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Identification of Ligands for Two Human Bitter T2R Receptors

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Abstract

Earlier, a family of G protein-coupled receptors, termed T2Rs, was identified in the rodent and human genomes through data mining. It was suggested that these receptors mediate bitter taste perception. Analysis of the human genome revealed that the hT2R family is composed of 25 members. However, bitter ligands have been identified for only three human receptors so far. Here we report identification of two novel ligand–receptor pairs. hT2R61 is activated by 6-nitrosaccharin, a bitter derivative of saccharin. hT2R44 is activated by denatonium and 6-nitrosaccharin. Activation profiles for these receptors correlate with psychophysical data determined for the bitter compounds in human studies. Functional analysis of hT2R chimeras allowed us to identify residues in extracellular loops critical for receptor activation by ligands. The discovery of two novel bitter ligand–receptor pairs provides additional support for the hypothesis that hT2Rs mediate a bitter taste response in humans.

Key words: bitter taste, denatonium, G protein-coupled receptor, signal transduction, T2R, transducin

Introduction

The physiology of taste is incompletely understood. However, recent studies started to shed light on the biology of taste (Lindemann, 2001; Margolskee, 2002). It is believed that humans can recognize five basic taste modalities: salt, sour, bitter, sweet and umami, i.e. savory taste characterized by the taste of monosodium glutamate (MSG). Among the recent breakthroughs was the identification of receptors, termed T1Rs, which mediate sweet and umami taste (Nelson *et al.*, 2001, 2002; Li *et al.*, 2002). These receptors belong to the family of seven transmembrane domains receptors that interact with intracellular G proteins (G protein-coupled receptors, or GPCRs). Identification of the receptor selectively activated by MSG provided mechanistic support for the idea that umami is a distinct taste recognized by humans (Li *et al.*, 2002; Nelson *et al.*, 2002). It was long believed that many bitter compounds produce bitter taste by interacting with cell surface receptors. A novel family of GPCRs, termed T2Rs, was identified in humans and rodents (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000; Matsunami *et al.*, 2000). Several lines of evidence suggested that T2Rs mediate response to bitter compounds. First, T2R genes are specifically expressed in subsets of taste receptor cells of the tongue and palate epithelia. Second, the gene for one of the human T2Rs (hT2R1) is located in a chromosomal locus that was linked to sensitivity to the bitter compound, 6-*n*-propyl-2-thiouracil (PROP), in humans (Adler *et al.*, 2000; Matsunami *et al.*, 2000). Third, one of the mouse T2Rs (mT2R5) is located in a chromosomal locus that is linked to sensitivity to cycloheximide in mice—a bitter tastant for rodents. When mT2R5 was expressed in HEK293 cells, the addition of cycloheximide stimulated calcium release via overexpressed promiscuous G protein, Gα15 (Chandrashekar *et al.*, 2000). It was also shown that mT2R5 can activate gustducin, a G protein selectively expressed in taste cells and linked to bitter taste signaling (Wong *et al.*, 1996; Ming *et al.*, 1998). Gustducin activation by mT2R5 occurs only in response to cycloheximide (Chandrashekar *et al.*, 2000). Thus, it has been proposed that the mT2R family mediates bitter taste response in mice, whereas hT2R family mediates bitter taste response in humans. Strong support for this hypothesis was provided when first ligands were identified for specific hT2Rs. Using the HEK 293/G15 assay system Bufe *et al.* (2002) demonstrated that bitter β-glucopyranosides, such as salicin, activate hT2R16. Evidence was also provided that hT2R10 is activated by strychnine (Bufe *et al.*, 2002). Most recently genetic linkage analysis identified a member of hT2R family as a human receptor for phenylthiocarbamide (PTC; Kim *et al.*, 2003). However, no functional data was provided to demonstrate this receptor activation in response to PTC. And the other 22 hT2Rs remained 'orphaned' with no identified ligands.

Nothing is currently known about regions of the receptors that are involved in bitter ligand recognition and signaling. It is not known whether extracellular loops or transmem-

brane domains, or both contribute to ligand binding. Two published reports have shown that mutations in T2R sequences can affect bitter receptor signaling. Strains of mice, which differ in sensitivity to cycloheximide ('taster' and 'nontaster'), have two different variants of mT2R5 gene (Chandrashekar *et al.*, 2000). When expressed in heterologous system, the 'nontaster' variant of mT2R5 demonstrated lower sensitivity to cycloheximide. In human studies, people with different alleles of one of the hT2R genes have different sensitivity to PTC (Kim *et al.*, 2003). However, in both mT2R5 and human PTC receptor cases, all 'nontaster' variants had several amino acid changes compared to the 'taster' alleles. These differences were located in extracellular loops, transmembrane domains as well as in the intracellular loops. Thus, which particular amino acid substitution had an effect on receptor signaling was not established.

One of the difficulties in studying T2R function is that these receptors are not readily expressed in cultured mammalian cell lines. To improve T2R expression an N-terminal sequence tag from another GPCR, rhodopsin, was attached to T2R sequences (Chandrashekar *et al.*, 2000). This method was employed earlier in an attempt to facilitate olfactory receptor expression in heterologous systems (Krautwurst *et al.*, 1998). Whereas the rhodopsin tag improved expression of some T2Rs in HEK293 cells, many receptors were not expressed well enough for functional studies. For example, even though Bufe *et al.* (2002) tested 24 hT2Rs with various bitter compounds (including denatonium and quinine) using the HEK 293/G15 assay system, ligands were identified for only two receptors. In a different approach, mT2R5 was successfully expressed in insect Sf9 cells and used for functional studies using a biochemical GTPγS binding assay (Chandrashekar *et al.*, 2000). We decided to apply this approach to study human T2Rs. This assay has one apparent advantage over cell-based assays—it allows the use of gustducin (or its homolog, transducin), which is the G protein shown to be involved in mediating bitter response (Wong *et al.*, 1996; Ming *et al.*, 1998). Whereas Gα15 can evidently successfully interact with mT2R5 and some hT2Rs, it is unclear whether other T2Rs can efficiently couple to this G protein. To further characterize the hT2R family we performed extensive analysis of the human genome and identified 25 members. Here we report that screening of 25 hT2Rs using GTPγS binding assay with five bitter compounds resulted in identification of two novel ligand–receptor pairs. Activation profiles for these receptors correlate with psychophysical data for the determined bitter compounds in human studies. Functional analysis of hT2R chimeras allows us to begin to understand the structural basis of bitter ligand recognition. Identification of these two novel bitter ligand–receptor pairs provides strong additional support for the hypothesis that hT2Rs mediate bitter taste response in humans.

Materials and methods

Materials

The following compounds were synthesized as previously described: 6-nitrosaccharin (Rose, 1969); 6-aminosaccharin (Kamogawa *et al.*, 1982), benzyldiethyl[(carboxymethyl) methyl]-ammonium chloride (Terakawa, 1954); benzyldimethyl[(2,6-xylylcarbamoyl)methyl]ammonium chloride; and benzylmethylethyl[(2,6-xylylcarbamoyl)methyl]ammonium chloride (Saroli, 1985).

N-Isopropyl-2-methyl-5-nitrobenzenesulfonamide was synthesized by modification of the procedure of Rose (1969). 6-Carboxysaccharin was purchased from ChemDiv Inc. (CA), PROP was obtained from ICN Biomedical Research Products. All other reagents were obtained from Sigma (MO) or other sources previously described.

Data deposition

The sequences reported in this paper have been previously published (Adler *et al.*, 2000, 2004; Adler, 2002).

Molecular cloning and expression of hT2Rs

The hT2Rs were identified by reiterated BLAST-based analysis of the human genome database as described in Adler *et al.* (2000). Full-length hT2Rs and hT2R fragments were named by order of discovery; gaps in the hT2R numbering scheme reflect coalescence of hT2R fragments and identification and exclusion of spurious hT2Rs that represented database contig assembly artifacts. Full-length potentially functional hT2Rs were cloned by PCR amplification from genomic DNA or BAC genomic clones corresponding to databank intervals that contained hT2Rs (ResGen and BACPAC Resources). Flanking 5′ *Asc*I and 3′ *Not*I sites were introduced using mutagenic PCR primers and used to introduce the hT2Rs and mT2R5 into a pFastBac-1-derived vector (Invitrogen) engineered to generate translational fusions to the N-terminus of bovine rhodopsin (39 amino acids). In rhodopsin-tagged T2Rs, rhodopsin codon M39 corresponded to the hT2R start codon and the Asc I junction introduced F37R and S38A missense mutations in the rhodopsin tag. Chimeric and mutant forms of hT2R61, hT2R64, hT2R44 and hT2R51 were generated using either nesting PCR techniques or QuickChange Site Directed Mutagenesis kit (Stratagene). The structure of all constructs was confirmed by nucleotide sequencing.

Generation of T2R containing membranes

Insect *Spodoptera frugiperda* Sf9 cells were infected for 48 h at 2×10^6 cells/ml with the appropriate baculoviruses at multiplicity of infection 1–2. Cells were harvested, washed with Hanks' balanced salt solution and lysed on ice with a Polytron homogenizer (three cycles of 20 s each at 20 000 r.p.m.) in a buffer A containing 25 mM tris–HCl, pH 8, 2 mM EDTA, 1 mM DTT with a cocktail of protease inhibitors (Roche). After particulate removal by centrifugation at

1000 *g* for 5 min, cell membranes were washed with 5M urea in buffer A and collected by centrifugation at 40 000 g for 40 min. Pelleted membranes were washed with a buffer B (20 mM Tris–HCl, pH8, 0.5 mM EDTA, 0.5 mM DTT) and collected by centrifugation. Membranes were then resuspended in buffer B and further homogenized by passing through a 29-gauge needle. Membrane concentration was adjusted to 3 mg of total protein/ml. Aliquots were flash frozen in liquid nitrogen and stored at –80°C.

Expression of all T2Rs was confirmed by Western blot with anti-rhodopsin mAb B6-30 (Hargrave *et al.*, 1986). Bovine rhodopsin was used as a standard to quantitate T2R protein expression in membrane preparations. Most T2Rs were expressed at a similar level of ~0.6 nmol/mg total membrane protein.

GTPγS binding assay

Transducin was purified from bovine retinas as previously described (Stryer *et al.*, 1983) or purchased from CytoSignal (CA). GTPγS binding assay was carried out essentially as previously described (Wessling-Resnick and Johnson, 1987). The assay reactions contained ~10 pmol of T2Rs from Sf9 cell membranes, 0.3 μM transducin in 30 μl of buffer BB (30 mM Tris–HCl, pH 8, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT). Some reactions also included 0.05–5 mM of bitter compounds. Reactions were initiated by the addition of 0.3 μM [γ-^{35}S]GTPγS (50 Ci/mmol) (NEN), carried on at 30°C for 1 h, stopped by the addition of 170 μl of 0.5 mM GTP in buffer BB and then filtered through nitrocellulose filters. The amount of bound [γ-^{35}S]GTPγS was determined by using a liquid scintillation counter. The amount of nonspecifically bound GTPγS was determined in the absence of transducin and subtracted from the total (typically ~20–40 fmol). When bitter compounds were tested in the assay, the amount of GTPγS bound to transducin in the absence of added compounds (control) was subtracted from the amount bound in the presence of compounds. Under the assay conditions the final GTPγS occupancy of transducin in the presence of any T2R and ligand did not exceed 35%, with basal binding to transducin in the absence of T2Rs of ~0.7% (80 fmol).

Results and discussion

Characterization of hT2R family in the human genome

Earlier, 12 hT2R pseudogenes and 25 potentially functional hT2Rs were identified in the genome databank (Adler, 2002). Here, we confirmed the structure of the identified hT2Rs by PCR amplification of genomic DNA and sequencing (Figure 1). One hT2R is located on chromosome 5 and nine hT2Rs (plus three pseudogenes) are located in an extended cluster on chromosome 7. The remaining 15 hT2Rs (and nine pseudogenes) are located in a dense cluster on chromosome 12. Analysis by others produced a similar number of full-length human hT2R genes (Bufe *et al.*, 2002; Conte *et al.*, 2002).

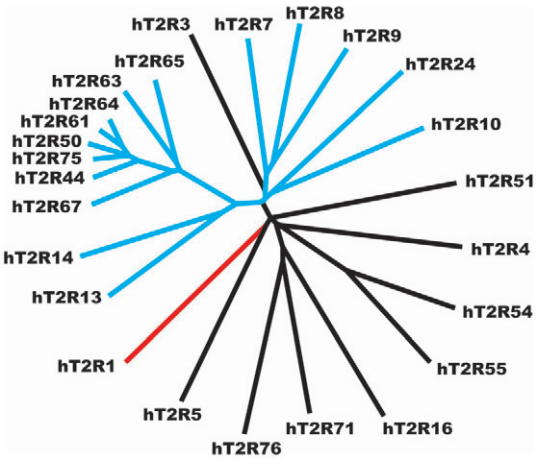

Figure 1 Unrooted phylogenetic tree of the human T2Rs. Twenty-five hT2R amino-acid sequences were aligned using ClustalX, with 1000 bootstraps. Sequence identity of hT2Rs varies from ~25% for the most distant members (e.g. hT2R3 and hT2R16) to 89% for the most closely related ones (hT2R61 and hT2R64). Color of branches indicates the chromosomal location of hT2R genes: red, chromosome 5; black, chromosome 7; blue, chromosome 12.

Expression of T2Rs and the GTPγS binding assay with transducin

One of the objectives of this study was to match specific human T2Rs to known bitter ligands. Because of the difficulties with T2R expression and assaying in mammalian cells, we focused on an alternative approach to monitor T2R activation. Chandrashekar *et al.* (2000) showed that mT2R5 can be successfully expressed in insect Sf9 cells. Membranes generated from Sf9 cells were then used to monitor gustducin activation using GTPγS binding assay. Because gustducin is not readily available in the large quantities needed for biochemical studies, we used a visual G protein, transducin, instead. Transducin is highly similar to gustducin. It was shown that, in addition to retina, transducin is expressed in taste epithelial cells, suggesting its possible role in taste signal transduction (Ruiz-Avila *et al.*, 1995). This possibility was further confirmed when it was shown that transducin is able to couple to bitter receptors. When purified transducin was mixed with membranes prepared from bovine taste epithelium, transducin would exchange GDP to GTPγS in response to bitter stimuli as monitored by partial trypsin digest (Ming *et al.*, 1998). It was also demonstrated that transducin overexpression is able to partially restore sensitivity to bitter compounds in gustducin-null mice (He *et al.*, 2002). To test whether transducin could be activated by T2Rs in a GTPγS binding assay we expressed mT2R5 in Sf9 cells and performed GTPγS binding assay with the purified transducin. Figure 2A shows that mT2R5 stimulates GTPγS binding by transducin in response to cycloheximide. The cycloheximide dose–response curve obtained in our experi-

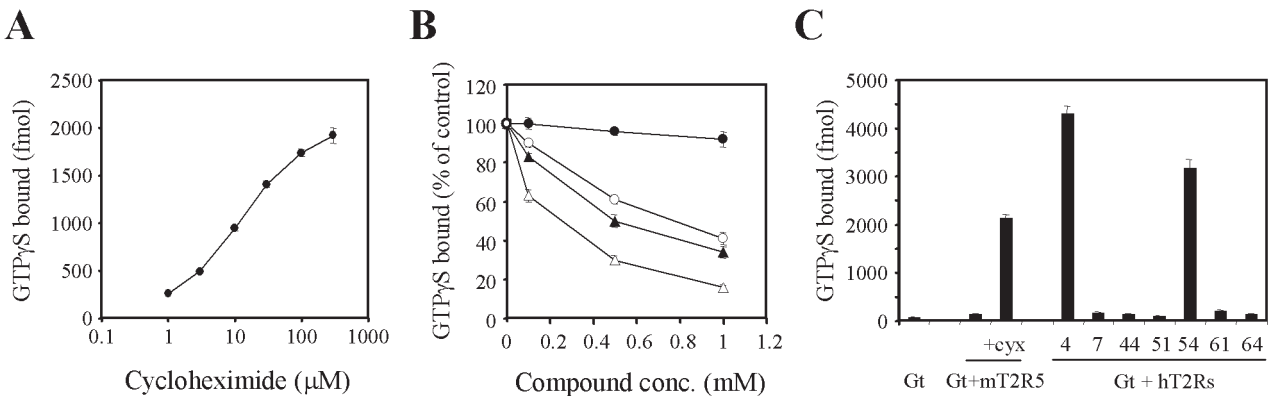

Figure 2 GTPγS binding assay with transducin and T2Rs. **(A)** Cycloheximide concentration dependence of transducin activation by mT2R5. Results are expressed as the amount of GTPγS bound above binding by transducin in the absence of mT2R5 and cycloheximide. **(B)** Inhibitory effects of compounds in the GTPγS binding assay. The assay was performed in the presence of mT2R5, 10 μM cycloheximide and the indicated concentrations of either PROP (filled circle), 6-nitrosaccharin (open circle), denatonium (filled triangle) or quinine (open triangle). Results are expressed as the percentage of control. **(C)** Two of the hT2Rs display a high constitutive activity. The GTPγS binding assay with transducin was performed either in the absence or presence of either mT2R5 [in the absence or presence of 300 μM cycloheximide (cyx)] or indicated hT2Rs.

ments with transducin is virtually identical to the dose–response curve generated with gustducin (Chandrashekar *et al.*, 2000) using the same assay. Thus we believe that transducin can functionally substitute for gustducin in the GTPγS binding assay with this and potentially other T2Rs.

All 25 hT2Rs were expressed in Sf9 cells using a baculovirus expression system. These T2R constructs had N-terminal tag consisting of 38 N-terminal rhodopsin amino acid residues added upstream of the coding sequences. Expression of all tested T2Rs was confirmed by immunoblotting using anti-rhodopsin tag antibodies. Using FACS analysis we also demonstrated that all hT2Rs were present on the cell surface when expressed in Sf9 cells (data not shown).

All expressed hT2Rs were tested in the GTPγS binding assay with transducin and five compounds known as bitter to humans: PTC, PROP, denatonium, quinine and 6-nitrosaccharin (a bitter derivative of saccharin). Typical GPCRs bind their ligands with nanomolar to micromolar affinity. However, many bitter compounds produce bitter taste in humans at relatively high concentrations (millimolar or higher) that may interfere with the assay. To determine if selected compounds interfere with the assay, we performed the GTPγS binding assay with transducin, mT2R5 and cycloheximide in the absence or presence of various concentrations of bitter compounds. None of the tested compounds displayed a significant effect on the GTPγS binding by transducin in the absence of cycloheximide (data not shown). However, at concentrations of 1 mM and higher, denatonium, quinine and 6-nitrosaccharin significantly inhibited cycloheximide-stimulated transducin activation (Figure 2B) . Similarly, these compounds also inhibited activation of transducin by rhodopsin (data not shown). Thus, it appears that these compounds inhibit the assay independently of the nature of the receptor, possibly affecting membrane lipid state. This result indicates that if the test concentration of the compound in this assay is too high, receptor activation could be missed due to a nonspecific inhibitory effect of the compound. PROP and PTC (not shown) did not significantly affect this assay. Thus, we selected 0.5 and 2 mM PTC, 0.1 and 1 mM PROP, 0.1 and 1 mM denatonium, 0.1 and 0.5 mM quinine, 0.2 and 2 mM 6-nitrosaccharin for the initial hT2R testing.

When mixed with transducin in the absence of any bitter compounds most hT2Rs displayed relatively low basal activity (0.5–2 fold increase in GTPγS binding compared to transducin alone). However, two hT2Rs (hT2R4 and hT2R54 [hT2R54 is also termed TAS2R39 (Bufe *et al.*, 2002) and TAS2R57 (Conte *et al.*, 2002)] activated transducin in the absence of any added bitter ligands (Figure 2C). The basal activity of hT2R4 and hT2R54 is as high as the activity of mT2R5 in the presence of maximal cycloheximide concentration. It is extremely unlikely that any of the assay buffer components (Tris, GTPγS, $MgCl_2$ or NaCl) could activate these receptors and these components are not known to be bitter to human at the concentrations used. These receptors displayed the same high basal activity when the assay was performed using phosphate buffer instead of Tris-based buffer (data not shown). Thus, our results suggest that a large fraction of hT2R4 and hT2R54 in Sf9 generated membranes is in a constitutively active conformation. The reason for this is unknown. It is also unclear whether this phenomenon is specific to T2Rs expressed in insect cells or observed in other cell types as well. Such high constitutive activity makes identification of agonists for hT2R4 and hT2R54 using the GTPγS binding assay difficult.

hT2R61 is activated by 6-nitrosaccharin

Two of the tested hT2Rs displayed increased activity in response to added bitter compounds. We demonstrated that hT2R61—TAS2R43 in Bufe *et al.* (2002) and TAS2R52 in

Conte *et al.* (2002)—activity is increased in the presence of 6-nitrosaccharin (Figure 3A). Of all tested hT2Rs only one other receptor (hT2R44, see below) was activated in the presence of 6-nitrosaccharin, whereas even the most closely related hT2R, hT2R64—TAS2R44 in Bufe *et al.* (2002) and TAS2R53 in Conte *et al.* (2002) (89% amino acid sequence identity to hT2R61)—was not activated. This indicates that activation of hT2R61 in the presence of 6-nitrosaccharin is specific. 6-nitrosaccharin is a bitter analog of saccharin (Hamor, 1961). It activates hT2R61 at concentrations (0.1–0.5 mM) that humans also perceive as bitter. At concentrations of 6-nitrosaccharin above 2 mM, the amount of bound GTPγS begins to decrease due to a nonspecific inhibitory effect of the compound in the assay.

Saccharin, a potent sweetener, has a bitter aftertaste to some people. However, at 0.2–0.5 mM concentrations, most humans do not taste saccharin as bitter (Bartoshuk, 1979). To determine whether saccharin and 6-nitrosaccharin differ in their effect on hT2R61 we included saccharin in the assay. We also included other derivatives of saccharin (6-aminosaccharin and 6-carboxysaccharin). Similar to saccharin, neither compound is bitter at 1 mM (Hamor, 1961). Figure 3A shows that none of the three compounds significantly activated hT2R61 at concentrations up to 4 mM. This suggests that the nitro group of 6-nitrosaccharin is very important for activation of the receptor. Moreover, the position of the nitro group is also critical for receptor activation. Unlike 6-nitrosaccharin, 4-nitrosaccharin does not activate hT2R61 (Figure 3A) and is not bitter to humans (Hamor, 1961). The correlation between biochemical and human psychophysical data, combined with the lack of activation of most other hT2Rs by 6-nitrosaccharin, argues that hT2R61 is a human receptor for the bitter taste of 6-nitrosaccharin.

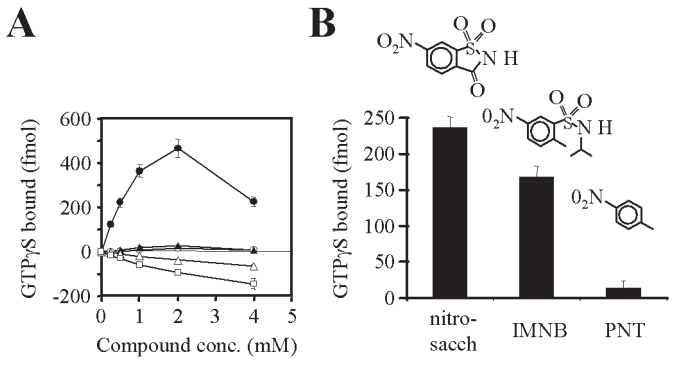

Figure 3 hT2R61 is activated by 6-nitrosaccharin. **(A)** The GTPγS binding assay with transducin was performed in the presence of hT2R61 and indicated concentrations of either 6-nitrosaccharin (filled circle), 6-aminosaccharin (open circles), saccharin (filled triangle), 6-carboxysaccharin (open triangle) or 4-nitrosaccharin (open square). Results are shown as the amount of GTPγS bound after the control amount (in the absence of any compound) was subtracted. **(B)** Activity of hT2R61 in the presence of 0.5 mM of either 6-nitrosaccharin, IMNB or *p*-nitrotoluene (PNT).

To further investigate a structure-activity relationship of the compounds that activate hT2R61 we tested *N*-isopropyl-2-methyl-5-nitrobenzenesulfonamide (IMNB), a compound structurally related to 6-nitrosaccharin with the open five-membered ring. We also tested *p*-nitrotoluene, a structural precursor of IMNB. Due to limited solubility both compounds were tested at 0.5 mM final concentration. At this concentration IMNB is bitter, whereas *p*-nitrotoluene is not. As Figure 3B shows, *p*-nitrotoluene has virtually no effect on hT2R61, while IMNB activates the receptor similarly to 6-nitrosaccharin. This result demonstrates that the nitrobenzene group is not sufficient to activate hT2R61 and that, in addition to the nitro group, the sulfonamide group is likely to be involved in the interaction with the receptor. It also provides an additional correlation between perceived bitterness of the compounds and their ability to activate hT2R61.

hT2R44 is activated by denatonium and 6-nitrosaccharin

6-Nitrosaccharin also activates hT2R44 [TAS2R47 in Bufe *et al.* (2002) (Figure 4A)]. Activation of hT2R44 by 6-nitrosaccharin is less efficient than activation of hT2R61 (~5-fold lower increase in GTPγS binding compared to hT2R61 at 2 mM 6-nitrosaccharin). Thus, it is unlikely that hT2R44 is the primary mediator of bitter taste of 6-nitrosaccharin. Similar to hT2R61, hT2R44 is not activated by 4-nitrosaccharin, suggesting that activation of hT2R44 by 6-nitrosaccharin is specific. IMNB, which activates hT2R61, does not activate hT2R44 (data not shown). This indicates that, whereas 6-nitrosaccharin and IMNB share some determinants that interact with bitter receptors, other parts of the molecules, which are different, are also involved in receptor binding.

We also demonstrated that hT2R44 activity is increased in the presence of denatonium benzoate (benzyldiethyl[(2,6-xylylcarbomoyl)methyl]ammonium benzoate). No other tested hT2R displayed elevated activity in the presence of this compound, indicating specific nature of this effect. Activation of hT2R44 by denatonium is concentration dependent (Figure 4A). At concentrations of denatonium >0.3 mM the amount of bound GTPγS is decreased due to the nonspecific inhibitory effect of denatonium in the assay. The active ingredient of denatonium benzoate is denatonium, not benzoate, because in the presence of 100 μM sodium benzoate the activity of hT2R44 was unchanged (data not shown).

Denatonium benzoate is one of the most potent bitter compounds known to humans, and it is used widely to study bitter taste. In a series of studies Saroli (1984, 1985) looked at the relationship between the structure of denatonium derivatives and their bitter taste in humans. That study demonstrated that selective changes in the denatonium molecule result in the specific reduction of the compound bitterness, which is reflected in the increased bitter thresholds for humans. To confirm that hT2R44 may mediate a bitter taste

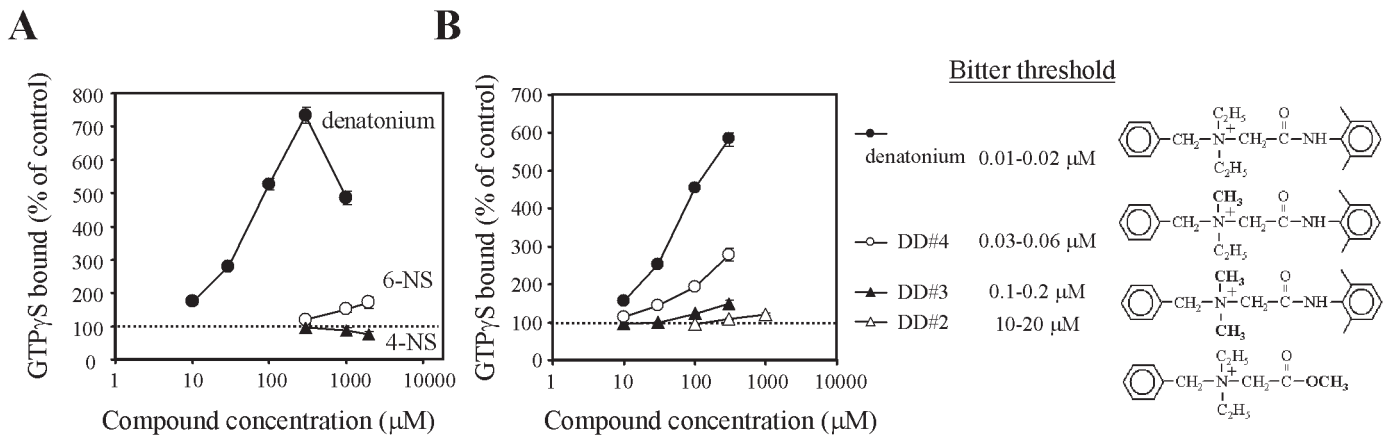

Figure 4 hT2R44 is activated by denatonium and 6-nitrosaccharin. (A) The GTPγS binding assay with transducin was performed in the presence of hT2R44 and indicated concentrations of either denatonium benzoate (filled circle), 6-nitrosaccharin (6-NS, open circle) or 4-nitrosaccharin (4-NS, filled triangle). Results are shown as the amount of GTPγS bound after the control amount (in the absence of any compound) was subtracted. (B) Effect of denatonium and denatonium derivatives on hT2R44 activity. Human bitter thresholds are from Saroli (1984, 1985).

of denatonium and its derivatives in humans we synthesized three of the compounds described in Saroli's papers—benzyldiethyl[(carboxymethyl)methyl]ammonium chloride (DD2), benzyldimethyl[(2,6-xylylcarbamoyl)methyl]ammonium chloride (DD3) and benzylmethylethyl[(2,6-xylylcarbamoyl)methyl]ammonium chloride (DD4). These compounds were tested for their ability to activate hT2R44 and compared to that of denatonium.

As Figure 4B shows, the denatonium derivatives were less effective at activation of hT2R44 compared to denatonium. It also shows that the relative abilities of compounds to activate hT2R44 are identical to their relative bitterness in human taste studies: denatonium > DD4 > DD3 > DD2. For example, compound DD4 is less potent than denatonium in activating hT2R44 in our assay but more potent than compound DD3. Similarly, compound DD4 is less bitter than denatonium to humans, but more bitter than compound DD3. These results demonstrate that hT2R44 likely mediates the bitter taste of denatonium and its derivatives in humans. It was previously suggested that denatonium activates hT2R4 when this receptor is expressed in HEK293 cells (Chandrashekar *et al.*, 2000). Because hT2R4 has high constitutive activity in our hands we were unable to demonstrate any further activation of the receptor by denatonium and compare its effective concentration to the one that activates hT2R44. However, the effective denatonium concentration (1.5 mM) used by Chandrashekar *et al.* (2000) was unusually high. It is 10^5-fold higher than the reported bitter threshold for denatonium in humans (Saroli, 1984). It is also at least 100-fold higher than the concentration of denatonium, which is readily detectable in the GTPγS binding assay with hT2R44 reported here (Figure 4). Thus, it is possible that denatonium had a nonspecific effect on calcium release in HEK293 cells in the presence of expressed hT2R4. Alternatively, hT2R4 may represent a low affinity receptor for denatonium. Although the concentration of denatonium needed for detectable activation of hT2R44 in the GTPγS binding assay is still significantly higher than the reported 'bitter threshold' in humans, we do not expect it to match precisely human taste sensitivity. The 'bitter threshold' is a concentration at which humans begin to differentiate between water alone and water plus a compound (i.e. 'barely detectable'). Sensory detection thresholds are often much lower than observed in heterologous assays. For example, the reported bitter threshold for cycloheximide in mice is 0.25 μM, whereas EC_{50} for mT2R5 activation in the GTPγS binding assay is ~25 μM (Chandrashekar *et al.*, 2000).

Identification of hT2R regions important for receptor activation

Interestingly, both receptors for which we identified ligands belong to a subclass of closely related hT2Rs (Figure 1). The amino acid sequence of hT2R61 is 82% identical (92% homologous) to hT2R44. The closest relative of hT2R61 is hT2R64, which is 89% identical (95% homologous). hT2R64 is also 82% identical (90% homologous) to hT2R44. Yet hT2R64 is not activated by either 6-nitrosaccharin, IMNB or denatonium. Western blot and FACS analysis showed that hT2R61 and hT2R64 expressed in Sf9 cells display virtually identical levels of expression (data not shown). Thus, inability of hT2R64 to respond to any of the identified ligands could be attributed to a sequence difference in these receptors. Sequence analysis demonstrates that 15 of 34 amino acid differences between hT2R61 and hT2R64 are concentrated in the predicted extracellular loops (EL) EL-1 and EL-2, while EL-3 is completely conserved (Figure 5).

To identify regions of hT2R61 that might be responsible for ligand recognition we generated chimeric receptors where EL-1 and EL-2 of hT2R61 and hT2R64 are swapped. As Figure 6A shows, activation of the receptor by IMNB is completely dependent on EL-1 from hT2R61. There are

only four amino acid differences between hT2R61 and hT2R64 in EL-1. However, replacing only these four residues with residues from hT2R64 (hT2R61–64/EL-1) is sufficient to render hT2R61 insensitive to IMNB. Conversely, after introduction of these four residues from hT2R61, hT2R64–61/EL-1 can now be activated by IMNB. Swapping EL-2 (hT2R61–64/EL-2 and hT2R64–61/EL-2) had no effect on IMNB-mediated receptor activation. These results suggest that while other regions of the receptors can also be involved in ligand binding, the four residues within EL-1 play a critical role in receptor signaling in response to IMNB.

The effect of loop swapping on 6-nitrosaccharin binding is more complex. Replacing both EL-1 and EL-2 in hT2R61 with hT2R64 loops eliminates most of the activation by 6-nitrosaccharin (hT2R61–64/EL-1+2; Figure 6B). Conversely, after introduction of hT2R61 residues into hT2R64 extracellular loops, hT2R64–61/EL-1+2 can now be activated by 6-nitrosaccharin as well as hT2R61. These results indicate that most of the residues that are important for 6-nitrosaccharin signaling and different in hT2R61 and hT2R64 lie within EL-1 and EL-2. However, low but clearly detectable activation of hT2R61–64/EL-1+2 suggest that other parts of the receptor also contribute to its activation. Replacing only EL-2 in hT2R61 causes noticeable reduction in the ability of 6-nitrosaccharin to activate this receptor. However, the remaining receptor activity in response to 6-nitrosaccharin is still much higher than the activity of the receptor with both EL-1 and EL-2 replaced. Introduction of hT2R61 EL-2 residues renders hT2R64 weakly responsive to 6-nitrosaccharin. Introduction of hT2R61 EL-1 residues has a much larger effect. hT2R64–61/EL-1 is activated by 6-nitrosaccharin almost as well as hT2R61. These data indicate that residues in EL-1 play a critical role in 6-nitrosaccharin signaling. The only paradoxical exception to this is hT2R61–64/EL-1. Even if residues in EL-1 of hT2R61 are replaced with the residues from hT2R64, the mutant receptor is still activated by 6-nitrosaccharin to a similar extent as the wild type receptor. Taken together, these results suggest that both EL-1 and EL-2 contribute to the receptor activation by 6-nitrosaccharin with EL-1 playing more important role. But the contribution of the specific residues also depends on the context of the rest of the receptor sequence, underscoring the importance of a three-dimensional conformation of the ligand binding site.

To investigate the contribution of specific EL-1 residues to the receptor activation we generated mutant hT2R61 and hT2R64 constructs with single amino acid substitutions. The substitution of N76, I87 or I91 in EL-1 of hT2R61 with hT2R64 residues had very little effect on hT2R61 activation by 6-nitrosaccharin or IMNB (Figure 7). Similarly, the replacement of the reciprocal residues in hT2R64 with hT2R61 residues had a very minor effect on hT2R64

```
                    EL-1
                                *
hT2R64  73-PAFYSVEVRTTAYNVWAVTGHFSN
hT2R61  73-PAFNSVEVRTTAYNIWAVINHFSN
hT2R44  73-PAFYSVEVRITAYNVWAVTNHFSS

                    EL-2

hT2R64 150-KEIVRTKEYEGNLTWKIKLRSAVYLSDATVTTLGN
hT2R61 150-NEIVRTKEFEGNMTWKIKLKSAMYFSNMTVTMVAN
hT2R44 150-DETVWTKEYEGNVTWKIKLRSAMYHSNMTLTMLAN

                    EL-3

hT2R64 250-WSFGSLENKPV
hT2R61 250-WSFGSLENKPV
hT2R44 250-CNLGRLEKQPV
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Figure 5 Alignment of hT2R61, hT2R64 and hT2R44 predicted amino acid sequences for the extracellular loops 1 (EL-1), 2 (EL-2) and 3 (EL-3). Residues, which are different from hT2R61, are boxed in gray and bold. N92 is marked with the asterisk.

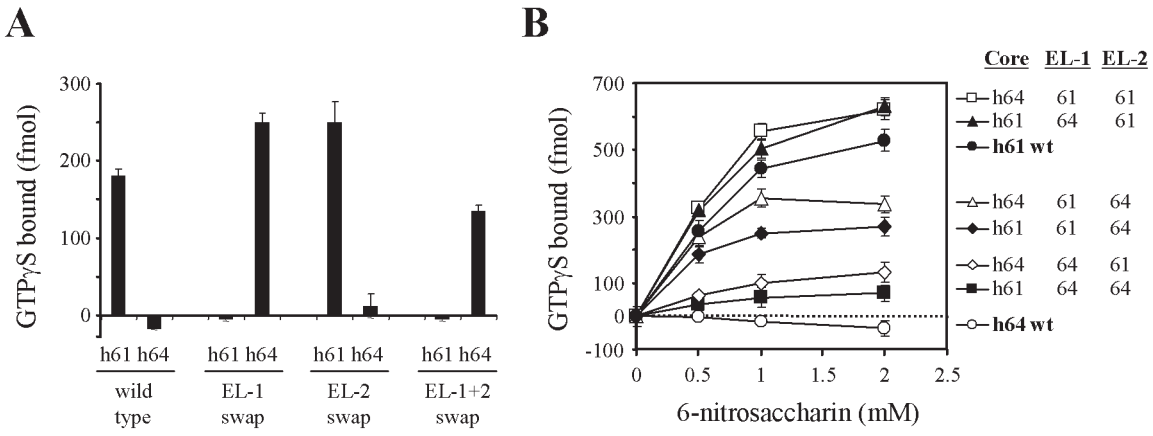

Figure 6 Effects of swapping the extracellular loops 1 or 2 on hT2R61 and hT2R64 activities. (A) Either wild type hT2R61 and hT2R64 or chimeric receptors with the indicated extracellular loops swapped were tested in the GTPγS binding assay with transducin and 0.5 mM IMNB. Results are shown as the amount of GTPγS bound after the control amount (in the absence of any compound) was subtracted. (B) Either wild type hT2R61 and hT2R64 or chimeric receptors were tested with the indicated concentrations of 6-nitrosaccharin. Fragments of receptors that constitute each construct are indicated.

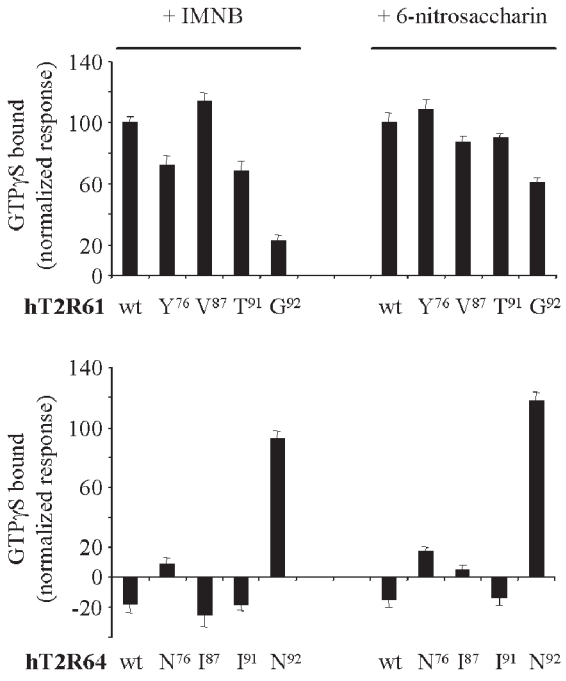

Figure 7 Effects of mutations in the extracellular loop 1 of hT2R61 and hT2R64 on receptor activities. Wild type (wt) hT2R61 and hT2R64 or the constructs with the indicated mutations were tested in the presence of either 0.5 mM IMNB or 1 mM 6-nitrosaccharin as shown. Changes relative to the control in the absence of compounds are presented as the percentage of the wild type hT2R61 response (100%).

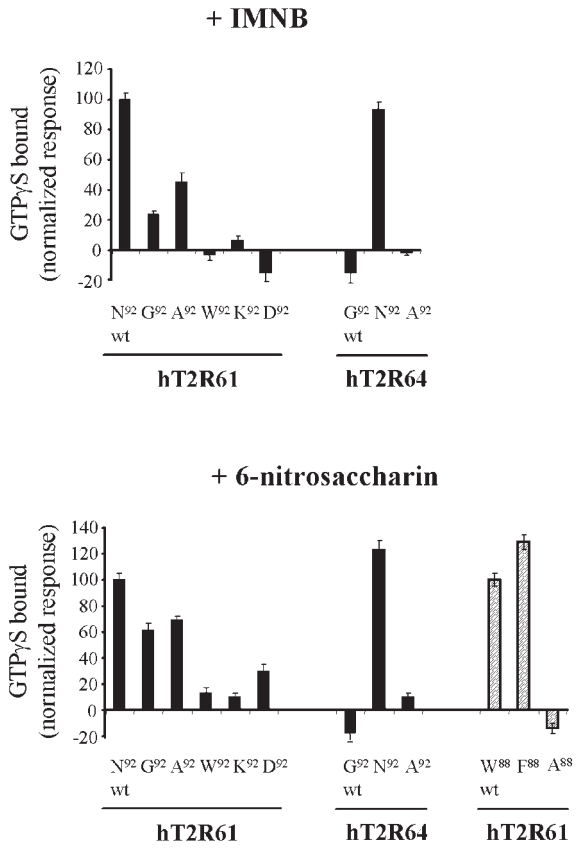

Figure 8 Effects of substitutions of the conserved residues in EL-1 with various amino acids on hT2R61 and hT2R64 activities. Wild type (wt) hT2R61 and hT2R64 or the constructs with the indicated mutations were tested in the presence of either 0.5 mM IMNB or 1 mM 6-nitrosaccharin as shown. Changes relative to the control in the absence of compounds are presented as the percentage of the wild type hT2R61 response (100%).

activity. However, the replacement of N92 with hT2R64 residue (G92) dramatically reduced activation of hT2R61 by IMNB and noticeably reduced activation by 6-nitrosaccharin. More importantly, the replacement of G92 with asparagine from hT2R61 renders hT2R64 sensitive to activation by both ligands. These results suggest that N92 plays a critical role in hT2R61 ligand activation. Interestingly, this asparagine located at the EL-1–TM-3 junction is very conserved among hT2Rs. It is found in 21 out of 25 hT2R.

Introduction of glycine in place of asparagine might provide significant rotation freedom resulting in a considerable change of the overall protein folding in that region. The fact that hT2R61-N92G retains ~60% of responsiveness to 6-nitrosaccharin argues against this possibility. To determine the effect of other residues in this position we mutated N92 into four additional residues (Figure 8). Mutation into alanine reduced activation of hT2R61 by both IMNB and 6-nitrosaccharin but the effect was relatively mild (~30–60%). In contrast, a replacement of the asparagine with either a bulky aromatic (tryptophan) or a charged (lysine or aspartic acid) residue almost or completely eliminated receptor responsiveness to these ligands. The mutation N92D also had a dramatic effect on hT2R61 basal activity—it was increased over 4-fold (data not shown). Interestingly, one of the hT2Rs that has very high constitutive activity in our assay system, hT2R4, also has aspartic acid in place of N92. However, hT2R54 has asparagine in the same position.

The mutation N92A had only a mild effect on hT2R61 activity, suggesting that alanine may functionally substitute asparagine in this position. However, the mutation of G92 into alanine did not significantly increase hT2R64 responsiveness to either IMNB or 6-nitrosaccharin.

Another residue in EL-1 which is highly conserved in hT2Rs (21 of 25) is W88. To determine if this tryptophan plays any role in hT2R61 activity we mutated it into either phenylalanine or alanine (Figure 8). Mutation of tryptophan into another aromatic residue, phenylalanine, had virtually no effect on activation of hT2R61 by 6-nitrosaccharin. However, a replacement with the smaller residue, alanine, completely abolished receptor activation.

To determine the effect replacement of these two conserved residues may have on another hT2R, we mutated N92 and W88 in hT2R44 (Figure 9). A replacement of these residues had much bigger effect on hT2R44 compared to hT2R61. The mutation of N92 into either glycine or alanine

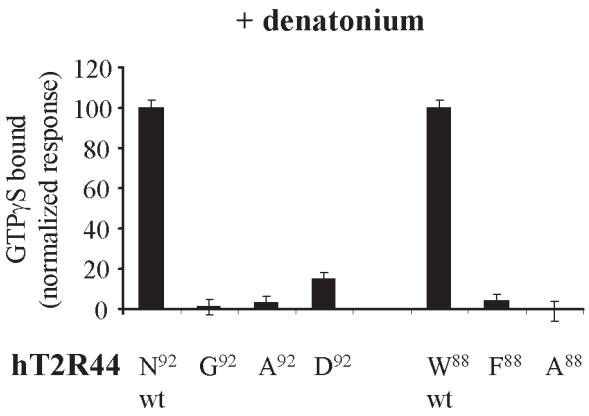

Figure 9 Effects of substitutions of the conserved residues in EL-1 with various amino acids on hT2R44 activities. Wild type (wt) hT2R44 or the constructs with the indicated mutations were tested in the presence of 100 μM of denatonium as shown. Changes relative to the control in the absence of denatonium are presented as the percentage of the wild type hT2R44 response (100%).

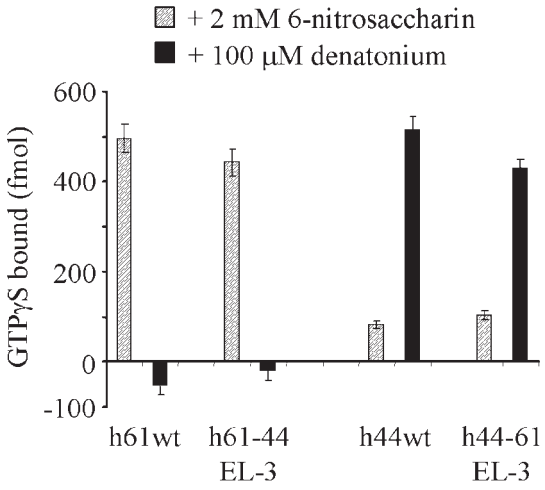

Figure 10 Effects of swapping extracellular loops 3 on hT2R61 and hT2R44 activities. Either wild type hT2R61 and hT2R44 or chimeric receptors with the EL-3 swapped were tested in the GTPγS binding assay with transducin and either 2 mM 6-nitrosaccharin or 100 μM denatonium as shown. Results are shown as the amount of GTPγS bound after the control amount (in the absence of any compound) was subtracted.

completely abolished hT2R44 activation by denatonium. Only N92D mutant retained ~16% of the wild type activity. Whereas the mutation W88F had no visible effect on hT2R61, it completely abolished activation of hT2R44. These results suggest that hT2R44 may have a much tighter binding pocket for denatonium, which may explain denatonium's high affinity for this receptor.

The exact role of the conserved asparagine and tryptophan residues in EL-1 is unclear. They might be directly involved in ligand interaction. Alternatively, they could interact with the other amino acid residues of the receptor, contributing to the formation of a ligand-binding pocket. It is also possible that these residues are critical for protein folding and changing them results in misfolding of the receptor.

Because hT2R61 and hT2R64 have identical extracellular loops-3, we could not use 'loop swap' approach with these receptors to determine a contribution of this region to a ligand binding. In contrast, hT2R44 has EL-3, which is very different from hT2R61 (Figure 5). This suggested that this loop might contribute to the interaction with denatonium. We generated mutant variants of hT2R61 and hT2R44 with EL-3 loops swapped and tested them with 6-nitrosaccharin and denatonium (Figure 10). Surprisingly, the replacement of the entire six residues in EL-3, which are different, with the residues from another receptor had no apparent effect on either hT2R61 or hT2R44 activity. This result strongly suggests that EL-3 is not directly involved in binding of either 6-nitrosaccharin or denatonium by hT2R61 or hT2R44.

Conclusions

In summary, our analysis here confirmed previous findings that the hT2R family consists of 25 members. Functional analysis of hT2Rs identified two novel human receptor–bitter ligand pairs—hT2R61 is activated by 6-nitrosaccharin and hT2R44 is activated by denatonium and 6-nitrosaccharin. De-orphaning of two bitter receptors provides additional support to the hypothesis that hT2R receptor family mediates a bitter response in humans. Our data also support the idea that T2Rs can recognize multiple structurally unrelated compounds. When the hT2R family was identified it was speculated that bitter receptors are broadly tuned. This was based on the fact that hT2R family has only two dozen identified members, whereas humans can recognize hundreds of different compounds as bitter. Indeed, here we demonstrated that the same receptor (hT2R44) can be activated by two structurally unrelated compounds—denatonium and 6-nitrosaccharin. Similarly, mT2R5 can be activated by lidocaine in addition to cycloheximide (S. O'Connell and G. Servant, personal communication). However, our results also suggest that hT2Rs display significant ligand specificity. hT2R61 was tested with 12 different compounds (including denatonium, quinine, PROP, PTC, imipramine, caffeine, caffeic acid, lidocaine, saccharin and acetaminophen), yet only 6-nitrosaccharin and structurally related IMNB activate this receptor. Similar finding was previously reported for mT2R5, which was activated only by cycloheximide out of twelve tested compounds (Chandrashekar *et al.*, 2000) and for hT2R16, which was activated by several β-D-glucopyranosides but not by phenyl-α-D-glucopyranoside or several other bitter compounds (Bufe *et al.*, 2002). Additionally, we showed here that even the receptor, which shares 89% sequence identity with hT2R61, hT2R64 is not activated by the same ligands. This observation makes it challenging to predict

ligand specificity of hT2Rs based solely on a sequence analysis.

Our results also provide the first insight into a structural basis of bitter ligand recognition. We demonstrated that the first extracellular loop of the receptor is very important for the receptor activation by ligands, whereas the third extracellular loop probably is not involved in signaling by the identified ligands. We showed that the mutation of a single residue in the extracellular loop of the receptors can change their ligand specificity. Thus, individuals that have different variants of the same receptor may have different sensitivity to a particular bitter compound. This was emphasized recently by the discovery that human sensitivity to PTC is determined by one or a few missense mutations in a single hT2R gene [hT2R51 in our nomenclature; TAS2R38 in Bufe *et al.* (2002) and TAS2R61 in Conte *et al.* (2002)] (Kim *et al.*, 2003). Interestingly, hT2R51 variant we used in our initial screening of receptors with PTC was a 'nontaster' variant (with three amino acid differences from the 'taster' allele) and we did not observe activation of any hT2R in the presence of PTC. When the paper by Kim *et al.* (2003) was published we generated 'taster' variants of hT2R51 gene. However, even the putative 'taster' variant of hT2R51 demonstrated no activation in the presence of PTC in our assay system. Thus, a direct functional confirmation of hT2R51 as the receptor for PTC awaits further investigation. It is possible that, unlike other hT2Rs, this receptor does not couple well to transducin, which we used in GTPγS binding assay, or may require an additional protein partner to function. A recent study demonstrated that coupling of T2Rs to G proteins can be improved by using specific G protein chimeras (Ueda *et al.*, 2003). This underscores the importance of using a variety of approaches to study the family of human bitter receptors.

Note added in proof

While this manuscript was in preparation, identification of several novel and previously described bitter ligand–hT2R pairs was reported [Bufe, B., Hofmann, T., Krautwurst, D., Kuhn, C. and Meyerhof, W. (2004) *Bitter taste receptors.* Patent application WO2004/029087A2]. One of the described receptors is TAS2R43 (hT2R61 in our nomenclature), for which saccharin was identified as one of the agonists.

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