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Comprehensive Study on G protein α-Subunits in Taste Bud Cells, with Special Reference to the Occurrence of Gαi2 as a Major Gα Species

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Abstract

Previous studies have identified many cDNA species that encode a variety of G protein α-subunits occurring in taste buds. These include the cDNA encoding a taste-bud-specific Gα, gustducin (G_{gust}). Here we carried out comprehensive analyses of Gα species that occur in the taste buds of rat circumvallate papillae and also in their single cells isolated from the taste buds. Reverse transcriptase–polymerase chain reaction showed the presence of 10 kinds of Gα cDNAs, including a splice variant of Gαs, among which G_{gust}, Gαs, Gαi2 and Gαi3 cDNAs were shown to be major species. *In situ* hybridization and immunohistochemistry showed that Gαi2, as well as G_{gust}, expressed in a subset of taste bud cells, and the frequency of Gαi2-expression appears to be higher than that of G_{gust}. Southern analyses of the amplified cDNA from single cells showed that each taste bud cell expresses multiple Gα mRNA species. For example, some Gαi2-positive cells also express one or more other Gα species, including Gαs, Gαi3 and G_{gust}, and there is no apparent correlation in expression among the three Gα species.

Introduction

In vertebrates, taste chemoreception takes place in taste bud cells (Lindemann, 1996). These comprise at least three types of elongated, spindle-shaped cells and a type of globular basal cell, all of which are derived from epithelial cells (Stone *et al.*, 1995). These cell types are thought to differ physiologically as well as morphologically (Delay *et al.*, 1986; Lindeman, 1996).

Sweet and bitter taste senses in humans and other mammals are believed to be mediated by a G-protein-coupled signaling system (Akabas *et al.*, 1988; Avenet *et al.*, 1988; Striem *et al.*, 1989; Brand *et al.*, 1991; Spielman *et al.*, 1994) that involves G-protein-coupled receptors (GPCRs), coupling G proteins, related effectors and other intracellular signaling factors. Various molecules have been identified and proposed as constituents involved in the system. Many rhodopsin-type GPCRs (Abe *et al.*, 1993a,b; Matsuoka *et al.*, 1993; Tal *et al.*, 1995; Thomas *et al.*, 1996) and taste-specific Ca^{2+}-sensing receptors (TR1 and TR2) (Hoon *et al.*, 1999) have been isolated from tongue epithelia. Meanwhile, G_{gust}, a Gαi-type G protein α-subunit closely related to transducin (Gαt), has been recognized as an essential component involved in sweet and bitter taste transduction (McLaughlin *et al.*, 1992; Wong *et al.*, 1996). Also, there are reportedly other Gα species in taste tissues (McLaughlin *et al.*, 1992, 1994; Kusakabe *et al.*, 1998), although their physiological significance has yet to be elucidated. The possibility exists that these Gα species may function in parallel with G_{gust}-mediated taste signaling. Relevant to this, it has been postulated that Gαq and Gα15 mediate Ca^{2+} signaling (Kusakabe *et al.*, 1998) in parallel with a G_{gust}-mediated cAMP pathway (McLaughlin *et al.*, 1994). It is also possible that Gα species may be involved in other signaling pathways, such as a cell-to-cell communication between taste bud cells. However, the total understanding of

such receptor-mediated signalings in taste bud cells requires comprehensive studies to describe the G proteins in taste bud cells to be performed.

In this study, we adopted four methods to analyze the expression of Gα species in taste buds and their constituent cells: reverse transcriptase–polymerase chain reaction (RT–PCR), *in situ* hybridization, immunohistochemistry and Southern analyses using amplified cDNA from single taste bud cells. By these procedures we have newly identified two Gα subunits, Gαi2 and Gαi3, as relatively abundant Gα subunits, in addition to the Gαs and G_{gust} already reported (McLaughlin *et al.*, 1994). We also show that Gαi2-expression occurs in a subset of taste bud cells, suggesting the presence of a Gαi2-mediated signaling pathway in the taste bud.

Materials and methods

Isolation of taste buds and single cells from taste buds

Five-week-old male rats (Wistar) were killed by injection of sodium pentobarbital. Tongues were dissected immediately and put into Ringer solution (150 mM NaCl, 4.7 mM KCl, 3.3 mM $CaCl_2$, 0.1 mM $MgCl_2$, 2 mM HEPES and 7.8 mM glucose). Ringer solution containing Type I collagenase (2 mg/ml, Sigma-aldrich) (collagenase solution) was injected beneath a circumvallate papilla. After incubation for 30 min at room temperature, epithelia containing the circumvallate papillae were peeled off. Epithelial sheets were again soaked in collagenase solution for 10 min at room temperature, transferred to Ca^{2+}-free Ringer solution containing 2 mM EDTA and incubated for 10 min. Taste buds were isolated in Ringer solution containing 0.1% bovine serum albumin (fraction V, Sigma-aldrich) (BSA solution) by suction through a 50-μm-diameter pipette and transferred into a new plate containing BSA solution. The isolated taste buds were dispersed by pipetting and individual spindle-shaped cells were collected for single cell analyses.

Identification of Gα members expressed in taste buds

mRNA was purified from 100 taste buds by the usual method (Sambrook *et al.*, 1989). RT–PCR was carried out using the following primers: 5′-GGIAA(AG)(AT)(GC)-IACITT(TC)(CA)TIAA(AG)CA(AG)ATG-3′ (primer A), and 5′-GGIAA(AG)AG(TC)ACIATIGTIAA(GA)CA(G-A)ATG-3′ (primer B) for sense, and 5′-(TC)(TC)ITGICCI-CCIAC(GA)TC(GA)AA(GC)AT-3′ (primer C) and 5′-TCI-(TG)(CA)IC(TG)(CT)TGICCICCIAC(GA)TC-3′ (primer D) for antisense. These primers were made based on the amino acid sequences GKSTFLKQM, GKSTIVKQM, M/IFDVGGQ/HR/E and DVGGQRS/DE, which were found as consensus sequences common to most of the known Gα members. PCR was carried out for 30 cycles under the following conditions: 94°C for 1 min, 52°C for 1 min and 72°C for 2 min. The resulting 480 bp fragments were gel-isolated, cloned into pBluescrip SK (Stratagene) and sequenced.

RT–PCR of the mRNA obtained from single taste bud cells, and Southern blot analysis of Gα cDNAs

The cDNA from each of the single cells was synthesized and amplified by the method of Dulac and Axel (Dulac and Axel, 1995). Aliquots of cDNA samples prepared from single cells were electrophoresed in a 1% agarose gel and blotted onto nylon membranes (Hybond-N, Amersham, UK). The membranes were processed for hybridization using ^{32}P-labeled DNA probes corresponding to the 3′-noncoding regions of Gαs, Gαi2, Gαi3, G_{gust} and β-actin mRNAs. The 3′-noncoding regions of Gαs, Gαi2, Gαi3 and G_{gust} were obtained by RT–PCR using the taste bud mRNA as a template and the specific primers, 5′-CACCTGAAT-TCTATGAGCAT-3′ (+429–+448) and 5′-TTAAGCTT-ACTAAATTTGGA-3′(+1375–+1356) for Gαs (Jones *et al.*, 1987); 5′-AT*AAGCTT*CGGGGCAGTGGGCCTG-GCAGG-3′(+1068–+1087) (added *Hin*dIII site italicized) and 5′-AT*GAATTC*GCCTCCAAGCGGCAGAGAGT-3′ (+1517–+1498) (added *Eco*RI site italicized) for Gαi2 (Jones *et al.*, 1987); 5′-AT*AAGCTT*GAGGATGGCA-TAGTAAAAGC-3′ (+1066–+1085) (added *Hin*dIII site italicized) and 5′-TC*TCTAGA*GTGGAATATTACTC-CTGACC-3′ (+1584–+1565) (added *Xba*I site italicized) for Gαi3 (Jones *et al.*, 1987); and 5′-ATGCCGTGACAGAT-ATAATA-3′(+1027–+1047) and 5′-TGCTTGTGGCAG-CCTAATTG-3′ (+1510–+1491) for G_{gust} (McLaughlin *et al.* 1992). The β-actin DNA fragment was obtained by RT–PCR using two primers, 5′-GAACCCTAAGGC-CAACCGTG-3′(+1658–+1677) and 5′-ATGGTGGTG-CCACCAGACAG-3′ (+2775–+2794) (Nudel *et al.*, 1983). Hybridization and washing were carried out under the usual stringent conditions at 68°C, with the final wash done in 0.1 × SSC containing 0.1% sodium dodecyl sulfate at the same temperature.

In situ hybridization

Digoxigenin-labeled anti-sense RNA probes for Gαs, Gαi2, Gαi3 and G_{gust} were synthesized using T3 or T7 RNA polymerase. The circumvallate papillae layer from the tongue of a 5-week-old male rat (Wistar) was removed, frozen in OCT compound, and sectioned into 10 μm slices. *In situ* hybridization was performed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993; Asano-Miyoshi *et al.*, 1998; Yasuoka *et al.*, 1999). The sections were post-fixed for 10 min in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, washed twice in PBS containing 1% diethylpyrocarbonate for 15 min, washed in 5 × SSC for 15 min and prehybridized in prehybridization solution containing 50% formamide and 5 × SSC, 40 μg/ml denatured herring sperm DNA for 1 h at room temperature. Next, 0.1 ml of hybridization buffer containing 50%

Table 1 Number of taste bud Gα cDNA clones isolated by RT–PCR using two different primer sets

Gα species	Primer set	
	A–C	B–D
G_{gust}	4	3
$G_{t\text{-}cone}$	0	1
Gαil	2	0
Gαi2	6	3
Gαi3	4	5
Gαq	2	0
Gα14	1	0
Gα15	2	0
Gαs	6	6
Gαs3	3	0

For RT–PCR, a template was prepared from the taste buds of rat circumvallate papillae. Sense primers (A and B) and antisense primers (C and D) were used. For details see Materials and methods. A–D and B–C gave results similar to those obtained using A–C and B–D, respectively.

formamide, 5 × SSC, 5 × Denhardt's solution, 500 μg/ml sonicated, denatured herring sperm DNA, 250 μg/ml torula tRNA, 1 mM dithiothreitol, and ~5–10 ng of probe were added to the slide. The slide was covered with a siliconized coverslip and hybridized for 36 h at 58°C. The coverslips were removed by soaking in 5 × SSC at 58°C and the slides were washed twice in 0.2 × SSC for 30 min at the same temperature. The sections were washed in Tris-buffered saline (TBS) for 5 min, blocked in 0.5% blocking reagent (Boehringer) in TBS for 1 h at room temperature and incubated in the same solution containing a 1:1000 dilution of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase for 1 h. The slides were then washed three times in TBS for 15 min and once in alkaline phosphatase buffer containing 100 mM Tris–HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$ for 5 min. The color reaction was performed by adding the color developing solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer onto the slide.

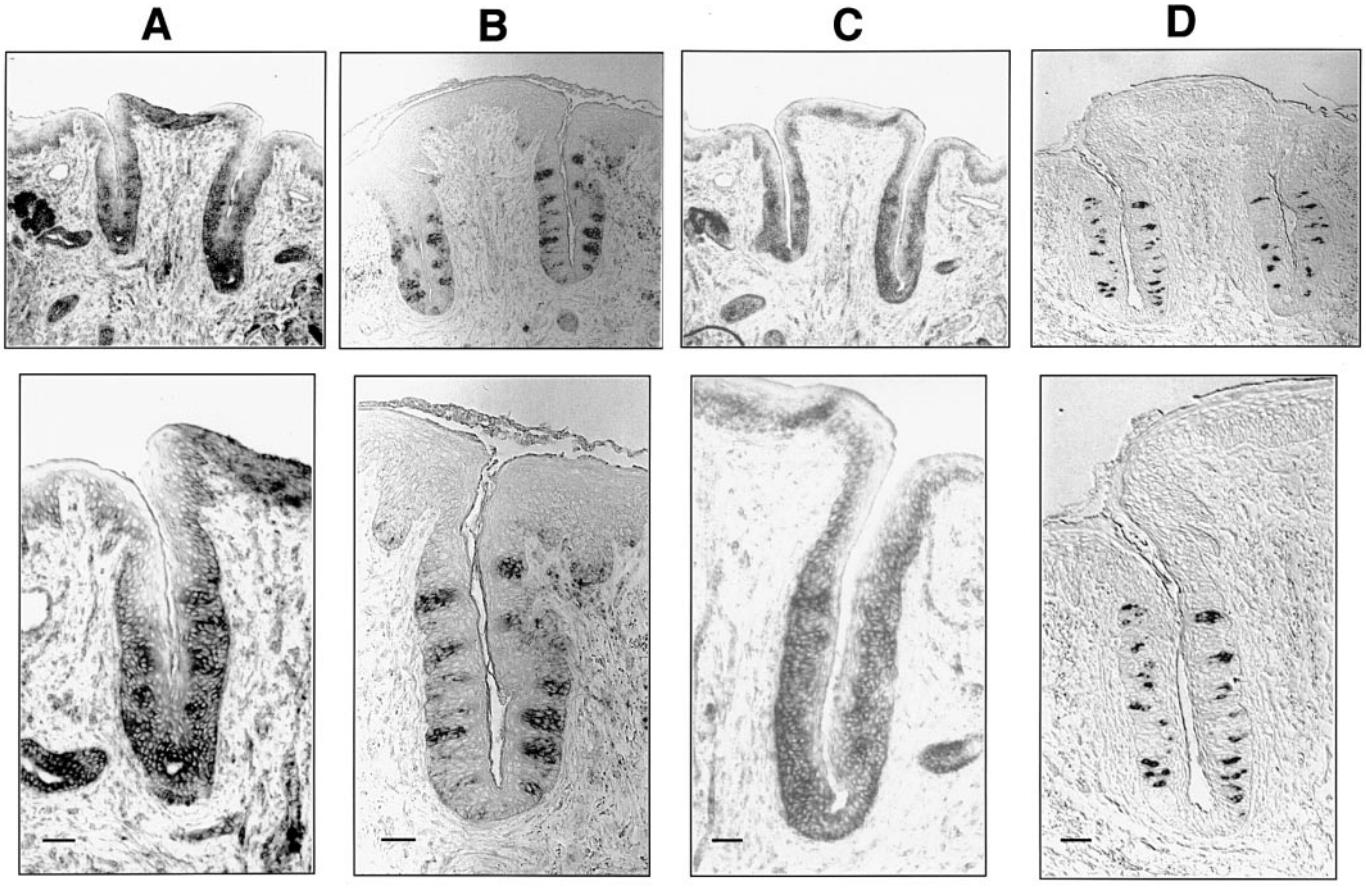

Figure 1 *In situ* hybridization showing the expression of Gαs, Gαi2, Gαi3 and G_{gust} in circumvallate papillae. Coronal sections, 10 μm each in thickness, were prepared from the circumvallate papillae of a 5-week-old male rat. Each section was hybridized with digoxigenin-labeled probes for the detection of Gαs **(A)**, Gαi2 **(B)**, Gαi3 **(C)** and G_{gust} **(D)**. Note that Gαi2 (B) as well as G_{gust} (D) are distinctly expressed in a subset of taste bud cells. Bars represent 50 μm.

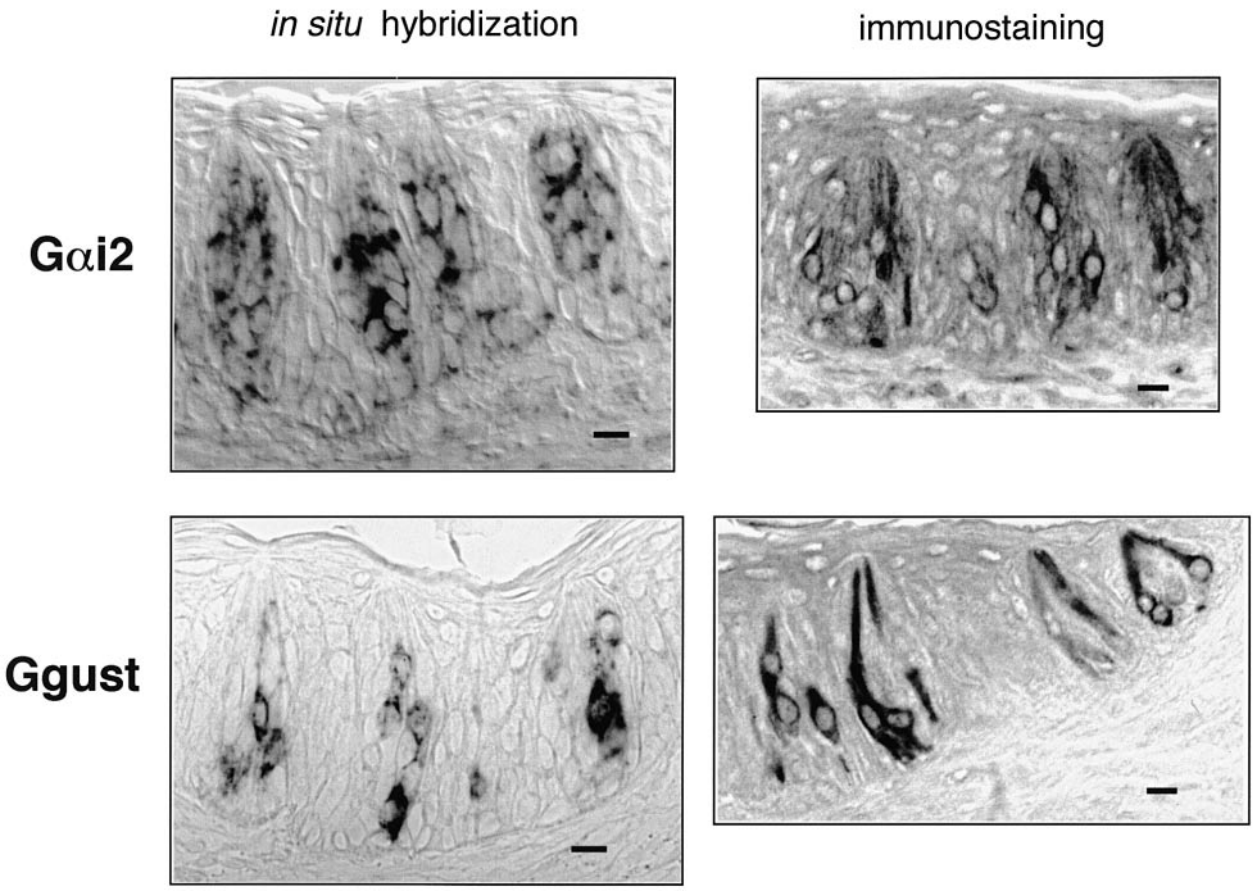

Figure 2 *In situ* hybridization and immunostaining showing the expression of Gαi2 and G_{gust} in taste buds. Conditions for *in situ* hybridization, methods for preparing coronal sections and details of the hybridization are the same as in Figure 1. For immunostaining, circumvallate papillae were excised from a 5-week-old male rat and fixed in Bouin's solution. Each section, 6 μm in thickness, was immunoreacted with anti-Gαi2 antibody (1:50) or anti-G_{gust} antibody (1:100). Signals are observed in particular cells that constitute each taste bud. The frequency of the Gαi2 signals in taste buds appears to be larger than that of G_{gust} signals. Bars represent 10 μm.

Immunostaining of circumvallate papillae

Anti-Gαi2 antibody, a gift from Dr T. Asano (Ueda *et al.*, 1998), was further purified by preabsorption with recombinant G_{gust} protein. Anti-G_{gust} antibody was purchased from Santa Cruz Biotechnology Corp. We produced Gαi1, Gαi2 and Gαi3 proteins by expression in *Escherichia coli.* We confirmed by Western blotting that anti-Gαi2 and anti-G_{gust} antibodies cross-reacted only with Gαi2 and G_{gust}, respectively. Tongues were fixed in Bouin's solution fixative (Sigma), dehydrated, embedded in paraffin and sectioned at 6 μm thickness. Each section was immunoreacted with each of the anti-Gαi2 antibody and anti-G_{gust} antibody, at dilutions of 1:50 and 1:100 respectively. After rinsing in PBS, the immunoreacted sections were incubated with biotin-labeled anti-rabbit IgG at a dilution of 1:500 and then treated with a complex of avidin-and-biotin-conjugated peroxidase (Vector Laboratories). The signals were developed by treatment with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 10 mM sodium phosphate, pH 7.4.

Results

Identification by RT–PCR of multiple Gα species expressed in taste buds

We carried out RT–PCR using the cDNAs prepared from isolated taste buds and identified 10 kinds of Gα cDNA clones (Table 1). Among them, G_{gust}, Gαq and Gα15 have been reported to exist in taste buds (McLaughlin *et al.*, 1992; Kusakabe *et al.*, 1998). We newly identified an exon 3-splice variant (Gαs3) that lacks part of the N-terminal region of Gαs (Feinstein *et al.*, 1992). In addition, we identified three Gαi species—Gαi1, Gαi2 and Gαi3— and a Gαq species—Gα14—although the frequencies of Gαi1 and Gα14 in the cDNA clones were lower. Since the cDNA used was synthesized from isolated taste buds freed from adjacent tissues, these Gα species identified were derived from taste bud mRNA, and the numbers of cDNA clones of each cDNA species may reflect the relative abundancy of Gαs species occurring in taste bud cells.

Next, in order to examine the cellular localization of

each Gα species in taste buds, we carried out *in situ* hybridization experiments using the Gα probes obtained above. The results show that the antisense probes for Gαs, Gαi2, Gαi3 and G_{gust}, all of which show higher values in Table 1, gave significant signals (Figure 1). On the other hand, the other probes gave faint signals in taste buds, and in some cases showed ubiquitous signals throughout the non-sensory epithelial tissues (data not shown). The Gαs probe gave distinct signals in taste buds, but there was no specific pattern (Figure 1A). The signals for Gαi3, although its expression frequency is similar to the others, were generally weak and ubiquitous in the basolateral regions of both taste buds and other parts of the epithelia (Figure 1C). Gαi2 and G_{gust} showed characteristic expression features (Figure 1B,D). The signals for Gαi2 were observed specifically in taste buds, but not in papillary epithelium lacking taste buds. The profile of G_{gust} was essentially the same as previously reported (Yang *et al.*, 1999). Gαi2-positive cells appeared to be larger in number than G_{gust}-positive cells.

Since our *in situ* hybridization experiment showed that Gαi2 and G_{gust} show taste-bud-specific expression (Figure 1), we next performed immunostaining with specific antibodies and compared the results with those of *in situ* hybridization experiments. As shown in Figure 2, subpopulations of taste bud cells were stained by both *in situ* hybridization and immunostaining. The results from both experiments are essentially the same. Gαi2-positive cells were more than G_{gust}-positive cells, although the signals for G_{gust} were higher and clearer than those for Gαi2. Gαi2- and G_{gust}-positive cells are estimated to be ~30 and 20%, respectively, in the taste bud cells.

Multiple Gα species expressed in single taste bud cells

As shown above, at least four Gα species (Gαs, Gαi2, Gαi3 and G_{gust}) are expressed in substantial amounts in taste buds, and their expression profiles appear to differ from one another. To obtain more precise information about Gα expression at the cellular level, we examined how many and which Gα species are expressed in individual cells by Southern blot analysis using amplified cDNA from single taste bud cells according to the method described for olfactory neurons (Dulac and Axel, 1995). We obtained cDNA from individual spindle-shaped taste bud cells and used four Gα probes. However, we noticed that the RNA extraction efficiency and PCR amplification efficiency as estimated from the signal intensity of β-actin varied during the experiment (data not shown). Therefore, we focused on Gαi2, which was found to be expressed in a subset of taste bud cells. We used Gαi2-positive cDNA preparations and looked at whether or not the other three Gα species were expressed in the Gαi2-positive cell. As shown in Figure 3, Gαi2-positive cells were positive to probes for one to three of the other Gα species, indicating that multiple Gα species are co-expressed in each taste bud cell. It was also shown that there is no apparent correlation among the expression patterns of Gαs, Gαi3 and G_{gust}; in some cases (nos 7 and 12 in Figure 3) all four Gα species were expressed and in others (nos 1 and 3) G_{gust} was not expressed. Further, different cells showed different relative abundance with respect to the expression of the four Gα species. An example is offered for two cells, no. 15, which is abundant in Gαi3, and no. 12, which is abundant in Gαi2 and Gαs, although these two cells expressed β-actin equally.

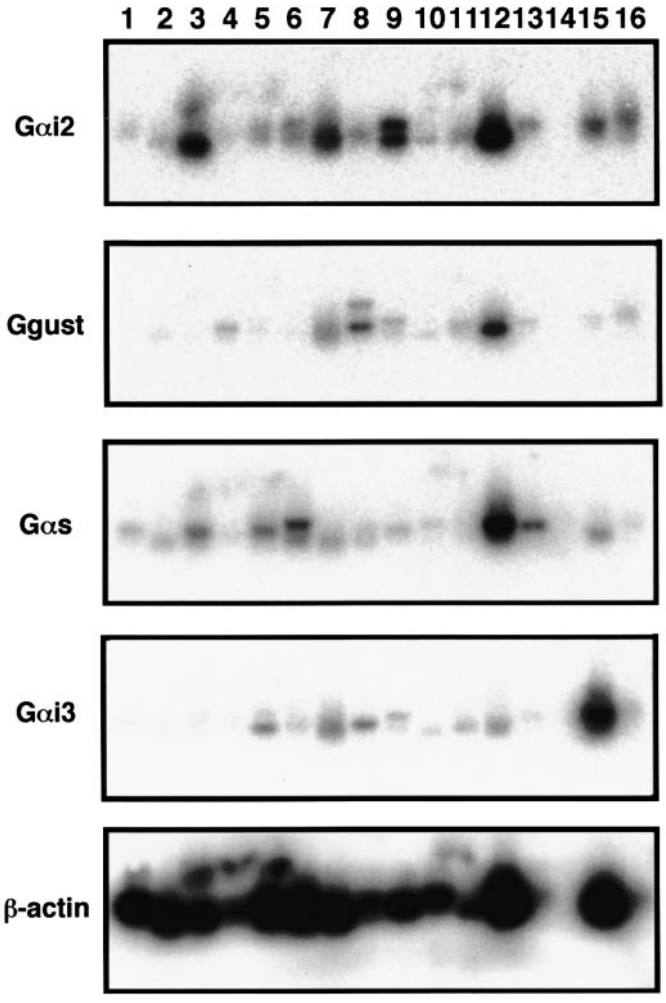

Figure 3 Southern blot analysis showing the expression of Gαs, Gαi2, Gαi3 and G_{gust} in single taste bud cells. Amplified cDNA samples from 16 single taste bud cells positive to the Gαi2 probe were electrophoresed, blotted and hybridized at 68°C with radiolabeled probes for Gαs, Gαi2, Gαi3, G_{gust} and β-actin. Lane 14 shows the result with amplified cDNA prepared without using reverse transcriptase as a negative control. Some lanes have plural bands with different sizes. This phenomenon is apparently due to the steric hindrance of mRNA molecules.

Discussion

Here we have newly identified several Gα species expressed in taste tissues. This study, together with previous papers (McLaughlin *et al.*, 1992, 1994, Kusakabe *et al.*, 1998), demonstrates that 12 Gα species are expressed in taste

tissues. Our study also shows that, among the 12, G_{gust}, Gαs, Gαi2 and Gαi3 are the dominant species in taste buds; also, in particular, G_{gust} and Gαi2 are expressed in a subset of taste bud cells. Gαi2 is thus expected to play a role in taste signal transduction, as in the case of G_{gust}. It is also possible that Gαi3 and/or Gαs, in addition to Gαi2 and G_{gust}, may have physiological functions, such as the tuning of Gαi2 and G_{gust} in taste signaling, because some Gαi2-positive cells express Gαi3 and/or Gαs, while some G_{gust}-positive cells express Gαi3 and/or Gαs. On the other hand, the other minor Gα species, such as Gα14, $G_{t\text{-}cone}$ and Gαi1, may also be of some physiological importance, although their expression levels are lower and/or ubiquitous.

In conclusion, it appears that the composition of Gα species in taste bud cells is complicated and that multiple Gα species occur in individual cells. This may be natural in the sense that, within each taste bud cell and between two of them, there are a number of cellular events other than the taste signaling, including cell-to-cell interaction, cell division, cell differentiation and signaling from gustatory nerves (Lindeman, 1996).

One intriguing observation obtained in this study is the cell-type-specific expression of Gαi2, which is known to be involved in the negative regulation of adenylyl cyclase to mitigate cAMP signaling (Taussig *et al.*, 1993). In addition, Gαi2-associated Gβγ subunits are capable of mediating Ca^{2+} signaling as an activator of PLC-β (Camps *et al.*, 1992), and of changing an ion current as an activator of an inward-rectifying K-channel (Kofuji *et al.*, 1995). Since both Ca^{2+} signaling and ion channel modulation are believed to occur in taste signal transduction (Hwang *et al.*, 1990, Berhardt *et al.*, 1996, Spielman *et al.*, 1996, Ogura *et.al.*, 1997), it is probable that there are some GPCR molecules that function by coupling with Gαi2 for taste signaling. Further studies will reveal further details to explain the significance of various Gα species, especially Gαi2, that exist in taste bud cells.

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