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Characterization of Bitter Taste Responses of Intestinal STC-1 Cells

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Abstract

Cellular responses of STC-1 cells to two bitter tastants (denatonium and caffeine) were investigated using a calcium-imaging technique and compared with the response to bombesin. Caffeine is known to stimulate taste receptor cells, but the properties of its signaling have not been well studied. STC-1 cells responded to all three molecules in a dose-dependent manner, and when a reverse transcriptase–polymerase chain reaction (RT–PCR) for denatonium receptor was performed, the product of predicted size was detected in STC-1 cells. Furthermore, all three signaling pathways were blocked by a phospholipase C (PLC) inhibitor, demonstrating the essential involvement of PLC in cellular responses. To study the regulatory system of G protein signaling in STC-1 cells, we searched G protein-coupled receptor kinases (GRKs) by the degenerate-primer PCR method and found that GRK2 is expressed. We also demonstrated that three GRKs (GRK2, GRK3 and GRK5) are differentially distributed in the circumvallate papilla while only GRK2 is present in taste bud cells. Finally, we overexpressed GRK2 in SCT-1 cells and found that bombesin-induced response was strongly inhibited by GRK2 but denatonium-activated signaling was not affected. In the case of caffeine, response was decreased by expression of GRK2 only when cells were activated by 1 mM caffeine. Thus, we showed that STC-1 cells emerge as a cell model for studying the molecular mechanism of bitter taste signaling, and could indicate properties of caffeine-induced signaling in comparison with other signaling.

Key words: denatonium, caffeine, dose-response relationship, U-73122, GRK, circumvallate papilla

Introduction

In vertebrates, tastants are detected mainly by specialized cells in taste buds on the tongue surface. Recent advances in our understanding of taste transduction have come from the identification of molecular components governing these processes. During the past few years, several G protein-coupled receptors have been identified in taste receptor cells and implicated in taste responses to bitter, sweet, and umami compounds (for review, see Gilbertson *et al.*, 2000; Lindemann, 2001). Umami tastes are mediated by T1r3 plus T1r1 and a truncated type 4 metabotropic glutamate receptor, and sweet tastes are mediated by T1r3 plus T1r2 (Nelson *et al.*, 2001, 2002; Li *et al.*, 2002). Also, bitter tastes are detected by the T2R family (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000). Heterotrimeric G protein complex containing α-gustducin has been shown to play significant roles in taste responses to bitter, sweet, and umami (Wong *et al.*, 1996; Ruiz *et al.*, 2003). It has been demonstrated that phospholipase C subtype β2 (PLCβ2) and the TRP channel subtype m5 (TRPM5) act as key effector enzymes of the taste transduction (Zhang *et al.*, 2003). Further studies, however, are required to determine which members of the T1R and T2R families recognize individual tastants and how sweet, umami and bitter transduction, despite relying on different receptors, converge on common signaling molecules such as PLCβ2 and TRPM5. Thus, G protein signaling pathways of taste receptors and their regulatory mechanism are still unclear.

The gustatory system is known to detect nutritive and beneficial compounds as well as harmful or toxic substances (Herness and Gilbertson, 1999; Katz *et al.*, 2000). In addition, chemosensory information perceived during the gastric and intestinal phases of digestion is important for the regulation of various aspects of gastrointestinal function, such as the secretory activity of gastrointestinal glands, the

resorptive activity, motility and blood supply of the intestinal tract, and satiation (Dockray, 2003). The enteroendocrine cells are specialized transducers of luminal factors. They are particularly important for the control of digestion and food intake. In addition, these cells are integrators, since they also respond to many different neurohumoral factors, growth factors and cytokines. STC-1 cells are well known as an enteroendocrine cell line. This cell line was derived from an endocrine tumor that developed in the small intestine of a double transgenic mouse expressing the rat insulin promoters linked to SV40 large T antigen and to the polyomavirus small T antigen (Rindi *et al.*, 1990). As cells of the upper small intestine, STC-1 cells express gastrin-releasing peptide receptors (GRP-R or BB2) and are activated by a neuropeptide, bombesin, through the IP3 pathway to elicit secretin secretion (Snow *et al.*, 1994; Chang *et al.*, 1998). Furthermore, Wu *et al.* (2002) recently demonstrated that STC-1 cells express T2R family members and respond to bitter taste substances.

To characterize cellular responses initiated by bitter tastant molecules, we examined the processes of cellular activation by monitoring responses in the intracellular calcium using the Fura-2 calcium-indicator dye. Bombesin signaling pathway is well characterized; therefore the bitter responses were compared with bombesin response. We could determine dose-dependent activation of STC-1 cells by bombesin and two bitter tastants of caffeine and denatonium. Their signaling pathways were also characterized by analyzing the effects of a PLC inhibitor. Especially, the signaling induced by caffeine as a bitter tastant was clearly detected and it was completely blocked by a PLC inhibitor.

G protein-coupled receptors (GPCRs) are regulated via activation-dependent phosphorylation by a family of G protein-coupled receptor kinases (GRKs). This process, which modulates the coupling state of GPCRs to their G proteins, is critically involved in GPCR desensitization. Seven distinct GRK genes are known, named *GRK1–7* (for review, see Ferguson, 2001). We also searched GRKs to investigate the regulatory system of G protein signaling in STC-1 cells and identified GRK2. We further examined how cellular responses induced by the three compounds are regulated by GRK2 expression in STC-1 cells.

Materials and methods

Materials

Caffeine and bombesin were from Sigma. Denatonium benzoate was from Fluka. Fura-2 AM was from Molecular Probes. U-73343 was from Wako. U-73122 was from Calbiochem. Polyclonal antibodies against gustducin (I-20), GRK2 (C-15), GRK3 (C-14) and GRK5 (C-20) were from Santa Cruz Biotechnology. Block Ace was from Dainippon Pharmaceutical. Growth factor reduced Matrigel® matrix was from Becton Dickinson. Lipofectamine 2000 and M-MLV reverse transcriptase were from Invitrogen. The STC-1 cell line was a gift from Dr D. Hanahan (University of California, San Francisco, CA). The vector pDsRed-N1 encoding the bovine GRK2 sequence (pDsRed-N1-GRK2) was provided by Dr R. Schulz (University of Munich, Munich, Germany). The vector pCMV5 encoding the human GRK5 sequence was provided by Dr J.L. Benovic (Thomas Jefferson University, Philadelphia, PA).

Cell culture and transient transfection of STC-1 cells

The culture medium consisted of DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin plus 100 μg/ml streptomycin). For transfection, cells were seeded onto Matrigel-coated coverslips at a density of 1×10^5 cells/well in six-well plates. After 24 h at 37°C, pDsRed-N1-GRK2 (1 μg/well) was transfected into STC-1 cells using Lipofectamine 2000 reagent (1.25 μl/well). The cells were used for calcium imaging analysis between 48 and 72 h after transfection.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

We isolated 0.5 μg of poly(A) RNA by mRNA Isolation Kit (Roche). First-strand cDNA was synthesized by random primers and M-MLV reverse transcriptase. As previously reported, degenerate primers to two absolutely conserved stretches of catalytic domains of G protein-coupled receptor kinase (G(R/K)GGFGE: 5′-CACCGGCTCGAGGGIMGIGGIGGITTYGGIGA-3′ and G(Y/F)MAAPEV: 5′-GCCCTTCTCGAGGACYTCIGGIGCCATRWAICC-3′) were used to amplify a 500 bp product (Premont *et al.*, 1994). Using fist-strand cDNA, PCR was performed with 500 nM primers, 250 μM concentration of each dNTP and 2.5 units of TAKARA Ex Taq DNA polymerase for 35 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 3 min. The 500 bp product was purified with Wizard SV Gel and PCR Clean-Up System (Promega) and subcloned into pGEM-T (Promega) using TAKARA DNA Ligation Kit. Recombinant plasmids were subjected to DNA sequencing.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Subconfluent STC-1 and HEK293T cells grown on 10 cm dishes were washed with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and lysed with sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol). Lysates were subjected to 13.5% 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. For Western blot analysis, non-specific binding sites were blocked with 5% skim milk in TBST (26.8 mM Tris, pH 8.0, 1.37 M NaCl, 250 mM KCl and 0.1% Tween 20) for 90 min at room temperature; the blots were incubated

overnight at 4°C with the GRK2 polyclonal antibody (at 1:1000 dilution in TBST). After washing with TBST, horseradish peroxidase-conjugated donkey anti-rabbit IgG was applied as the secondary antibody (at 1:100,000 dilution with 5% skim milk in TBST) for 90 min at room temperature. Immunoreaction was visualized using the ECL-system (Amersham Pharmacia Biotech).

Immunohistochemistry

A tongue was obtained from a male ICR mouse aged 44 weeks. To prepare cryosections, fresh tissues were placed in OCT and snap-frozen in dry ice. Cryosections (10 μm) were collected on aminopropylsilane-coated slides, and stored at −80°C until use. Slides were fixed with 4% paraformaldehyde in PBS for 20 min and treated with 0.5% Triton X-100 in PBS for 5 min. When gustducin was immunostained, sections were soaked in 5% skim milk in PBS for 90 min, and incubated in gustducin antibody diluted 1:500 in PBS containing 10% Block Ace for 90 min. After repeating washing in PBS, it was incubated for 90 min in Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) diluted 1:800 in PBS containing 10% Block Ace. For the staining of GRKs, sections were incubated in normal goat serum diluted 1:50 in ICC buffer (PBS containing 0.5% BSA, 0.2% Triton X-100, pH 7.3) for 1 h at room temperature. Antibody to GRK2, GRK3 or GRK5 was diluted to 1:400 in ICC buffer and applied to sections overnight at 4°C. After washing, they were incubated for 90 min in Cy3-conjugated donkey anti-rabbit antibody diluted 1:800 in PBS containing 10% Block Ace.

Calcium imaging analysis

STC-1 cells grown on Matrigel-coated coverslips were washed with Tyrode's solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM pyruvic acid) and then incubated in Tyrode's solution containing 5 μM Fura-2 AM for 30 min at room temperature. Cells were then washed with Tyrode's solution and left at room temperature for an additional 30 min to allow cleavage of the AM ester. Each coverslip was placed in a recording chamber filled with 200 μl of Tyrode's solution. To achieve abrupt changes in ligand concentration, 400 μl of 150% concentrated ligand solution was applied by pipette. As previously reported, $[Ca^{2+}]_i$ was monitored as a function of the ratio of 510 nm Fura-2 emission excited by illumination at 340 and 380 nm applied using a Lamda DG-4 light source and filter exchanger (Sutter, Novato, CA). Fura-2 fluorescence was recorded every 2.0 s using a MicroMAX 512BFT cooled CCD camera (Roper Sceintific, Tucson, AZ) and Metafluor imaging software (Universal Imaging, Downingtown, PA) (Abe *et al.*, 2003). All calcium imaging experiments were repeated two or three times.

Results

Calcium Responses of STC-1 cells to bombesin and bitter tatstants

We monitored responses in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ of the intestinal STC-1 cell line by using Ca^{2+} imaging of individual cells and tested the effect of bombesin and bitter tatstants. Bombesin is known to activate the IP3 pathway and induced a rapid increase in $[Ca^{2+}]_i$ in STC-1 cells (Snow *et al.*, 1994; Chang *et al.*, 1998). As expected, addition of bombesin to cultures of STC-1 cells, loaded with the fluorescence Ca^{2+} indicator Fura-2 AM, induced rapid and dose-dependent elevation in $[Ca^{2+}]_i$ (Figure 1A,D). The concentration of bombesin required to induce half-maximal response was 2.69 nM. A similar value (EC_{50} of 1 nM) was reported by examining bombesin-induced secretion of secretin in STC-1 cells (Chang *et al.*, 1998). We next examined the effects of bitter taste substances on STC-1 cells. As bitter tastant molecules, several compounds containing denatonium benzoate and caffeine are reported to induce calcium response in STC-1 cells (Wu *et al.*, 2002), but a substantial dose–response relationship of responses was not examined. Moreover, Wu *et al.* observed robust response of STC-1 cells to denatonium, but they could not detect the expression of known denatonium receptor (mT2R8). When denatonium was added to STC-1 cells, we observed a rapid and dose-dependent response of $[Ca^{2+}]_i$. In the case of denatonium, the concentration of half-maximal response was 4.74 mM (Figure 1B,E). Since mT2R8 was reported to function as a receptor for denatonium (Chandrashekar *et al.*, 2000), we also investigated the presence of the mT2R8 transcript by RT–PCR analysis using RNA prepared from STC-1 cells. PCR products of the expected size (707 bp) was detected in taste papillae and STC-1 cells (data not shown). Since Wu *et al.* (2002) did not describe the detailed procedures for RT–PCR of mT2R8, we cannot make a conclusion. PCR conditions of Wu *et al.* might be different from ours. Addition of caffeine to STC-1 cell cultures induced a sharp increase in $[Ca^{2+}]_i$. The effect of caffeine was also dose-dependent, with an EC_{50} of 0.528 mM (Figure 1C,F). Thus, all three responses were concentration dependent and had distinctive time course. When HEK293T cells were activated by bitter tatstants, neither denatonium nor caffeine induced significant calcium response, even at 10 mM (Figure 2). Thus it is apparent that STC-1 cells have unique ability to respond to bitter compounds, and it was considered that thees responses were not due to mechanical artifacts. Additionally, the concentration response of the bitter tastants and the use of U-73343 described below further eliminated possibility of mechanical artifacts (Figures 1 and 3).

Role of the phospholipase C pathway in signaling of STC-1 cells

It has been reported that the PLC/IP3 pathway is involved in bombesin-induced activation of SCT-1 cells (Chang *et al.*,

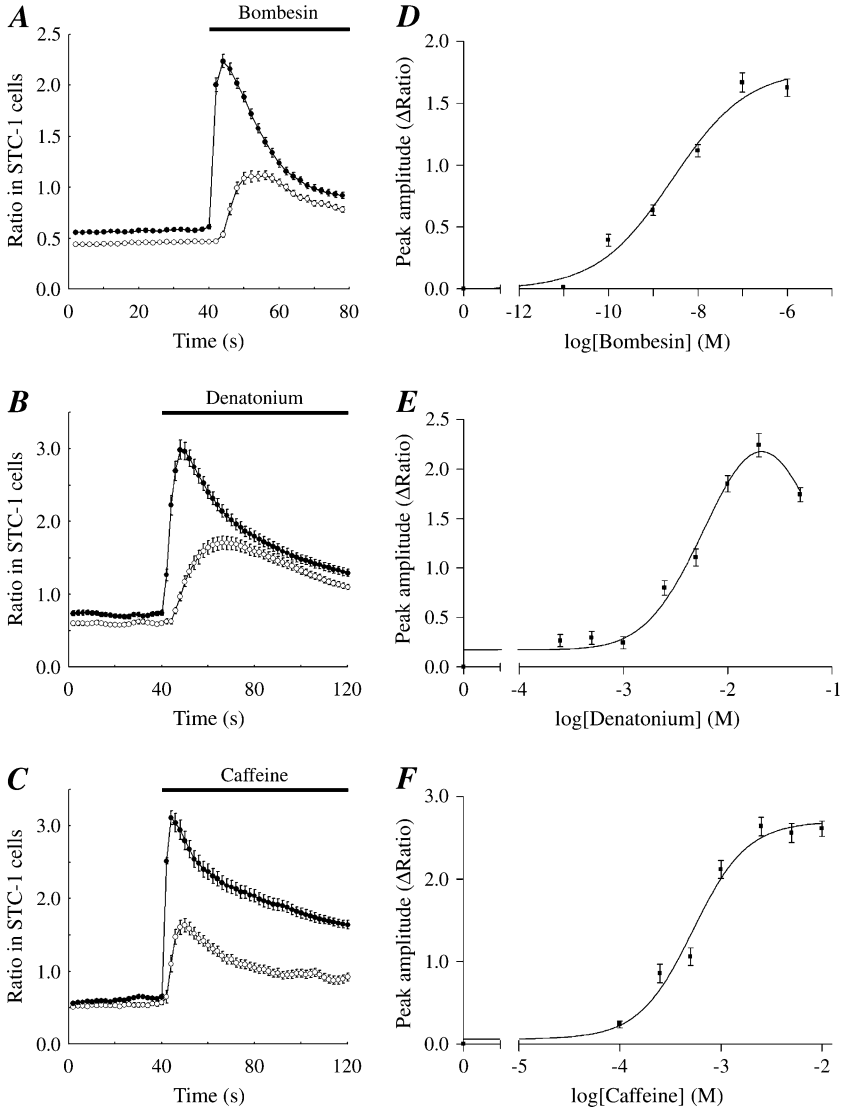

Figure 1 Ca^{2+} responses of STC-1 cells elicited by bombesin, denatonium and caffeine. STC-1 cells preloaded with 5 μM Fura-2 AM were challenged with ligands. **(A–C)** Mean fluorescence ratio ± SEM of 40–70 cells evoked by bombesin (A, 1 nM, open circles; 1 μM, filled circles), denatonium benzoate (B, 5 mM, open circles; 20 mM, filled circles) and caffeine (C, 0.5 mM, open circles; 5 mM, filled circles). Horizontal bars above traces indicate the duration of ligand applications. **(D–F)** Concentration response relationships for bombesin (D), denatonium benzoate (E) and caffeine (F). Responses are from at least 28 cells. Data are curve-fitted with GraphPad Prism 4. Error bars indicate SEM. The EC_{50} values of bombesin, denatonium and caffeine were 2.69mM, 4.74mM and 0.528 mM respectively.

1998; Hira *et al.*, 2004). On the other hand, membrane preparations from circumvallate and foliate taste regions of mouse tongues responded to the addition of known bitter taste stimuli, including denatonium and caffeine, by increasing the amount of inositol phosphates (Spielman *et al.*, 1994). Furthermore, using knockout mice, it has been demonstrated that knockout of PLCβ2, a PLC selectively expressed in taste tissue, abolished bitter taste reception to compounds including denatonium. Therefore, to investigate the roles of PLC in signaling pathways in STC-1 cells, we examined the effect of pretreatment with a PLC inhibitor, U-73122. This agent fully blocked bombesin-induced calcium response (Figure 3A, open circles). Similarly, both calcium responses activated by denatonium and caffeine were completely abolished by the pretreatment with U-73122 (Figure 3B,C, open circles). On the other hand, an inactive analog U-73343 could not block all three calcium responses (Figure 3A–C, filled circles). As for bombesin-induced signaling, we clearly showed the essential role of the PLC pathway in denatonium and caffeine responses in STC-1 cells. To our knowledge, there is no cell line except STC-1 cells, which respond to bitter tastants through PLC pathway.

Expression of GRK2 in STC-1 cells

To investigate the regulatory system of G protein signaling in STC-1 cells, we searched STC-1 cells to determine which G

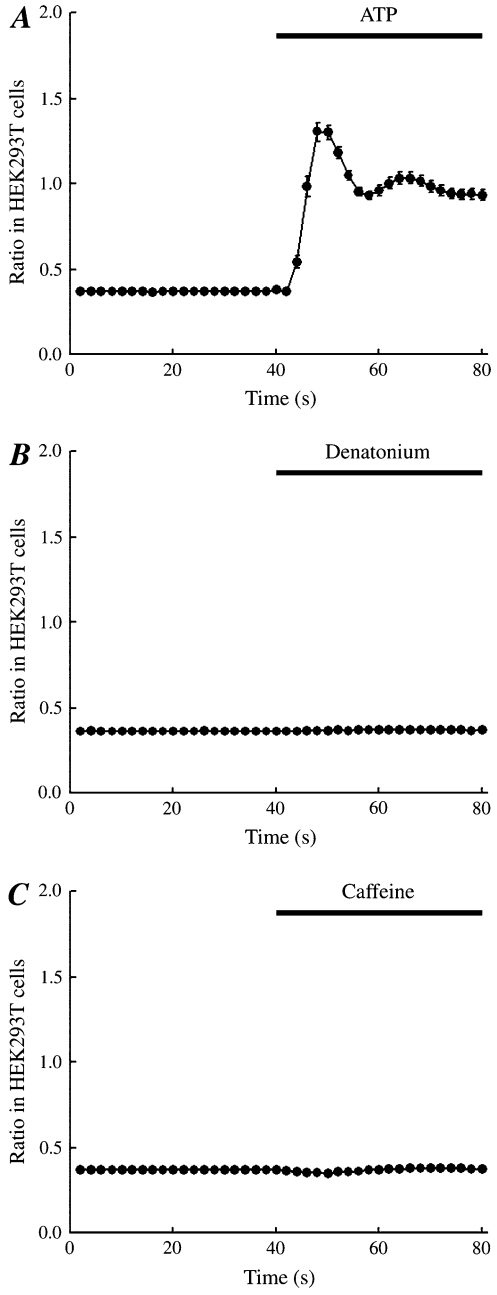

Figure 2 Exposure of denatonium and caffeine to HEK293T cells. HEK293T cells were exposed to 100 μM ATP **(A)**, 10 mM denatonium benzoate **(B)** and 10 mM caffeine **(C)**. Values are mean fluorescent ratio ± SEM from 85–115 cells.

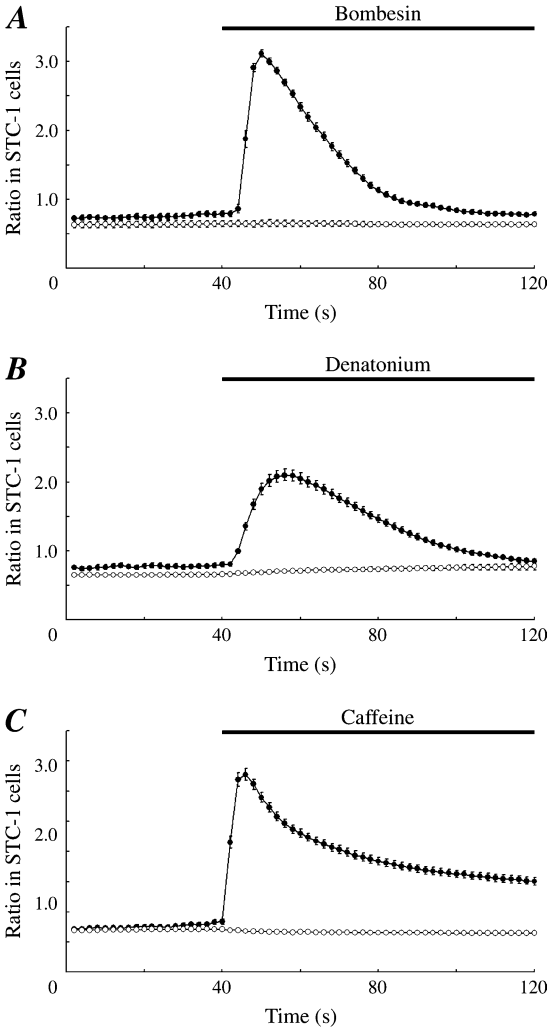

Figure 3 Effect of phospholipase C inhibitor on Ca^{2+} transients induced by bombesin, denatonium and caffeine. STC-1 cells treated with 5 μM U-73343 (filled circles) or 5 μM U-73122 (open circles) for 15 min were challenged with 100 nM bombesin **(A)**, 10 mM denatonium benzoate **(B)** or 10 mM caffeine **(C)**. Data are means ± SEM of 30–60 cells.

protein-coupled receptor kinases are present. Using degenerate primers corresponding to kinase catalytic domain sequences conserved only among known members of GRKs, RT–PCR was performed as previously reported (Premont *et al.*, 1994). An ~500 bp product was amplified from RNA of STC-1 cells (Figure 4A). The amplified DNA was cloned into pGEM-T vector, resulting in the isolation of seven independent clones. Sequencing analysis revealed that STC-1 cells expressed only GRK2. We next examined the protein expression of GRK2 by Western blotting using anti-GRK2 antibody. As previously reported (Freedman *et al.*, 1995), the GRK2 protein was detectable in HEK293T cells (Figure 4B). Similarly, a protein band with the same molecular weight size was detected in STC-1 cells. The results indicated that GRK2 is expressed in STC-1 cells.

Distinct distribution of GRK2, 3 and 5 in mouse circumvallate papilla

Premont *et al.* showed that GRK2, 3 and 5 were expressed in bovine taste epithelium using RT–PCR with the same degenerate primers, but they concluded that their mRNAs do not show apparently specific distributions (Premont *et al.*, 1994). On the other hand, our results suggested that GRK2 might be expressed in cells possessing a system of

bitter taste transduction. To determine the distributions of three GRKs in tongue, we first examine the specificity of antibodies by Western blotting (Figure 5). Immunoreactive bands of appropriate molecular weights were observed in cerebellum extracts for GRK2 and GRK3. Although GRK2, 3 and 5 mRNA are expressed in the cerebellum (Erdtmann-Vourliotis *et al.*, 2001), we could not detect any GRK5 signal (data not shown). This raised the possibility that expression levels of GRK5 protein in the cerebellum might be too low to be detected with this antibody. Therefore, the specificity of anti-GRK5 antibody was established using HEK293T cells transfected with GRK5 cDNA. Control cells (HEK293T + empty vector) produced no significant bands, but main (indicated with arrowhead) and minor multiple bands were specifically detected in HEK293T cells transfected with GRK5 cDNA. Multiple bands of lower molecular weights must be proteolytic products of GRK5, since they were only observed by transfection of GRK5 cDNA. Thus, GRK5 antibody was considered to be specific for GRK5 protein. The specificity of anti-GRK2 antibody was further confirmed using extract of STC-1 cells transfected with pDsRed-N1-GRK2. In STC-1 cells transfected with pDsRed-N1-GRK2, we could detect GRK2-DsRed in addition to endogenous GRK2. Using these antibodies, we performed immunostaining of frozen sections of circumvallate papilla containing taste buds. Surprisingly, staining with antibodies revealed completely distinct patterns of distributions of three GRKs in the circumvallate papilla. GRK2 was enriched in cells in the taste buds and the surrounding epithelium (Figure 6B). In the case of GRK3, immunostaining was detected in cells in the surface of the epithelium (Figure 6C). Moreover, expression of GRK5 was observed in cells of the apical epithelium (Figure 6D). The position of taste buds could be easily recognized by staining with antibody to gustducin specific for taste receptor cells (McLaughlin *et al.*, 1992).

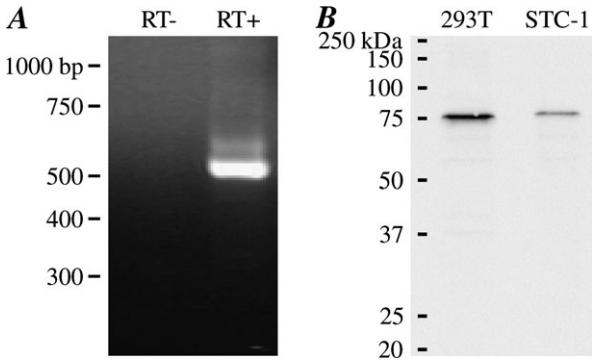

Figure 4 Expression of the GRK gene in STC-1 cells. **(A)** mRNA isolated from STC-1 cells was subjected to RT–PCR analysis by using degenerate primers to conserved stretches of catalytic domains of G protein-coupled receptor kinase. The PCR products were size fractionated on an agarose gel resulting in a band of 500 bp. RT–, without reverse transcriptase; RT+, with reverse transcriptase. **(B)** Lysates of HEK293T cells and STC-1 cells were subjected to SDS–PAGE and transferred onto a nitrocellulose membrane. Blots were processed by Western immunoblotting with anti-GRK2 antibody.

Differential effects of GRK2 expression on responses of STC-1 cells

We next focused on the roles of GRK2 in regulating cellular responses of STC-1 cells. GRK2 was expressed as a fusion protein with DsRed at the carboxyl terminus to identify cells expressing GRK2. Results of calcium imaging analysis were shown in Figure 7. In Figure 7A–C, closed circles represent the results of the control cells and open circles represent the

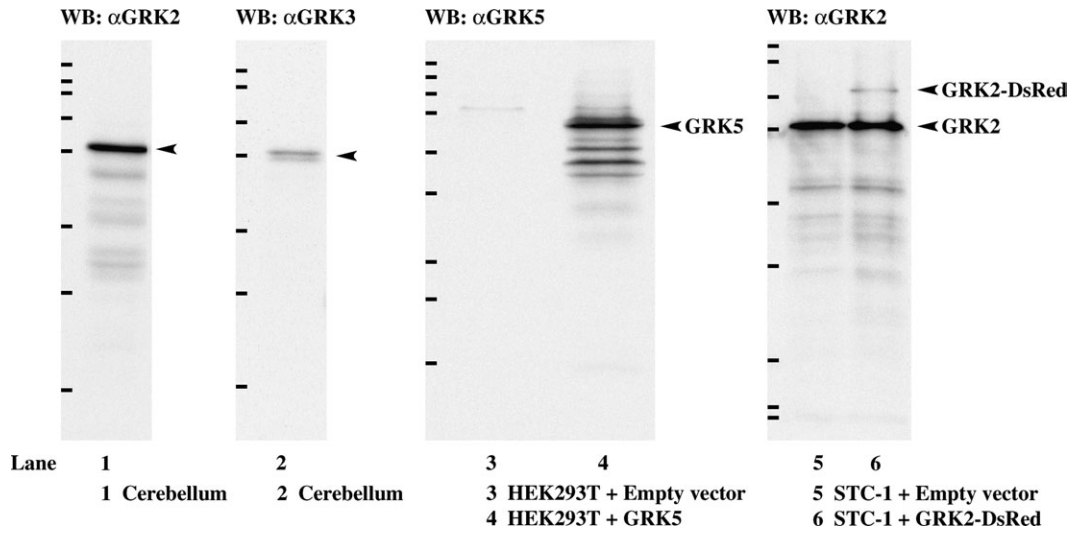

Figure 5 Specificities of the anti-GRK antibodies. Mouse cerebellum was dissected and solubilized in sample buffer. Whole cell lysates from HEK293T cells transfected with GRK5 and STC-1 cells transfected with GRK2 were prepared. The samples were electrophoresed on SDS–polyacrylamide gels, and transferred to nitrocellulose membrane. Antibodies raised against GRK2, GRK3, and GRK5 were used to detect each GRK. The signals corresponding to GRKs are indicated by arrowheads.

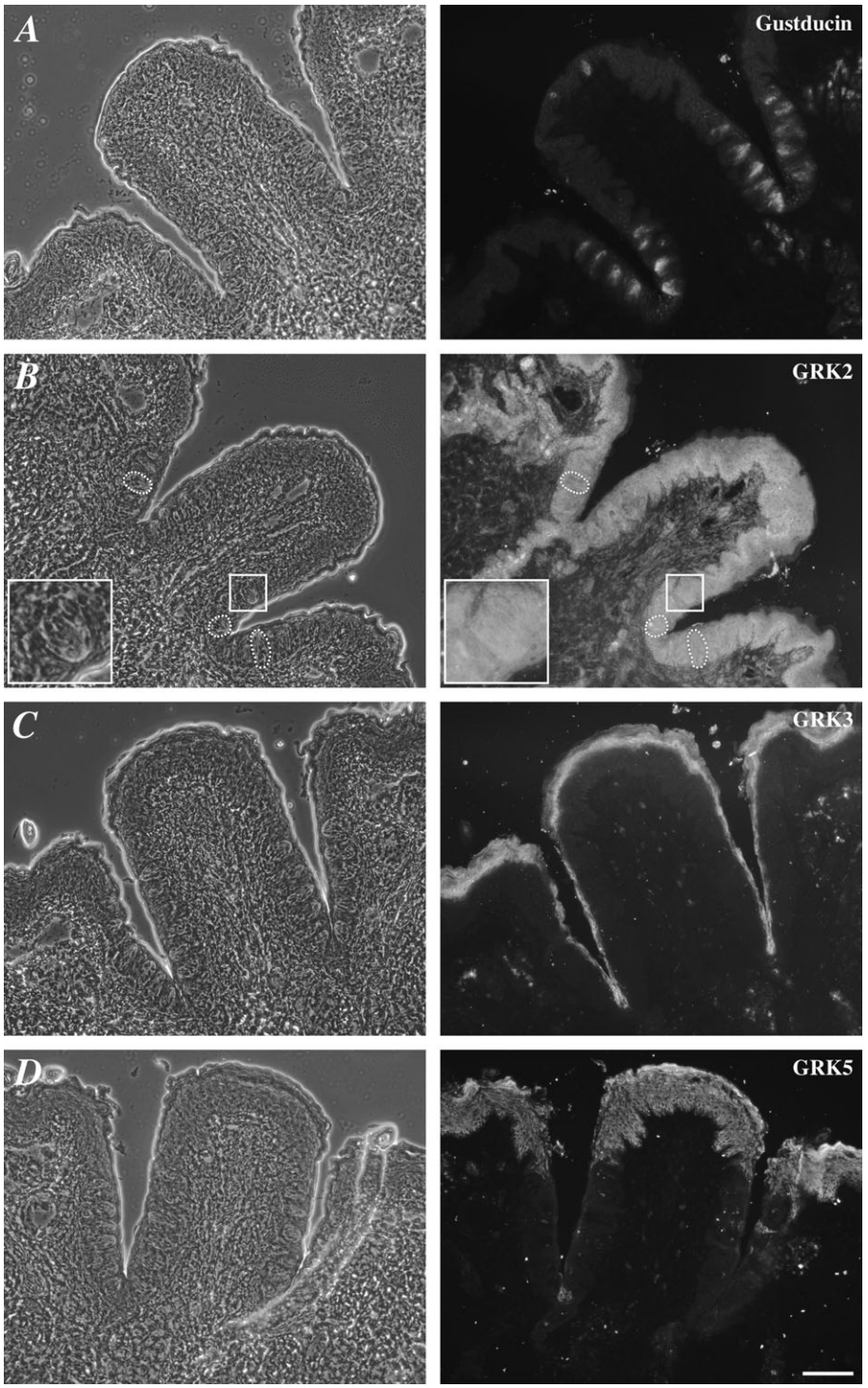

Figure 6 Immunohistochemistry of GRK2, GRK3 and GRK5 proteins in circumvallate papillae. Coronal sections (10 μm) of circumvallate papillae were immunostained with anti-gustducin **(A)**, anti-GRK2 **(B)**, anti-GRK3 **(C)** and anti-GRK5 antibody **(D)**. Phase-contrast (left) and corresponding immunofluorescence micrographs (right) are shown. Negative control omitted the primary antibodies did not produce significant staining (data not shown). Dotted lines in (B) indicate taste buds. Insets represent high magnification view of a taste bud indicated with a small square. Scale bar = 100 μm.

results of the cells expressing GRK2. In Figure 7D,E, the means of control results are indicated by solid bars and those of the results induced by GRK2 expression are indicated by open bars. The expression of GRK2 greatly inhibited calcium response induced with bombesin in STC-1 cells. A similar inhibitory function of GRK2 was observed in cells activated with 10 and 100 nM bombesin (Figure 7A,D). We could not detect any significant

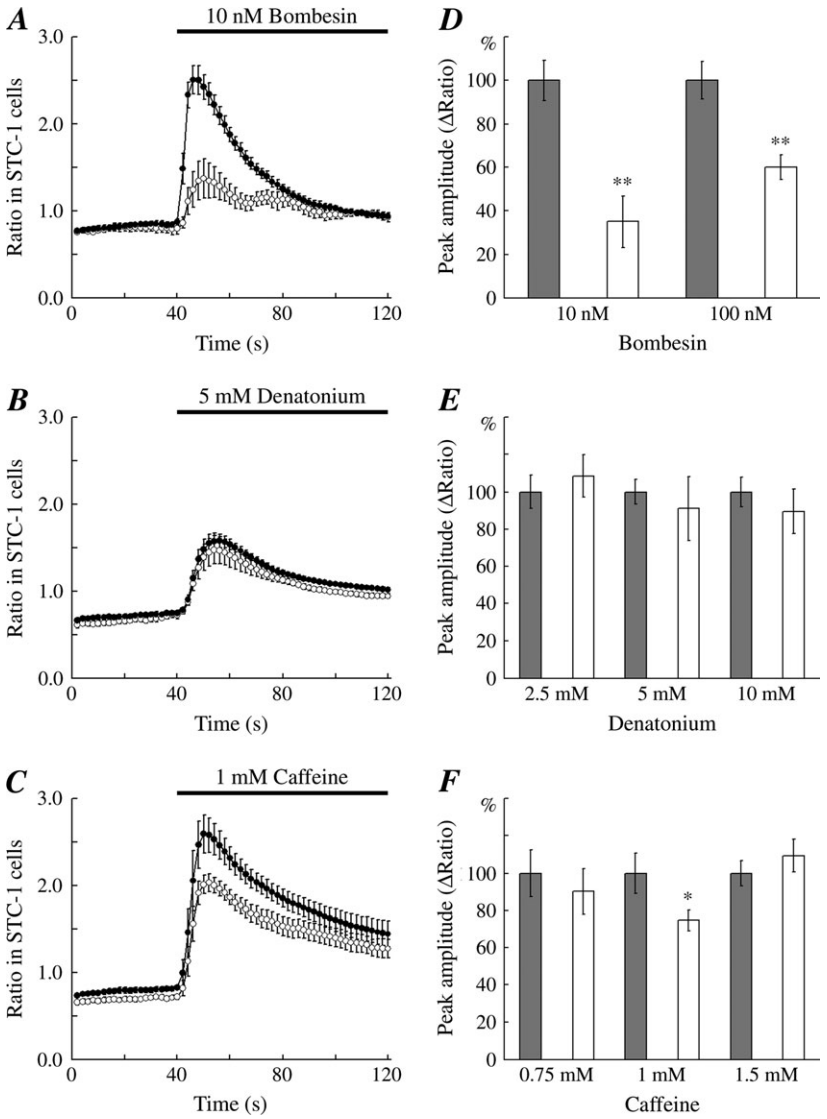

Figure 7 Effect of GRK2 on bombesin-, denatonium- and caffeine-induced calcium responses. STC-1 cells grown on 12 mm coverslips were transiently transfected with pDsRed-N1-GRK2. At 2 days after transfection, STC-1 cells were challenged with bombesin **(A, D)**, denatonium benzoate **(B, E)** and caffeine **(C, F)**. (A–C) Shown are the mean ratio ± SEM generated from five visual fields. DsRed-negative cells, filled circles; DsRed-positive cells, open circles. (D–F) Summarized data from five visual fields. Data are presented as mean ± SEM from DsRed-negative cells (open bar) and DsRed-positive cells (solid bar). $*P < 0.05$, $**P < 0.01$, compared with DsRed-negative cells, unpaired Student's *t*-test. (A–F) Each visual field contains more than four DsRed-positive cells and 25 DsRed-negative cells.

effects of GRK2 in denatonium-induced calcium response of STC-1 cells. Experiments were performed at three different concentrations (2.5, 5 and 10 mM) of denatonium, but statistically significant difference was not observed in control and GRK2-transfected cells (Figure 7B,E). When the caffeine-activated elevation of intracellular calcium was investigated, we found unique inhibitory effects of GRK2 expression. Although the GRK2 function was not recognized in cells treated with 0.75 and 1.5 mM caffeine, GRK2-dependent reduction was detected in cells activated by 1 mM caffeine (Figure 7C,F). Experiments were repeated three times for the caffeine response, with the same results being obtained. Thus, it was apparent that GRK2 has differential effects on cellular responses of STC-1 cells induced by bombesin and bitter tastants. Moreover, since the Gq signaling is known to be prevented strongly by GRK2 through its N-terminal RGS domain for Gαq, we suggest that the Gq-dependent system may not be involved in denatonium- and caffeine-induced activation of STC-1 cells.

Discussion

We investigated the cellular responses of STC-1 cells by monitoring the intracellular calcium using Fura-2 calcium-indicator dye. We focused on three signaling pathways initiated by bombesin and two bitter tastants of caffeine

and denatonium. All three molecules activated STC-1 cells in a dose-dependent manner, and all of their signaling pathways depended on PLC-induced Ca^{2+} release. We then investigated GRKs in STC-1 cells by the degenerate-primer PCR method, and found that STC-1 cells express GRK2. We also demonstrated that three GRKs (GRK2, GRK3 and GRK5) are differentially distributed in the circumvallate papilla of the tongue, and that only GRK2 is present in taste receptor cells. Finally, we examined how overexpression of GRK2 affects the responses of SCT-1 cells. The results indicated that the three cellular responses are differentially regulated by expression of GRK2. Bombesin-induced response was strongly inhibited by GRK2, but denatonium-activated signaling was not significantly affected. In the case of caffeine, response was decreased by expression of GRK2 only when cells were activated by 1 mM caffeine.

Previous biochemical studies showed that GRK2 phosphorylates bombesin receptor BB2 to uncouple it from Gq (Kroog *et al.*, 1999). Indeed, expression of GRK2 inhibited the bombesin-induced signaling in STC-1 cells that express BB2. In the case of the denatonium-induced response, IP3 production was reported to be blocked by the GST-3ct fusion protein, which contains the C-terminal domain of GRK3 (Rössler *et al.*, 2000). Both GRK2 and GRK3 contain a pleckstrin homology domain for binding to βγ subunits of G proteins in the C-terminus (Koch *et al.*, 1993). The expression of GRK2 was expected to attenuate the denatonium-dependent signaling. In this study, however, we observed that responses activated by denatonium were unaffected by GRK2 expression. It is possible that each C-terminus of GRK2 and GRK3 may form a specific complex containing different isoforms of Gβγ subunits. Indeed, it was reported that the GRK2 C-terminus binds $G\beta_1$ and $G\beta_2$ but not $G\beta_3$, while the GRK3 C-terminus binds all three Gβ isoforms (Daaka *et al.*, 1997). Therefore, the specific Gβγ isoforms that can be recognized only with GRK3 might be involved in the calcium elevation induced by denatonium in STC-1 cells.

Signaling pathways activated by caffeine as a bitter stimulus have not been studied in detail. However, as a pharmacological tool, caffeine has been well studied in cells other than bitter tasting cells. Its actions are mostly ascribed to three mechanisms: mobilization of intracellular calcium through ryanodine receptor, inhibition of phosphodiesterases and antagonism of adenosine receptors. On the other hand, our observed response with caffeine in STC-1 cells was completely attenuated by a PLC inhibitor. Therefore, it was demonstrated that the described three mechanisms may not be involved in caffeine-induced response in STC-1 cells. At present, it is not known how PLC-dependent calcium signaling is activated by caffeine in STC-1 cells. The expression of GRK2, however, induced some detectable effects on caffeine-response. Despite no significant difference at 0.75 and 1.5 mM, we observed a reduced response by GRK2 in STC-1 cells activated by 1 mM caffeine. It seems that an unknown G protein-coupled receptor might be responsible for caffeine-activation of STC-1 cells. Further investigation is required, but it is possible that this putative caffeine receptor might be phosphorylated by GRK2 to change the dose-dependency for caffeine. Otherwise, it is also possible that the expression of arrestin may be necessary for the full inhibitory function of GRK2 on caffeine-induced responses.

In the present study, we demonstrated that the properties of bitter taste responses of STC-1 cells can be easily characterized by using calcium imaging, RT–PCR, the application of inhibitors and an overexpression system. Thus, STC-1 cells emerge as a good cell model for studying the molecular mechanism of bitter taste signaling.

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References

Abe, H., Tateyama, M. and **Kubo, Y.** (2003) *Functional identification of Gb^{3+} binding site of metabotropic glutamate receptor 1α*. FEBS Lett., 545, 233–238.

Adler, E., Hoon, M.A., Mueller, K.L., Chandrashekar, J., Ryba, N.J.P. and **Zuker, C.S.** (2000) *A novel family of mammalian taste receptors*. Cell, 100, 693–702.

Chandrashekar, J., Mueller, K.L., Hoon, M.A., Adler, E., Feng, L., Guo, W., Zuker, C.S. and **Ryba, N.J.P.** (2000) *T2Rs function as bitter taste receptors*. Cell, 100, 703–711.

Chang, C.H., Chey, W.Y., Erway, B., Coy, D.H. and **Chang, T.M.** (1998) *Modulation of secretin release by neuropeptides in secretin-producing cells*. Am. J. Physiol., 275, 192–202.

Daaka, Y., Pitcher, J.A., Richardson, M., Stoffel, R.H., Robishaw, J.D. and **Lefkowitz, R.J.** (1997) *Receptor and Gβγ isoform-specific interactions with G protein-coupled receptor kinases*. Proc. Natl Acad. Sci. USA, 94, 2180–2185.

Dockray, G.J. (2003) *Luminal sensing in the gut: an overview*. J. Physiol. Pharmacol., 54, 9–17.

Erdtmann-Vourliotis, M., Mayer, P., Ammon, S., Riechert, U. and **Höllt, V.** (2001) *Distribution of G-protein-coupled receptor kinase (GRK) isoforms 2, 3, 5 and 6 mRNA in the rat brain*. Brain Res. Mol. Brain Res., 95, 129–137.

Ferguson, S.S.G . (2001) *Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling*. Pharmacol. Rev., 53, 1–24.

Freedman, N.J., Liggett, S.B., Drachman, D.E., Pei, G., Caron, M.G. and **Lefkowitz, R.J.** (1995) *Phosphorylation and desensitization of the human β1-adrenergic receptor.* J. Biol. Chem., 270, 17953–17961.

Gilbertson, T.A., Damak, S. and **Margolskee, R.F.** (2000) *The molecular physiology of taste transduction*. Curr. Opin. Neurobiol., 10, 519–527.

Herness, M.S. and **Gilbertson, T.A.** (1999) *Cellular mechanisms of taste transduction*. Annu. Rev. Physiol., 61, 873–900.

Hira, T., Elliott, A.C., Thompson, D.G., Case, R.M. and **McLaughlin, J.T.** (2004) *Multiple fatty acid sensing mechanisms operate in enteroendocrine cells: novel evidence for direct mobilization stored calcium by cytosolic fatty acid*. J. Biol. Chem., 279, 26082–26089.

Katz, D.B., Nicolelis, M.A.L. and **Simon, S.A.** (2000) *Nutrient tasting and signaling mechanisms in the gut IV. There is more to taste than meets the tongue.* Am. J. Physiol., 278, 6–9.

Koch, W.J., Inglese, J., Stone, W.C. and **Lefkowitz, R.J.** (1993) *The binding site for the βγ subunits of heterotrimeric G proteins on the β-adrenergic receptor kinase*. J. Biol. Chem., 268, 8256–8260.

Kroog, G.S., Jian, X., Chen, L., Northup, J.K. and **Battey, J.F.** (1999) *Phosphorylation uncouples the gastrin-releasing peptide receptor from Gq.* J. Biol. Chem., 274, 36700–36706.

Laemmli, U.K. (1970) *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature, 227, 680–685.

Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M. and **Adler, E.** (2002) *Human receptors for sweet and umami taste*. Proc. Natl Acad. Sci. USA, 99, 4692–4696.

Lindemann, B. (2001) *Receptors and transduction in taste*. Nature, 413, 219–225.

McLaughlin, S.K., McKinnon, P.J. and **Margolskee R.F.** (1992) *Gustducin is a taste-cell-specific G protein closely related to the transducins*. Nature, 357, 563–569.

Nelson, G., Hoon, M.A., Chandrashekar, J., Zhang, Y., Ryba, N.J.P. and **Zuker, C.S.** (2001) *Mammalian sweet taste receptors*. Cell, 106, 381–390.

Nelson, G., Chandrashekar, J., Hoon, M.A., Feng, L., Zhao, G., Ryba, N.J.P. and **Zuker, C.S.** (2002) *An amino-acid taste receptor.* Nature, 416, 199–202.

Premont, R.T., Koch, W.J., Inglese, J. and **Lefkowitz, R.J.** (1994) *Identification, purification, and characterization of GRK5, a member of the family of G protein-coupled receptor kinases*. J. Biol. Chem., 269, 6382–6841.

Rindi, G., Grant, S.G.N., Yiangou, Y., Ghatei, M.A., Bloom, S.R., Bautch, V.L., Solcia, E. and **Polak, J.M.** (1990) *Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Heterogeneity of hormone expression*. Am. J. Pathol., 136, 1349–1363.

Rössler, P., Boekhoff, I., Tareilus, E., Beck, S., Breer, H. and **Freitag, J.** (2000) *G protein βγ complexes in circumvallate taste cells involved in bitter transduction*. Chem. Senses, 25, 413–421.

Ruiz, C.J., Wray K., Delay, E.R., Margolskee, R.F. and **Kinnamon, S.C.** (2003) *Behavioral evidence for a role of α-gustducin in glutamate taste*. Chem. Senses, 28, 573–579.

Snow, N.D., Prpic, V., Mangel, A.W., Sharara, A.I., McVey, D.C., Hurst, L.J., Vigna, S.R. and **Liddle, R.A.** (1994) *Regulation of cholecystokinin secretion by bombesin in STC-1 cells*. Am. J. Physiol., 267, 859–865.

Spielman, A.I., Huque, T., Nagai, H., Whitney, G. and **Brand, J.G.** (1994) *Generation of inositol phosphates in bitter taste transduction.* Physiol. Behav., 56, 1149–1155.

Wong, G.T., Gannon, K.S. and **Margolskee, R.F.** (1996) *Transduction of bitter and sweet taste by gustducin*. Nature, 381, 796–800.

Wu, S.V., Rozengurt, N., Yang, M., Young, S.H., Sinnett-Smith, J. and **Rozengurt, E.** (2002) *Expression of bitter taste receptors of the T2R family in the gastrointestinal tract and enteroendocrine STC-1 cells*. Proc. Natl Acad. Sci. USA, 99, 2392–2397.

Zhang, Y., Hoon, M.A., Chandrashekar, J., Mueller, K.L., Cook, B., Wu, D., Zuker, C.S. and **Ryba, N.J.P.** (2003) *Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways*. Cell, 112, 293–301.