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G Protein βγ Complexes in Circumvallate Taste Cells Involved in Bitter Transduction

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Abstract

G protein βγ (Gβγ) complexes are considered to play an important role in second messenger signaling of phospholipase C (PLC). Monitoring the inositol 1,4,5-trisphosphate (IP_3) response in circumvallate tissue homogenates upon stimulation with denatonium benzoate, it was demonstrated that a glutathione S-transferase–GRK3ct fusion protein—a Gβγ scavenger—attenuates the bitter tastant-induced second messenger reaction. Towards an identification of the Gβγ complex involved in rat bitter taste transduction, it was found that the G protein β_3 subtype is specifically expressed in taste receptor cells of circumvallate papillae. $G\beta_3$-specific antibodies blocked the denatonium benzoate-induced IP_3 formation in a dose-dependent manner; the inhibitory effect was reversed by preincubation with the antigenic peptide. A less pronounced inhibition was observed using $G\beta_1$-specific antibodies. Analyzing individual taste cells by single cell reverse transcriptase–polymerase chain reaction approaches, overlapping expression patterns for $PLC\beta_2$, $G\alpha_{gust}$, $G\beta_3$ and $G\gamma_3$ could be demonstrated. Furthermore, the co-expression of all profiled signal transduction components in individual taste receptor cells could be detected. These data support the concept that the denatonium benzoate-induced IP_3 response is mediated by an activation of $PLC\beta_2$ via a Gβγ complex, possibly composed of $G\beta_3$ as the predominant β subunit and $G\gamma_3$, and imply that multiple second messenger pathways may exist in individual taste receptor cells.

Introduction

Given the chemical diversity of taste molecules, complex mechanisms are involved for their transduction, ranging from amiloride-sensitive ion channels and several types of K^+-channels to G protein-coupled receptors (Lindemann, 1996a). The perception of bitter stimuli is considered to be mediated by G protein-coupled reaction cascades involving the activation of at least two different effector enzymes (Kinnamon and Margolskee, 1996). One of the proposed mechanisms for signaling bitter tastants is the activation of phosphodiesterase (PDE), resulting in reduction of cAMP levels which cause activation of cyclic nucleotide-inhibited ion channels, thus leading to depolarization of bitter-reactive gustatory cells (Kolesnikov and Margolskee, 1995; Ruiz-Avila *et al.*, 1995; Wong *et al.*, 1996). In addition, there is compelling evidence for an alternative mechanism for bitter transduction which relies on activation of phospholipase C (PLC) leading to generation of IP_3 (Akabas *et al.*, 1988; Hwang *et al.*, 1990; Spielman *et al.*, 1994). A special subtype of PLCβ has recently been identified from rat circumvallate papillae which is likely to participate in sensory transduction of the bitter agent denatonium benzoate (Rössler *et al.*, 1998). Since this isoform is closely related to human $PLC\beta_2$, it may be activated in the same manner as proposed for the human isoform (Camps *et al.*, 1992; Park *et al.*, 1992; Sankaran *et al.*, 1998). In cotransfection assays, receptor-mediated release of G protein βγ (Gβγ) subunits from pertussis toxin-sensitive heterotrimeric G proteins was shown to result in activation of human $PLC\beta_2$ (Katz *et al.*, 1992). Possible sites for interaction with Gβγ complexes are the pleckstrin homology domain, a region separating the bipartite catalytic domain and the first half of the Y box (Wu *et al.*, 1993; Lee and Rhee, 1995; Kuang *et al.*, 1996; James and Downes, 1997).

Considering a role of Gβγ complexes in regulating $PLC\beta_2$ activity, we set out to identify and to characterize βγ subunits possibly involved in rat bitter transduction.

Material and methods

Isolation of taste buds, single taste cells and non-sensory lingual tissue

Tongues were isolated from freshly decapitated adult Sprague–Dawley rats (Charles River, Sulzfeld, Germany) and stored in ice-cold tyrode solution (140 mM NaCl, 5 mM

KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) for 10 min. Circumvallate papillae were collected using glass capillaries and immediately frozen in liquid nitrogen (Spielman *et al.*, 1989). Circumvallate sheets were prepared as described previously (Striem *et al.*, 1991; Bernhardt *et al.*, 1996; Kretz *et al.*, 1999). Single taste buds were harvested by suction with a 40 µm pipette containing low-Ca^{2+} tyrode solution (140 mM NaCl, 5 mM KCl, 1 mM EDTA, 10 mM HEPES, pH 7.4) by observation under an inverted microscope (Olympus IX70). For the isolation of single taste cells, individual taste buds were transferred to a Petri dish covered with cured Sylgard containing low-Ca^{2+} tyrode solution. Cells were observed under inverted microscope and taste receptor cells were identified by their characteristic morphology. Single taste cells were picked up under microscopic control with a micromanipulator by applying suction on a 10 µm microcapillary containing low-Ca^{2+} tyrode solution. Single cells were then transferred to PCR tubes by applying positive pressure, immediately frozen in liquid nitrogen and stored at –70°C. Non-sensory lingual tissue was obtained from epithelium around the circumvallate papilla and from the dorsal part of the tongue, just anterior to the circumvallate papilla, including underlying muscle and salivary tissue.

RNA preparation, cDNA synthesis and amplification of cDNA

Total RNA isolated from non-sensory control tissue was prepared by tissue homogenization in TRIzol reagent (Gibco BRL, Eggenstein, Germany) according to the manufacturer's instructions, including a DNase digestion (DNase I, Gibco BRL). Subsequently, mRNA was isolated from total RNA of control tissue and directly from single taste buds (Dynabeads, Dynal, Oslo, Norway), and reverse transcribed using a first-strand cDNA synthesis kit according to the manufacturer (Pharmacia, Freiburg, Germany). cDNA of individual taste buds was synthesized and re-amplified as described in the following for single taste cells. For lysis of single cells, frozen cells were incubated at 72°C for 2 min and rapidly chilled on ice. Single-stranded cDNA was synthesized using Superscript reverse transcriptase II (Gibco BRL) according to the manual provided with the SMART™ cDNA technology (Clontech Laboratories, Heidelberg, Germany). The amplification of the cDNA was performed using the SMART™ PCR cDNA synthesis kit (Clontech Laboratories) according to the manufacturer's recommendations. Cycling parameters were as follows: 95°C for 1 min; 95°C for 15 s, 65°C for 30 s and 68°C for 6 min (24 cycles); 68°C for 6 min. After ethanol precipitation of the amplified cDNA the resulting pellet was resuspended in 20 µl water.

Reverse transcriptase–polymerase chain reaction (RT–PCR), cloning and sequencing

For amplification of genes encoding Gβ subtypes, equal amounts (20–100 ng) of cDNA from single taste buds or non-sensory control tissue was used as the template. For analysis of Gβ gene expression, five independent amplifications were performed: four experiments employing an individual taste bud and one experiment with a pool of six taste buds. The sense primers were for $G\beta_1$ 5′-TGACACCAGACTGTTTGTCTC-3′; for $G\beta_2$ 5′-CCAGATCACAGATGGGCTG-3′; for $G\beta_3$ 5′-AGAAGACAGTGTTCGTGGGAC-3′; for $G\beta_4$ 5′-GGGGATATGATTCCAGGCTAC-3′; and for $G\beta_5$ 5′-TACAGAGCTTCCATGGGCA-3′. The antisense primers were common for $G\beta_{1–4}$ 5′-KCCWGTDGCCACAGCCATSC-3′ and for $G\beta_5$ 5′-GATCCCATGATCCCGAGCAG-3′. Amplification was carried out in 50 µl of 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 25 pmol of each primer and 2 U of Taq DNA polymerase (Gibco BRL). PCR was performed according to the following conditions: 94°C for 1 min; 94°C for 30 s, 62.5°C for 1.30 min and 72°C for 40 s (25 cycles); 72°C for 7 min. A 0.5–1 µl aliquot of the re-amplified cDNA from a single cell was used as template for specific RT–PCR amplifications. In all, 12 individual taste cells were subjected to single cell RT–PCR. PCR was carried out as described before. The specific primers for $PLC\beta_2$ were 5′-CTGGAGGCTGAAGTAAAGGAG-3′ (sense) and 5′-GCCCCTGCATGTATGTTAGG-3′ (antisense); for $G\beta_3$ 5′-GGACTCTCTGAAGTGTGAG-3′ (sense) and 5′-CACCTATAGTCATGGGCTG-3′ (antisense); and for $G\alpha_{gust}$ 5′-CAATCCGAGAAGTAGAGAGG-3′ (sense) and 5′-GCTGTTGAAGAGGTGAAGAC-3′ (antisense). In order to identify Gγ subtypes present in single cells, degenerate primers matching conserved regions of Gγ coding genes were used which had been tested for their ability to amplify common Gγ subtypes (Gallagher and Gautam, 1994; Gautam *et al.*, 1998). The primers were 5′-GTIGAICARCTIAARATI-3′ (sense) and 5′-YTCICKRAAIGGRTTYTC-3′ (antisense). The amplification was performed according to the following schedule: 94°C for 2 min, 50/60°C for 2 min and 72°C for 5 min (1 cycle); 94°C for 30 s, 50/60°C for 1.30 min and 72°C for 30 s (34 cycles); 72°C for 7 min. The annealing temperature for $PLC\beta_2$, $G\beta_3$ and $G\alpha_{gust}$ was 60°C; for genes encoding Gγ subtypes it was 50°C. To exclude an amplification of genomic DNA contamination, the quality of cDNA was monitored by using primers corresponding to distinct exons of the β actin gene in PCR reactions (Ziegler *et al.*, 1992). Actin exon 4 primer (5′-TCATGTTTGAGACCTTCAA-3′) and actin exon 5 primer (5′-GTCTTTGCGGATGTCCACG-3′) amplified a 512 bp fragment transcribed from RNA and a 607 bp fragment from genomic DNA. Following PCR, 10 µl of the reaction products were separated on 1.2% agarose gels. PCR products were subcloned into pGEM-5Zf(+) vector using the pGEM-T vector system I (Promega, Mannheim, Germany). Recombinant plasmids were subjected to DNA sequencing using the RR Dye Deoxy Terminator cycle sequencing kit (PE Biosystems, Weiterstadt, Germany).

Automatic sequencing was performed on an ABI 310 sequencer (PE Biosystems).

Analysis of sequence data

Analysis of sequence data was performed using HUSAR 3.0 software package based on the sequence analysis software package 7.2 from the Genetic Group (Madison, WI).

Stimulation experiments and second messenger determination

The circumvallate papilla from each rat tongue was dissected using glass capillaries and immediately frozen in liquid nitrogen (Spielman *et al.*, 1989, 1996). For a typical stimulation experiment, 10 circumvallate papillae were minced in a glass homogenizer in hypotonic buffer (10 mM Tris/HCl, 3 mM $MgCl_2$, 2 mM EGTA, pH 7.4) and centrifuged at 750 *g* for 10 min at 4°C. The supernatants were immediately employed in subsequent stimulation experiments. Therefore, denatonium benzoate was diluted in reaction buffer [200 mM NaCl, 10 mM EGTA, 50 mM 4-morpholinepropanesulphonic acid, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% sodium cholate, 1 mM ATP and 4 μM GTP, 12 nM free calcium calculated and adjusted as described elsewhere (Pershadsingh and McDonald, 1980), pH 7.4] containing denatonium benzoate at a final concentration of 100 μM. To prevent degradation of IP_3, stimulation experiments were performed in the presence of 20 mM LiCl (final concentration during incubation). The reaction was started by mixing 210 μl of prewarmed reaction buffer with 50 μl of the protein samples (0.59 ± 0.1 μg/μl), incubated for 2 min at 37°C in a shaking water bath and stopped by 7% ice-cold perchloric acid (105 μl) before the concentration of IP_3 was determined according to Palmer and Wakelam (Palmer and Wakelam, 1989). To determine the influence of the glutathione S-transferase (GST)–GRK3ct fusion protein, the GST control peptide and the subtype-specific Gβ antisera (Santa Cruz Biotechnology, Santa Cruz, CA) on the stimulus-induced second messenger responses, circumvallate protein samples were preincubated with the indicated dilution of the modulators for 10 min on ice. The GST–GRK3ct fusion protein and the GST control peptide were prepared and purified in a manner described previously (Koch *et al.*, 1993). In additional experiments, the specificity of the $G\beta_3$-specific antibodies was verified by incubating these antibodies with the corresponding peptide according to the specifications of the manufacturer (Santa Cruz Biotechnology) prior to stimulation.

In situ hybridization

Circumvallate papillae were dissected from 2- to 5-week-old rats, embedded in Tissue Tek (Miles Inc., Elkhart, IL) and rapidly frozen in a liquid N_2 cooled isopentane bath. Coronal sections of 10 μm were cut on a Leica cryostat (model CM 3000) at –30°C and thaw-mounted on silanated slides. Generation of antisense and sense digoxigenin-labeled probes from $G\beta_1$, $G\beta_2$ and $G\beta_3$ cDNAs previously amplified by RT–PCR and subsequent *in situ* hybridization were performed as described previously (Rössler *et al.*, 1998).

SDS–PAGE and Western blot analysis

Membrane fractions of the gustatory tissue were prepared for SDS–PAGE as described previously (Rössler *et al.*, 1998), subjected to 12.5% acrylamide gel electrophoresis and analyzed using the Laemmli buffer system (Laemmli, 1970). The separated proteins were transferred onto nitrocellulose using the semi-dry blotting system (Pharmacia). The blot was stained with Ponceau S and stored at 4°C until use. For Western blot analysis, non-specific binding sites were blocked with 8% non-fat milk powder (Naturaflor) in TBST (10 mM Tris, pH 8.0, 150 mM NaCl and 0.05% Tween 20); the blots were incubated overnight with the $G\beta_3$ polyclonal antibody (Santa Cruz Biotechnology) diluted in TBST, containing 3% non-fat milk powder. After three washes with TBST, horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG; 1:10 000 dilution in TBST with 3% milk powder) was applied and the enhanced chemoluminescence system (Amersham) was used to monitor immunoreactivity.

Immunohistochemistry

Circumvallate papillae were dissected from tongues of 2- to 5-week-old rats and placed in 4% paraformaldehyde, 1% picric acid in phosphate-buffered saline (PBS; 150 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4), pH 7.3, for 2 h at 4°C. Tissue was rinsed in PBS, cryoprotected by immersion in 25% sucrose/PBS for 2 h at 4°C and embedded as described before. Coronal sections (10 μm) adhered to Superfrost plus (Fisher, Orangeburg, NY) microslides were air-dried for 4 h. Slides were treated with 0,1% Triton X-100 in PBS for 3 min. After washing three times in PBS, unspecific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min in a wet chamber at room temperature. Excess of blocking solution was removed before application of the $G\beta_1$-, $G\beta_2$- and $G\beta_3$-specific antibody (Santa Cruz Biotechnology), diluted 1:200 in 1% BSA/PBS. After incubation at 37°C in a wet chamber for 2 h, sections were washed three times with PBS. The goat anti-rabbit IgG conjugated to the flourescent dye Cy 3 antibody (Jackson ImmunoResearch, West Grove, PA) was applied (1:500 in PBS) and incubated for 1 h at room temperature. After three washes with PBS the reaction was stopped with double distilled water. The slides were air-dried, embedded in Vectashield (Vector Laboratories, Burlingame, CA) and examined under a Zeiss Axiphot microscope. The specificity of the immunoreactivity obtained by the $G\beta_3$-specific antibody was confirmed in control experiments employing primary antibodies preincubated with the antigenic peptide according to the

specifications of the manufacturer (Santa Cruz Biotechnology).

Results

In order to approach the question whether Gβγ subunits may play a role in tastant-induced IP_3 responses, a fragment of the G protein receptor kinase GRK3 was used as a scavenger for βγ subunits with respect to its capacity to directly interact with βγ dimers and to block their ability to modulate corresponding effectors (Pitcher *et al.*, 1992; Boekhoff *et al.*, 1994; Daaka *et al.*, 1997). Therefore, tissue homogenates of circumvallate papillae were incubated with a GST–GRK3ct fusion protein containing the C-terminal 222 amino acid residues corresponding to the βγ binding domain of the GRK3 (Pitcher *et al.*, 1992) and subsequently analyzed for formation of IP_3 upon stimulation with denatonium benzoate. In such second messenger assays, the bitter agent causes a generation of IP_3 in taste tissue, but not in non-gustatory control tissue (Spielman *et al.*, 1996; Rössler *et al.*, 1998). As demonstrated in Figure 1, in the presence of GST–GRK3ct fusion protein the denatonium benzoate-induced IP_3 response was significantly attenuated. In contrast, a GST control peptide had no effect on the tastant-induced second messenger response, emphasizing the specificity of the inhibitory effect. Since the C-terminal domain of GRK3 has no enzymatic activity, it is conceivable that the inhibitory effect is due to its interaction with membrane-anchored βγ subunits, thus supporting the notion that Gβγ subunits may mediate a tastant-induced activation of phospholipase C.

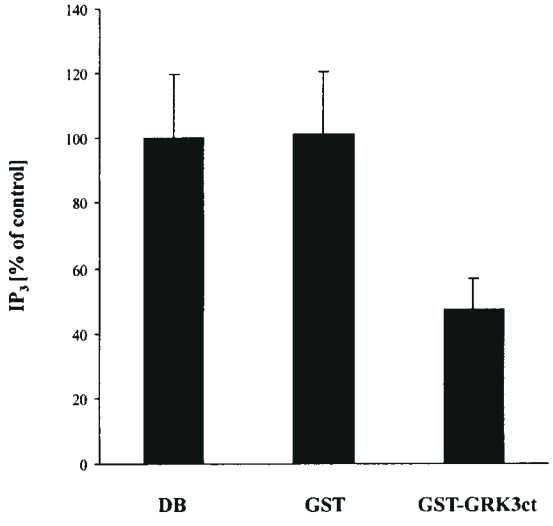

Figure 1 Denatonium benzoate-induced IP_3 response is blocked by GST–GRK3ct fusion protein containing the βγ binding domain. After stimulation with 100 μM denatonium benzoate an inhibition of 53% was observed using 10 μM GST–GRK3, whereas pretreatment with 10 μM GST control peptide had no effect (1%). The basal level of IP_3 in circumvallate homogenates was 207.4 ± 110.5 pmol/mg of protein; denatonium benzoate stimulation increased the IP_3 concentration to 331.6 ± 88.4 pmol/mg of protein. Data, calculated as the percentage of denatonium benzoate-induced IP_3 formation, are the mean values of three independent experiments with triplicate determinations ± SD. DB, denatonium benzoate-induced IP_3 response under control conditions.

As a first step towards an identification of the distinct β and γ subunits possibly involved in the transduction process for bitter compounds, RT–PCR assays were performed to identify the β subtypes expressed in gustatory tissue. To date, five β subunits have been identified in mammals, generally designated as $Gβ_1$–$Gβ_5$; in addition, a heart-specific rat $Gβ_3$ subtype as well as a retina-specific $Gβ_5$ splice variant have been described (Ray and Robishaw, 1994; Watson *et al.*, 1994; Gautam *et al.*, 1998). Specific oligonucleotide primer pairs targeting each known β subunit were employed in

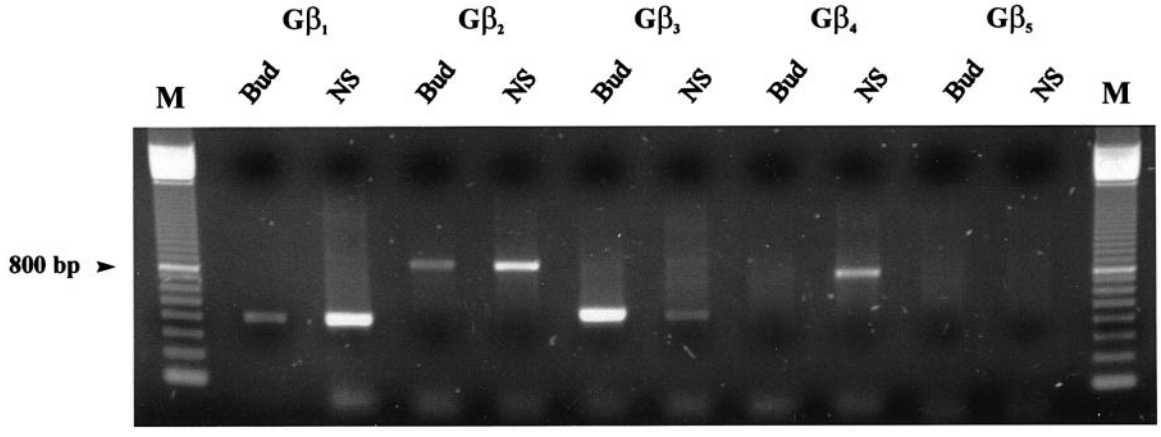

Figure 2 Differential expression of the Gβ genes evaluated by RT–PCR. RNA isolated from a circumvallate taste bud **(lanes 1, 3, 5, 7 and 9)** and non-sensory lingual tissue **(lanes 2, 4, 6, 8 and 10)** was subjected to RT–PCR analysis using specific primers for each known β subunit. The PCR products resulted in bands of predicted size (lanes 1 and 2, ±410 bp for $Gβ_1$; lanes 3 and 4, ±906 bp for $Gβ_2$; lanes 5 and 6, ±470 bp for $Gβ_3$; lanes 7 and 8, ±820 bp for $Gβ_4$; lanes 9 and 10, ±480 bp for $Gβ_5$). The position of the 100 bp mol. wt marker is shown on the left. Identical results were obtained in four independent experiments employing individual taste buds and an additional RT–PCR amplification of RNA isolated from a pool of six taste buds. Note the strong amplification signals for $Gβ_3$ in taste bud cDNA. Bud, circumvallate taste bud; NS, non-sensory lingual tissue.

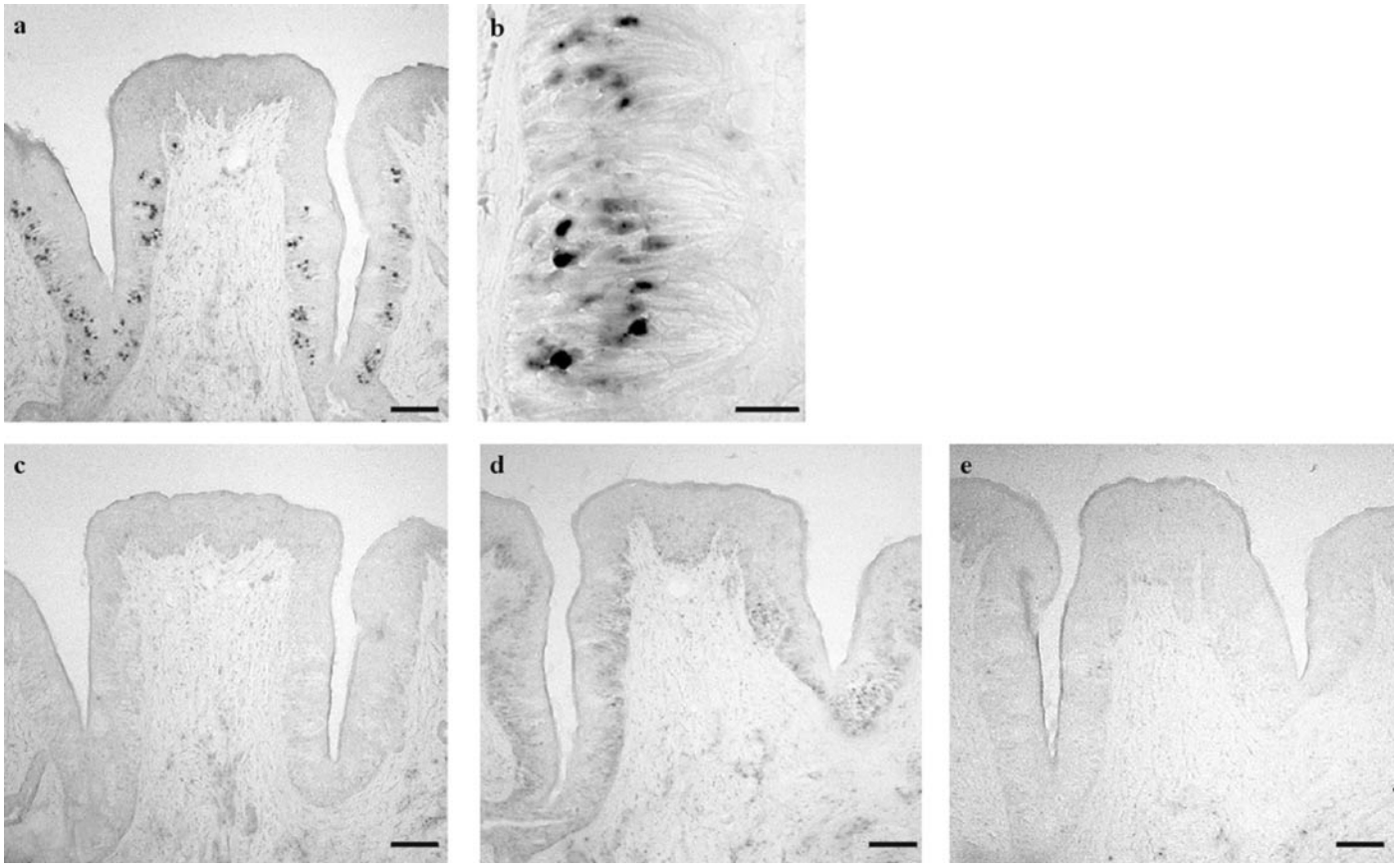

Figure 3 Cellular localization of $G\beta_3$, $G\beta_2$ and $G\beta_1$ expression in rat circumvallate papillae by *in situ* hybridization. **(a)** In the circumvallate taste buds individual gustatory cells were intensively labeled using the $G\beta_3$-specific antisense probe. Signals were absent from the adjacent non-sensory lingual epithelium (bar = 50 μm). **(b)** High magnification of the hybridization with the $G\beta_3$ antisense probe indicates the labeling of individual cells within the taste buds (bar = 20 μm). **(c)** No signals were observed in control experiments using the $G\beta_3$ sense probe (bar = 50 μm). **(d)** The $G\beta_2$ antisense probe diffusely labeled cells close to the taste buds basal membrane and cells in the surrounding epithelium (bar = 50 μm). **(e)** No hybridization signals were detected within taste buds using the $G\beta_1$ antisense probe (bar = 50 μm).

RT–PCR experiments using cDNA obtained from isolated taste buds of rat circumvallate papillae as template. For comparison, experiments with cDNA from non-sensory lingual tissue were performed. The results of these RT–PCR studies, shown in Figure 2, indicate highly amplified PCR fragments of the expected size for $G\beta_3$. In contrast, specific primers for $G\beta_1$ and $G\beta_2$ resulted in minor signals of predicted size, whereas no amplification products were detectable using either $G\beta_4$-or $G\beta_5$-specific primers. For the non-sensory lingual tissue, strong amplification signals for $G\beta_1$ and $G\beta_2$ and weak signals for $G\beta_3$ and $G\beta_4$ were obtained (Figure 2). Sequence analysis of the amplified PCR fragments confirmed their identity to the previously described $G\beta_1$, $G\beta_2$ and $G\beta_4$ subtypes, as well as to the $G\beta_3$ subtype that is highly expressed in rat heart (Ray and Robishaw, 1994).

To explore the topological expression pattern of specific Gβ subtypes in the lingual tissue, *in situ* hybridization experiments were performed using digoxigenin-labeled antisense probes of $G\beta_1$, $G\beta_2$ and $G\beta_3$. Only the $G\beta_3$ subtype was expressed in gustatory cells of the circumvallate taste buds (Figure 3a,b); transcripts for $G\beta_2$ were weakly detected in cells close to the basal membrane of the rat taste buds and in cells of the surrounding non-gustatory lingual epithelium (Figure 3d). The antisense probe for $G\beta_1$ failed to produce any hybridization signal within taste buds or adjacent tissue (Figure 3e). Since the RT–PCR led to the isolation of $G\beta_1$, the failure of the $G\beta_1$ antisense probe to hybridize within circumvallate buds may be due to a very low expression level.

To localize $G\beta_3$ protein and to monitor the subcellular localization, immunohistochemical experiments were performed. The specificity of the employed antibodies was first analyzed in Western blots. As depicted in Figure 4, antibodies raised against $G\beta_3$ visualized a polypeptide band with an apparent molecular mass of 37 kDa in circumvallate membrane preparations, corresponding to the expected molecular weight for $G\beta_3$. The specificity of the labeling was confirmed by preincubation of the primary antibody with the antigenic peptide, which inhibited immunoreactivity

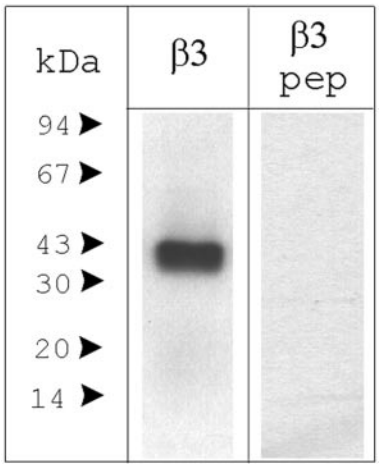

Figure 4 Western blot analysis of rat circumvallate papillae validating the specificity of $G\beta_3$-specific antibodies. The $G\beta_3$ antibody was applied at 1:1000 dilution. Left panel, in membrane preparations of circumvallate papillae a strongly labeled band at about 37 kDa was visualized. Right panel, absorption of the $G\beta_3$ antibodies with the corresponding synthetic peptide. The positions of the low molecular mass markers are shown on the left.

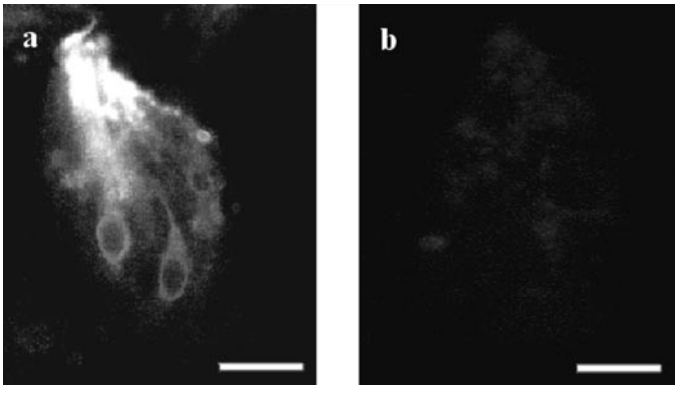

Figure 5 Immunohistochemical localization of $G\beta_3$ in rat circumvallate taste buds using the $G\beta_3$-specific antibodies. **(a)** Immunoreactivity is restricted to a subset of elongated cells within a taste bud with a particular strong labeling of the apical portion of the taste bud (bar = 20 μm). **(b)** No immunoreactivity was observed after preincubation of the $G\beta_3$ antibody with the antigenic peptide (bar = 20 μm).

(Figure 4). Subsequently, coronal sections through the tongue were subjected to immunostaining. It was found that the $G\beta_3$ protein was located in a subset of spindle-shaped cells; particular strong immunoreactivity was observed at the apical part of taste cells. No labeling was detectable in surrounding epithelial cells (Figure 5a). The possibility that the observed immunoreactivity represented artifactual labeling was excluded in a control experiment: when the primary antibody was blocked with the antigenic peptide, no labeling could be detected (Figure 5b). Employing specific antibodies for $G\beta_1$ and $G\beta_2$ gave no staining of elongated taste cells; $G\beta_2$ was restricted to cells in the basal region of

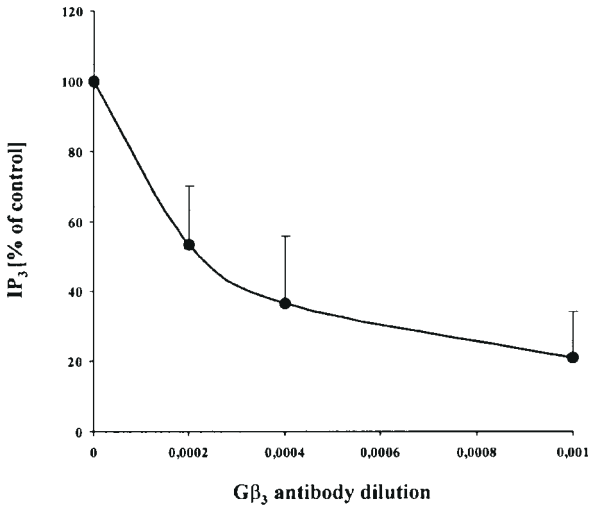

Figure 6 Concentration-dependent inhibition of denatonium benzoate-induced IP_3 formation by $G\beta_3$-specific antibodies. After stimulation with 100 μM denatonium benzoate an inhibition of 47% was obtained at an antibody dilution of 1:5000, 63% at a 1:2500 dilution and 79% at a 1:1000 dilution. Data, calculated as a percentage of denatonium benzoate-induced IP_3 formation, are the mean values of three independent experiments with triplicate determinations ± SD.

the taste buds and low immunoreactivity for $G\beta_1$ was found in the microvillar taste pore region (data not shown). These results confirm the *in situ* data and suggest that $G\beta_3$ may play a role in taste signal transduction.

To assess this notion, biochemical assays were performed monitoring the effect of $G\beta_3$-specific antibodies on denatonium benzoate-induced IP_3 responses in circumvallate preparations. As shown in Figure 6, preincubation with $G\beta_3$ antibodies attenuated the bitter tastant-induced IP_3 response in a dose-dependent manner. $G\beta_3$ antibodies caused a strong inhibition already at very low antibody concentrations; 47% inhibition was registered at a 1:5000 dilution. The specificity of this effect was confirmed in control experiments, employing $G\beta_3$ antibodies neutralized with the antigenic peptide, where no inhibitory effect was observed (Figure 7). Comparing the effects of the Gβ subtype-specific antibodies on the denatonium benzoate-induced IP_3 signaling, it was found that $G\beta_2$ antibodies did not affect the responsiveness; in contrast, antibodies specific to $G\beta_3$ and $G\beta_1$ reduced IP_3 formation elicited by the bitter agent; however, at a 1:1000 dilution of both subtype-specific antibodies, the inhibitory effect of $G\beta_1$ was less pronounced than blockage observed upon pretreatment with $G\beta_3$-specific antibodies (Figure 7).

Even though these results strengthen the hypothesis for a functional involvement of $G\beta_3$ in denatonium benzoate-induced IP_3 transduction, it still remains unclear whether $G\beta_3$ could communicate bitter signals from receptors to the effector enzyme $PLC\beta_2$. Therefore, individual taste cells were assessed by single-cell RT–PCR approaches in order to examine $PLC\beta_2$-positive cells for expression of G protein

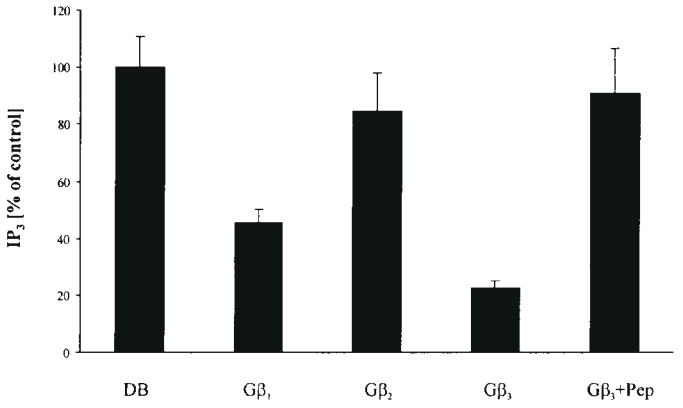

Figure 7 Effect of subtype-specific Gβ antibodies on IP_3 formation induced upon stimulation with denatonium benzoate. Comparing the same antibody dilution (1:1000) after stimulation with 100 μM denatonium benzoate a strong inhibition was registered using $Gβ_3$ antibodies (77%; **$Gβ_3$**), a less pronounced inhibition using $Gβ_1$ antibodies (54%; **$Gβ_1$**) and almost no inhibition using $Gβ_2$ antibodies (16%; **$Gβ_2$**). The inhibitory effect of $Gβ_3$ antibodies was neutralized after absorbing $Gβ_3$ antibodies with the synthetic peptide (9%; **$Gβ_3$ + Pep**). Data, calculated as percent of denatonium benzoate-induced IP_3 formation, are the mean values of three independent experiments with triplicate determinations ± SD. DB, denatonium benzoate-induced IP_3 response under control conditions.

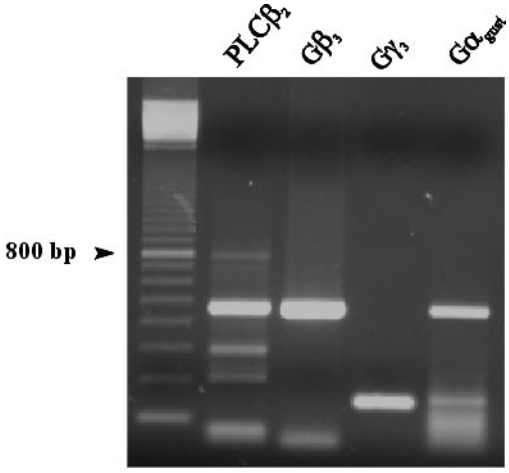

Figure 8 Identification of $Gβ_3$, $Gγ_3$ and $Gα_{gust}$ in a $PLCβ_2$-expressing circumvallate gustatory cell using a single cell RT–PCR approach. RT–PCR analysis resulted in PCR products of expected size; 474 bp for $PLCβ_2$ **(lane 1)**, 476 bp for $Gβ_3$ **(lane 2)**, 144 bp for $Gγ_3$ **(lane 3)** and 469 bp for $Gα_{gust}$ **(lane 4)**. The position of the 100 bp mol. wt marker is shown on the left.

subtypes. Twelve individual taste receptor cells were picked according to their typical elongated bipolar morphology. To monitor possible RNA degradation, cells were first tested for β actin expression. All 12 cells showed a positive actin amplification, indicating the integrity of the employed RNA (data not shown). Of these 12 cells, eight were $PLCβ_2$ positive. These eight cells were further analyzed employing a specific primer combination for $Gβ_3$, degenerate oligonucleotides for Gγ subunits and primers for the taste cell-specific G protein α subunit gustducin ($Gα_{gust}$) (McLaughlin *et al.*, 1992; Gallagher and Gautam, 1994; Clapham and Neer, 1997; Gautam *et al.*, 1998). In detail, two of the $PLCβ_2$-expressing taste cells showed amplification products of the expected size employing $Gβ_3$- (476 bp), $Gα_{gust}$- (469) and Gγ-specific primers (140 bp) (Figure 8). Two other cells showed positive amplification with $Gβ_3$- and $Gα_{gust}$-specific primers, and an additional two cells with primers targeting only Gγ subunits. Two cells showed amplification products either with $Gβ_3$- or with $Gα_{gust}$-specific primers. By subcloning and sequence analysis, the Gγ-specific amplification products were identified as $Gγ_3$-coding sequences in each cell, whereas the specificity of the other amplification products could be confirmed.

These results indicate that $PLCβ_2$, $Gα_{gust}$, $Gβ_3$ and $Gγ_3$ are coexpressed in individual cells and suggest that the denatonium benzoate-induced transduction cascade could be mediated by $PLCβ_2$ and the $Gβ_3γ_3$ complex.

Discussion

Several bitter-tasting compounds have been correlated with PLC activation and a raise of IP_3 levels in gustatory cells (Spielman *et al.*, 1996). With respect to the sensitivity of PLC to stimulation by βγ dimers (Park *et al.*, 1993; Wu *et al.*, 1993), the results of the present study point to a role for distinct Gβγ complexes as modulators of the PLC effector system. The specific expression of the $Gβ_3$ subtype in gustatory cells of circumvallate taste buds and the inhibitory effect of $Gβ_3$-specific antibodies on the denatonium benzoate-induced IP_3 formation suggest a major function for this β subunit in mediating second messenger signaling in bitter taste. So far, the rat $Gβ_3$ subtype has been found to be predominately expressed in heart, whereas the closely related human $Gβ_3$ (96% identity) is most abundantly expressed in retina and brain (Levine *et al.*, 1990; Ray and Robishaw, 1994), suggesting that each subtype is restricted to specialized systems and interacts with a limited set of effectors.

One proposed mechanism for transduction of the intensely bitter compound denatonium benzoate is an increase of IP_3 levels via activation of PLC (Akabas *et al.*, 1988; Hwang *et al.*, 1990; Spielman *et al.*, 1994). The possible effector enzyme likely involved in transducing Gβγ-mediated responses to bitter tastants is the recently identified novel $PLCβ_2$ subtype, which is selectively expressed in distinct taste sensory cells of the rat circumvallate papillae (Rössler *et al.*, 1998). In addition, it has been proposed that denatonium benzoate may activate an alternative transduction pathway mediated by the taste-specific Gα subunits gustducin and/or transducin, activating a phosphodiesterase; the resulting decreased levels of cyclic nucleotides might elicit taste cell depolarization through a cyclic nucleotide-suppressible conductance (Kolesnikov and Margolskee, 1995; Ruiz-Avila *et al.*, 1995; Wong *et al.*, 1996). Besides the given multiplicity of pathways involved in bitter taste

transduction, the molecular data of this study demonstrate that an individual cell expressing PLCβ_2 is also equipped with the G$\beta_3\gamma_3$ complex and Gα_{gust}. The notion that a similar portion of the monitored cells express either PLCβ_2, Gα_{gust} and Gβ_3 without Gγ_3 or, alternatively, PLCβ_2 and Gγ_3 without Gα_{gust} and Gβ_3 raises the possibility that other Gβ or Gγ subunits might be involved in bitter signal transduction processes. In this context, it is interesting to note that recent studies led to the identification of a novel Gγ subtype (Gγ_{13}) which is coexpressed with gustducin and which is functionally involved in bitter taste transduction (Huang *et al.*, 1999); furthermore, it was found that gustducin-positive cells always express Gγ_{13} and Gβ_3, whereas 79% also express Gβ_1. Thus, the results of the present study demonstrating that Gβ_3- as well as Gβ_1-specific antibodies block the denatonium benzoate-induced rise in IP_3 led to the suggestion that distinct compositions of Gβ and Gγ subtypes might take part in bitter taste transduction processes. As distinct β subunits have selective effects on the activity of effector enzymes (Clapham and Neer, 1997; Hamm, 1998), it is conceivable that the Gβ_1 and Gβ_3 subtypes could differ in their regulatory activities of downstream signaling molecules.

The finding that Gα_{gust} and PLCβ_2 are coexpressed in the same cell raises the possibility that particular taste sensory cells may respond to denatonium benzoate through the PDE- as well as the PLC-mediated signaling machinery, thus activating parallel pathways. This notion is supported by a recent study demonstrating that denatonium induces not only a rise in the IP_3 level but also a suppression of the cAMP level; preincubation with gustducin antibodies rescued the suppressive effect on cAMP but did not affect IP_3 responses (Yan *et al.*, 1999). Also, in mouse fungiform papilla denatonium elicited both IP_3 and cAMP responses (Nakashima and Ninomiya, 1999). These effects may account for a synchronous activation of the PDE cascade through Gα_{gust} and the PLC cascade through the associated βγ complex in a distinct taste cell subpopulation. Basically, the existence of multiple second messenger pathways in one taste cell may have an important role in fine-tuning and modifying the response to a given taste stimulus, as has been suggested for the olfactory system (Ache and Zhainazarov, 1995). Moreover, it has been proposed that an individual taste cell may respond to more than one stimulus (Lindemann, 1996b). Recent evidence that particular taste cells express both putative taste receptors, TR1 and TR2, as well as the observation that neither TR1 nor TR2 are uniformly coexpressed with gustducin strengthen this concept (Hoon *et al.*, 1999). Future studies should gain further insight how the sensory cells receive and process tastant stimuli.

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