3, respectively). T, tongue; SP, soft palate.

magnitude in neurophysiological response to sweet stimuli than that of the CT or GL, in rats and hamsters (Nejad 1986; Harada and Smith 1992; Harada et al. 2000). In spite of their functional importance, the molecular characteristics of SP taste buds have remained largely unexplored. In the present study, we showed that gustducin is expressed in almost all IP3R3-expressing cells in rat SP taste buds. Recent evidence suggests that IP3R3 is expressed in all cells responsive to sweet, umami, and bitter tastes and to be the common essential molecules for sensing these 3 kinds of tastes in rats and mice (Clapp et al. 2001; Miyoshi et al. 2001; Perez et al. 2002; Zhang et al. 2003). Further, our in situ hybridization of taste receptors and Trpm5 confirmed that the molecules associated with IP3R3-mediated taste sensing are expressed in the rat SP. Therefore, our data suggest that gustducin is involved in signal transduction of all the tastes of sweet, umami, and bitter in the rat SP.

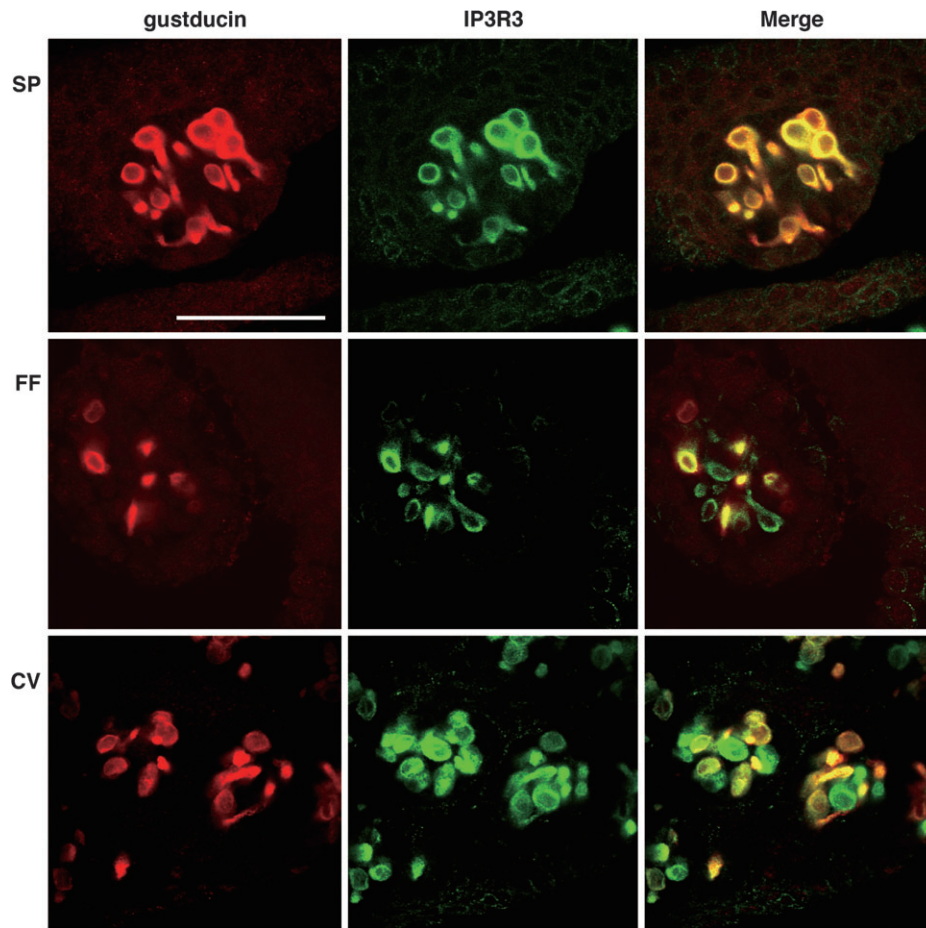


Figure 3 Optical sections of double-color whole-mount immunohistochemistry of gustducin and IP3R3 of the epithelia of the soft palate (SP), FF and CV in the rat. The scale bar indicates 50 μ m.

Previous histological studies showed the expressional combination of gustducin and taste receptors differed by location in the tongue. Gustducin is coexpressed exclusively in the CV in bitter taste receptor-expressing cells (in rats and mice), whereas in the FF, gustducin is expressed in sweet and umami taste receptor-expressing cells (in mice) (Adler et al. 2000; Kim et al. 2003). Also in humans, coexpression of gustducin and sweet taste receptor was reported in FF (Max et al. 2001). Our immunohistochemical data in rats showed that gustducin was expressed in 42.4% and 60.1% of IP3R3-expressing cells in the FF and CV, respectively, supporting the different expressional combination of gustducin and taste receptors in the rat tongue. Gustducin was originally considered to play an essential role in the transduction of sweet and bitter taste qualities because gustducin knockout mice showed significant reduced responses to both taste stimuli in 2-bottle choice tests and nerve recordings (Wong et al. 1996). Recently, a reduced response to umami taste stimuli in gustducin knockout mice was also reported (Ruiz et al. 2003; He et al. 2004; Glendinning et al. 2005). In these studies, reductions in taste nerve responses were evident for

sweet and umami in CT innervating FF and for bitter in GL innervating CV. A concordance of histological and neurophysiological analyses would be expected in the SP similar to the results in the tongue. The neurophysiological analysis of the GSP in the gustducin knockout mice would provide the evidence that gustducin-expressing cells contribute to the sense of sweet, umami, and bitter tastes in the SP because our preliminary immunohistological experiments in mice suggest that the expression pattern of gustducin in mouse SP is almost same as in rat SP.

Recently, it was reported that, in the mouse SP, gustducin and T1r3 were almost completely coexpressed and that gustducin was expressed in 91% and 50% of T1r2- and T1r1-expressing cells, respectively (Stone et al. 2007). These observations were consistent with our data in rats regarding coexpression of gustducin and sweet taste receptor (T1r2/3) but suggested that a half of umami receptor (T1r1/3)-expressing cells did not express gustducin in conflict with our data in rats. Because our preliminary immunohistological data in mice suggested the similar expression patterns in rats and mice regarding gustducin and IP3R3, the conflicting

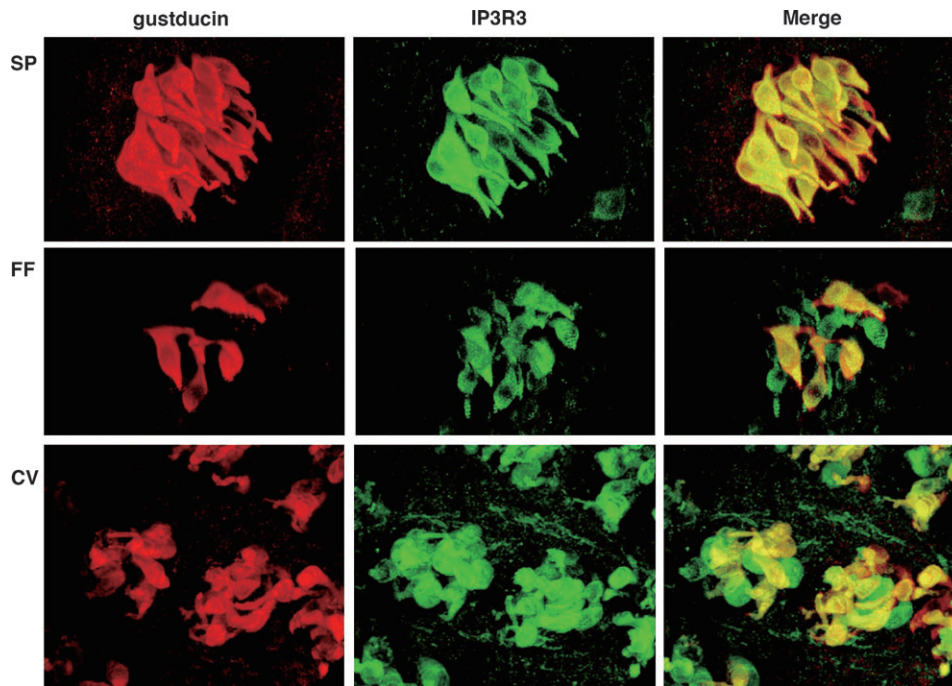


Figure 4 Three-dimensional projection images of gustducin- and IP3R3-expressing cells in the taste buds on the soft palate (SP), FF, and CV.

Table 1 Number of gustducin- and IP3R3-expressing cells in taste bud

	No. of rats	No. of taste buds	Total counts				Mean (\pm SEM) counts per taste bud		
			Total labeled cells	Gustducin cells	IP3R3 cells	Double-positive cells/IP3R3 cells (%)	Gustducin cells	IP3R3 cells	Double-positive cells/IP3R3 cells (%)
SP	3	52	799	769	799	96.2	14.8 \pm 0.7	15.3 \pm 0.8	96.7 \pm 0.8
FF	3	43	540	227	540	42.0	5.3 \pm 0.3	12.6 \pm 0.5	42.4 \pm 1.7
CV	3	69	910	536	910	58.9	7.8 \pm 0.4	13.2 \pm 0.6	60.1 \pm 1.7

results may be due to the method difference: they examined coexpression using the combination of immunohistochemistry for gustducin and in situ hybridization for T1r receptors. Alternately, it may be due to the regional difference of taste buds within the SP. We examined the taste buds in the GS, the most rostral part of the SP, as SP taste buds in the current study, whereas several taste buds were confirmed to have the same expression patterns of gustducin and IP3R3 in the midsection of the rat SP (data not shown). Further examination of the posterior part of the SP may be necessary.

We employed double-color whole-mount immunohistochemistry to compare the gene expression in the taste buds. This method enabled precise quantification of the number of the gustducin- and IP3R3-expressing cells and the ratio of the coexpression of these genes in individual taste buds because cell shapes and nuclear profiles of each gene-expressing cells were clearly recognized in the optical serial sections obtained by CLSM. Mean numbers of gustducin-expressing

cells per taste bud in the SP (9.56 ± 0.40) and FF (3.12 ± 0.27) estimated by Boughter et al. (1997) were about two-thirds of our estimation (SP [14.8 ± 0.7] and FF [5.3 ± 0.3]). The difference in the estimation may be due to the method difference. Their method using sections may cause oversights of positive cells between sections, whereas whole-mount immunohistochemistry seems to reduce the risk of oversight, especially in the scattered taste buds like those in the SP and FF, and to be an optimal method to compare the gene expression in individual taste buds at present. The present paper is the first report to apply the double-color whole-mount immunohistochemistry for the taste bud analysis, whereas a similar approach was taken to estimate the developmental change of gustducin-expressing cells in mouse CV using single immunofluorescent detection of gustducin (Bigiani et al. 2002).

In summary, our data provide the first molecular evidence suggesting that gustducin is involved in the signal transduction of all the tastes of sweet, umami, and bitter in the SP, in

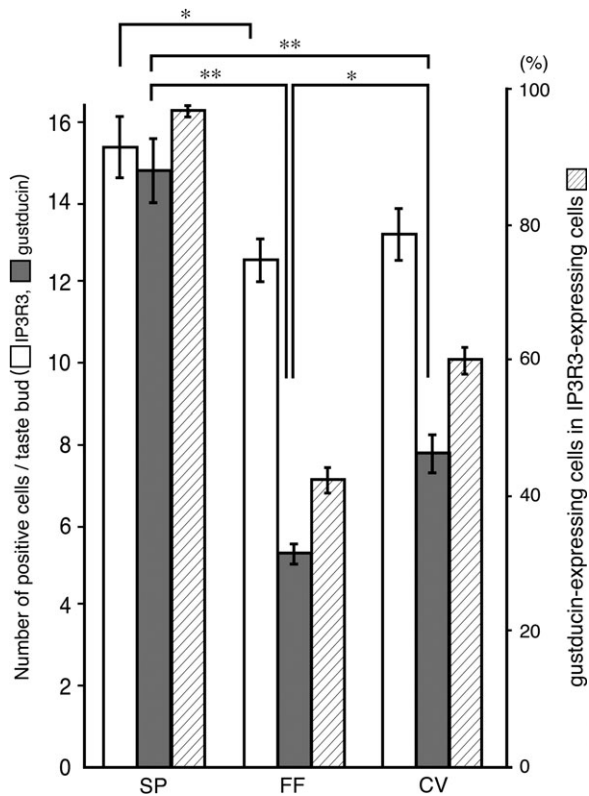


Figure 5 The mean number of gustducin- and IP3R3-expressing cells in individual taste buds and the mean ratio of the gustducin-expressing cells in IP3R3-expressing cells. Error bars are SEM. Asterisks indicate statistically significant difference (Scheffe's test: * $P < 0.005$, ** $P < 0.0001$). Gustducin-expressing cells were included in IP3R3-expressing cells, and the mean ratio of gustducin-expressing cells in IP3R3-expressing cells was significantly different among the SP, FF, and CV (Scheffe's test: $P < 0.0001$).

contrast to its limited function in the tongue papillae, in the rat. The usefulness of double-color whole-mount immunohistochemistry in precise analysis of gene expression in individual taste buds was also demonstrated. Further analyses using this method are expected to improve the accuracy in understanding of the molecular difference in individual taste buds.

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