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Oligomerization of TAS2R Bitter Taste Receptors

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Abstract

A family of 25 G protein–coupled receptors, TAS2Rs, mediates bitter taste in humans. Many of the members of this family are coexpressed in a subpopulation of taste receptor cells on the tongue, thereby allowing the possibility of receptor–receptor interactions with potential influences on their function. In this study, we used several experimental approaches to investigate whether TAS2Rs can form oligomers and if this has an effect on receptor function. Coimmunoprecipitations clearly demonstrated that TAS2Rs can physically interact in HEK293T cells. Further bioluminescence resonance energy transfer analysis of all 325 possible binary combinations of TAS2Rs established that the vast majority of TAS2R pairs form oligomers, both homomers and heteromers. Subsequent screenings of coexpressed bitter receptors with 104 different tastants did not reveal any heteromer-specific agonists. Additional studies also showed no obvious influence of TAS2R hetero-oligomerization on plasma membrane localization or pharmacological properties of the receptors. Thus, our results show that receptor oligomerization occurs between TAS2R bitter taste receptors; however, functional consequences of hetero-oligomerization were not obvious.

Key words: bitter taste receptors, BRET, calcium imaging, coimmunoprecipitation, heterologous expression, oligomerization

Introduction

G protein–coupled receptors (GPCRs) represent one of the largest gene families in the human genome and mediate a broad variety of physiological processes, for example, neurotransmission, cell metabolism, cell proliferation, and differentiation as well as sensory (visual, olfactory, and gustatory) perception (Pierce et al. 2002). Although it was long assumed that GPCRs are active as monomers, recent studies provided evidence that, like other membrane receptors, also GPCRs form dimers or oligomers (Milligan 2004; Maggio et al. 2005). The metabotropic receptor for the neurotransmitter gamma amino butyric acid, the $GABA_B$ receptor consisting of the 2 subunits $GABA_BR1$ and $GABA_BR2$, was the first GPCR to be identified as an obligatory functional heteromer (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998; Kuner et al. 1999). Both subunits are not functional on their own when expressed in a heterologous system, but upon coexpression, they form a functional receptor that is able to bind GABA with the same affinity as the previously characterized $GABA_B$ receptor of the brain, where both $GABA_BR1$ and $GABA_BR2$ subunits are coexpressed and interact with each other. Receptor oligomerization was subsequently shown to be necessary for other class C GPCRs, for example, metabotropic glutamate receptors (Kunishima et al. 2000), and is also discussed for class A GPCRs, for example, rhodopsin, β_2-adrenergic receptor, and opioid receptors (Hebert et al. 1996; Cvejic and Devi 1997; Fotiadis et al. 2003). Furthermore, also hetero-oligomerization of different receptor subtypes and even between distantly related class A GPCRs was described (Jordan and Devi 1999; Rocheville et al. 2000; McGraw et al. 2006). Functional consequences of receptor hetero-oligomerization vary from protein maturation and transport

to the plasma membrane, differences in signaling pathways, and internalization to pharmacological effects, for example, alterations in ligand binding affinities and positive or negative binding cooperativity (Hansen and Sheikh 2004; Terrillon and Bouvier 2004; Maggio et al. 2005). Notably, heteromer-specific agonists were discovered (Waldhoer et al. 2005). Thus, hetero-oligomerization may lead to the formation of new, functionally distinct receptors and thereby greatly increase receptor diversity (Park and Palczewski 2005).

The 3 members of the mammalian T1R/TAS1R family are remarkable examples of functional GPCR hetero-oligomerization: the combination of TAS1R1 and TAS1R3 is an umami taste receptor and TAS1R2 and TAS1R3 form a sweet taste receptor in vitro and in vivo (Nelson et al. 2001, 2002; Li et al. 2002; Damak et al. 2003; Zhao et al. 2003). Both respective receptor subunits interact in vitro (Nelson et al. 2002). Further in vitro studies combining the use of receptor chimeras and mutational analysis subsequently established that different ligands interact with distinct receptor sites, both at TAS1R3 as well as at TAS1R1 and TAS1R2, respectively (Jiang et al. 2004; Xu et al. 2004; Nie et al. 2005; Winnig et al. 2005). This is so far the only known case among GPCRs, in which 1 receptor (TAS1R3) forms functionally distinct receptors through hetero-oligomerization with different partners (TAS1R1 or TAS1R2). In addition, also a function of TAS1R3 alone (probably as homodimer) as low-affinity sweet taste receptor for high concentrations of sucrose was recently reported (Zhao et al. 2003).

T2Rs or TAS2Rs represent another, unrelated family of metabotropic taste receptors with 25 members in humans (Adler et al. 2000; Matsunami et al. 2000; Shi et al. 2003). Strong evidences from a variety of different approaches substantiated T2Rs as bitter taste receptors: they are expressed in a subset of taste receptor cells (Adler et al. 2000; Matsunami et al. 2000; Behrens et al. 2007), they respond to bitter tasting compounds in vitro (Chandrashekar et al. 2000; Bufe et al. 2002), polymorphisms in TAS2R genes correlate with variations in receptor protein function and taste perception (Chandrashekar et al. 2000; Kim et al. 2003; Bufe et al. 2005; Pronin et al. 2007), and receptor knockout mice have lost their ability to respond to specific bitter compounds, whereas transgenic mice engineered to express human bitter taste receptors became sensitive (i.e., averse) to their ligands (Mueller et al. 2005). Like TAS1Rs, also TAS2Rs are coexpressed in a subpopulation of taste receptor cells (Adler et al. 2000; Behrens et al. 2007). This makes it possible that also TAS2Rs hetero-oligomerize with potential effects on receptor function. The formation of receptor heteromers that have their own agonist spectrum could even be the basis for the perceptual ability of mammals to recognize a so large number and variety of compounds as bitter (Zhao et al. 2003; Andres-Barquin and Conte 2004).

We therefore set out to examine if TAS2Rs indeed form oligomers. We used 2 different and independent, that is, biochemical and biophysical, experimental approaches to identify TAS2R oligomers in a heterologous expression system and subsequently also explored possible functional consequences of TAS2R oligomerization.

Material and methods

Receptor constructs

We mostly used constructs coding for TAS2R sequences fused N-terminally to the first 45 amino acids of rat somatostatin type 3 receptor (sst) and C-terminally to a herpes simplex virus (HSV) glycoprotein D epitope tag (Bufe et al. 2002). For bioluminescence resonance energy transfer (BRET) experiments, the sequence for the C-terminal HSV tag was replaced by codon-humanized sequences for either a green fluorescent protein variant (GFP^2) or the luciferase of *Renilla reniformis* (Rluc), which had been amplified from the $BRET^2$ vector $pGFP^2$-MCS-Rluc(h) (PerkinElmer). For coimmunoprecipitation experiments, we replaced the C-terminal HSV epitope tag with a FLAG epitope tag. All constructs were cloned in pcDNA5/FRT, pcDNA5/FRT/TO, pcDNA3 (all Invitrogen GmbH) or pEAK10 (Edge Biosystems) vectors.

Functional expression studies of TAS2Rs

Calcium-imaging analysis

Functional expression studies were essentially carried out as described earlier (Bufe et al. 2002; Behrens et al. 2004; Kuhn et al. 2004). Briefly, HEK293T cells stably expressing the chimeric G protein α subunit Gα16gust44 (Ueda et al. 2003) were transiently transfected with sst-TAS2R-HSV encoding plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. In cotransfection experiments, equal amounts of DNA for both receptors or for 1 receptor and empty plasmid were transfected. After 22–32 h of transfection, cells were loaded with the calcium-sensitive dye Fluo-4-AM (Molecular Probes). Calcium-imaging experiments were carried out in a fluorometric imaging plate reader (FLIPR, Molecular Devices) using excitation at 488 nm and emission recording at 515 nm 1 min before and 5–10 min after bath application of ligands. A second application of 100 nM somatostatin 14 (Bachem), which activates endogenous somatostatin receptor type 2, was carried out to control for cell number and vitality.

Determination of EC_{50} values

Fluorescence signals were first corrected for fluorescence changes of mock-transfected control cells using the FLIPR software and then normalized to background fluorescence [$\Delta F/F = (F - F_0/F_0)$]. The following equation was used to calculate EC_{50} values by nonlinear regression: $f(x) = (a - d)/[1 + (x/EC_{50})^{nh}] + d$, with a being the minimum, d the maximum,

and nh the Hill coefficient. All calculations and plots were performed with SigmaPlot 9.0.

Coimmunoprecipitation

For coimmunoprecipitation (CoIP) experiments, HEK293T Gα16gust44 cells were cotransfected with plasmids encoding for sst-TAS2R-FLAG and sst-TAS2R-GFP^2 fusion proteins. After 22–26 h, cells were lysed in CoIP buffer (120 mM NaCl, 50 mM Tris, pH 8.0, 1 mM ethylenediaminetetraacetic acid, pH 8.0, 0.5% [v/v] Igepal, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 10 μg/ml aprotinin) in a glass homogenizer. In control experiments, cells were transfected separately with constructs for sst-TAS2R-FLAG and sst-TAS2R-GFP^2, respectively, and cell extracts mixed after cell lysis. Cell debris and membrane fragments were removed from solubilized proteins through ultracentrifugation (100 000 × *g* for 1 h 30 min, 4 °C). The supernatants were subjected to immunoprecipitation using anti-FLAG agarose beads (EZview Red ANTI-FLAG M2 Affinity Gel, Sigma-Aldrich) according to the manufacturer's protocol. Briefly, the supernatants were incubated with prewashed anti-FLAG beads at 4 °C overnight. After extensive washes with CoIP buffer, bound proteins were eluted with standard 2× Laemmli buffer (without reducing agents) for 5 min at 95 °C. Dithiothreitol was added to the samples to a final concentration of 200 mM before they were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and analyzed by western blot. Detection of FLAG and GFP^2 fusion proteins or other proteins was carried out with rabbit primary antibodies diluted 1:1000 unless noted otherwise (anti-FLAG M2, Sigma, 1:4000; anti-GFP, Abcam; anti-Gα, Cell Signaling; anti-Gβ, Santa Cruz Biotechnology; anti-ezrin-radixin-moesin (ERM) binding phosphoprotein of 50 kDa (EBP50), Abcam; anti-phospholipase C (PLC)-β3, Santa Cruz Biotechnology). A peroxidase-coupled donkey anti-rabbit antibody (Amersham, now GE Healthcare) was used as secondary antibody. Blots were developed with the ECL system (GE Healthcare), and bands were visualized using the imaging system LAS-1000 (Fujifilm).

Bioluminescence resonance energy transfer

BRET assay

We used the BRET^2 technology (PerkinElmer) with the Rluc as BRET donor and a modified GFP variant (GFP^2) as BRET acceptor.

HEK293T Gα16gust44 cells were seeded at ~10% confluence in white 96-well plates with clear bottom (μClear, Greiner). After ~48 h, at ~50% confluence, cells were transfected with plasmids encoding for sst-TAS2R-GFP^2 and sst-TAS2R-Rluc fusion proteins in a mix of 3:1 using Lipofectamine 2000 (Invitrogen). After 22–28 h of transfection, cells were washed twice with C1 solution (130 mM NaCl, 5 mM KCl, 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, 2 mM $CaCl_2$, and 10 mM glucose, pH 7.4). The clear bottom of the 96-well plate was covered with white tape before the BRET measurements were carried out in a Fluostar Optima (BMG Labtech). Using the device's dispenser, 5 μM (final concentration) of the luciferase substrate DeepBlueC (PerkinElmer) was added to a well, and Rluc and GFP^2 signals were detected simultaneously at 370–450 and 500–530 nm, respectively, 4–12 s after substrate application before the measurement moved on to the next well. The BRET ratio was calculated as light emission at 500–530 nm (corrected for light emission at 500–530 nm of mock-transfected cells) over light emission at 370–450 nm (corrected for light emission at 370–450 nm of mock-transfected cells).

Expression analysis of BRET fusion proteins

Expression of receptor-Rluc fusion proteins was analyzed via luciferase activity after application of 5 μM (final concentration) DeepBlueC (PerkinElmer) as described above. Expression of receptor-GFP^2 fusion proteins was controlled visually under a fluorescence microscope using an excitation wavelength of 360 nm. Quantification of the GFP^2 fluorescence was performed using the FLIPR with an excitation at 488 nm and emission detection at 515 nm.

Results

Identification of TAS2R oligomers by coimmunoprecipitation

Coimmunoprecipitation can be used to demonstrate physical interactions of GPCRs indicative of receptor oligomerization. (Hebert et al. 1996; Milligan and Bouvier 2005). If specific antibodies are not available for the investigated proteins, differently epitope-tagged receptors are coexpressed in heterologous expression systems. An interaction of the 2 receptors is considered to be shown if both proteins are detected in immunoprecipitations obtained with an antibody against 1 of the epitopes (Hebert et al. 1996; Milligan and Bouvier 2005).

We therefore coexpressed various pairs of TAS2Rs that were differently tagged at their C-termini with either a GFP variant (GFP^2) or a FLAG epitope in HEK293T Gα16gust44 cells. Transfected cells were lysed in detergent-containing buffer and membrane proteins solubilized. To remove insoluble cell debris and remaining membrane fragments that could contain both of the coexpressed monomeric receptors (Milligan and Bouvier 2005), cell lysates were subjected to ultracentrifugation prior to immunoprecipitation with an anti-FLAG antiserum; the obtained proteins were analyzed via western blot.

Figure 1 shows that FLAG-tagged TAS2R14 and TAS2R44 were successfully precipitated. The sizes of the detected proteins corresponded well with the predicted values for TAS2Rs (~40 kDA); the multiple discrete bands at

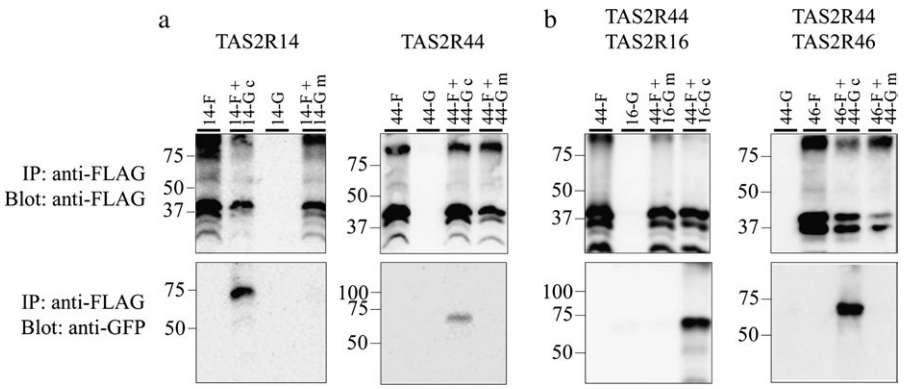

Figure 1 Coimmunoprecipitation experiments reveal physical interactions between TAS2Rs in HEK293T cells. Constructs for differently tagged TAS2Rs were transfected into HEK293T Gα16gust44 cells as indicated by receptor number and tag abbreviation (F, FLAG; G, GFP^2; c, DNAs for both receptors were cotransfected; m, receptors were expressed separately and solubilized membrane protein extracts mixed prior to immunoprecipitation). Membrane proteins were extracted, subjected to immunoprecipitations with anti-FLAG antiserum, and then analyzed via SDS–polyacrylamide gel electrophoresis and western blot with anti-FLAG (upper panel) and anti-GFP (lower panel) antisera. **(a)** Homo-oligomerization of TAS2R14 and TAS2R44, respectively. **(b)** Hetero-oligomerization of TAS2R44 and TAS2R16 as well as TAS2R44 and TAS2R46.

around 40 kDa most likely represent distinct glycosylation states of the receptors (Reichling et al. 2008). The bands at ~80 kDa probably correspond to SDS-resistant TAS2R dimers and may already indicate that TAS2Rs indeed form oligomers. In contrast, GFP^2-tagged TAS2Rs (TAS2R14, TAS2R16, TAS2R44, and TAS2R46) were not detected when expressed alone in HEK293T Gα16gust44 cells, confirming that they could not be precipitated with the anti-FLAG antiserum. If, however, GFP^2-tagged TAS2Rs were coexpressed with FLAG-tagged receptors, they were detected with protein sizes similar to the predicted values of ~67 kDa (Figure 1, lanes marked with c). These data therefore clearly demonstrate a physical interaction of TAS2Rs and show that the investigated receptors exist both as homomers (TAS2R14 and TAS2R44, Figure 1a) and heteromers (TAS2R44/TAS2R16 and TAS2R44/TAS2R46, Figure 1b) in HEK293T Gα16gust44 cells. We also show that this interaction was already formed in living cells and did not arise artificially during the experimental manipulations as we were not able to detect GFP^2-tagged TAS2Rs in mix controls where differently tagged receptors were expressed separately and the solubilized membrane protein extracts were mixed before the FLAG immunoprecipitation (Figure 1, lanes marked with m).

We also ruled out the possibility that TAS2R oligomerization was induced by the plasma membrane targeting sequence of the rat sst attached to the N-termini of the TAS2Rs by showing that TAS2Rs lacking the sst tag also formed receptor homo- and hetero-oligomers (Supplementary Figure S1). We also investigated the possibility that we obtained positive results in coimmunoprecipitation experiments not because of a direct physical interaction between the TAS2Rs but because of an indirect interaction, for example, because the receptors were part of larger signaling complexes. We therefore analyzed the obtained FLAG immunoprecipitates for the presence of EBP50 (Na^+/H^+ exchanger regulatory factor), a PDZ domain–containing protein expressed in kidney cells and previously shown to interact with GPCRs (Weinman et al. 1995; Hall et al. 1998), PLC-β3, a PLC-β subtype efficiently activated by Gαq subunits (Rebecchi and Pentyala 2000) and therefore most likely the effector enzyme of the bitter receptor transduction cascade in HEK293T Gα16gust44 cells, as well as G protein α or β subunits. Although all these proteins were easily detected in HEK293T Gα16gust44 cell lysates, we were unable to find any of these proteins in FLAG immunoprecipitates of TAS2R44/TAS2R16 (Supplementary Figure S2). Although we cannot with certainty rule out the possibility of larger signaling complexes that include different TAS2Rs, we did not find any evidence for this in our experiments.

We then continued to investigate if other members of the TAS2R bitter taste receptor family also form oligomers. In total, we performed coimmunoprecipitation experiments of 21 TAS2R combinations with 12 structurally divergent receptors. TAS2R16 and TAS2R44 were tested for hetero-oligomerization in combination with 8 and 7 other TAS2Rs, respectively. Both receptors as well as TAS2R5, TAS2R14, and TAS2R46 were also analyzed for homo-oligomerization. All the investigated combinations gave positive results in coimmunoprecipitation experiments and thereby demonstrated oligomerization of TAS2Rs (Table 1).

Evidence for general TAS2R homo- and hetero-oligomerization by BRET

The preceding analyses of TAS2R oligomerization by coimmunoprecipitation have revealed that various TAS2Rs indeed form oligomers in HEK293T cells. However, based on its limited throughput, this method is inappropriate to examine all 25 TAS2Rs in all 325 possible combinations

Table 1 Summary of coimmunoprecipitation experiments with differently epitope-tagged TAS2Rs

Receptor-FLAG	Receptor-GFP^2	CoIP
TAS2R5	TAS2R5	+
TAS2R14	TAS2R14	+
TAS2R16	TAS2R5	+
TAS2R16	TAS2R7	+
TAS2R16	TAS2R14	+
TAS2R16	TAS2R16	+
TAS2R16	TAS2R40	+
TAS2R16	TAS2R43	+
TAS2R16	TAS2R47	+
TAS2R16	TAS2R49	+
TAS2R44	TAS2R5	+
TAS2R44	TAS2R7	+
TAS2R44	TAS2R10	+
TAS2R44	TAS2R16	+
TAS2R44	TAS2R43	+
TAS2R44	TAS2R44	+
TAS2R44	TAS2R50	+
TAS2R46	TAS2R7	+
TAS2R46	TAS2R14	+
TAS2R46	TAS2R44	+
TAS2R46	TAS2R46	+

+, Positive result obtained in 1–2 experiments.

for their ability to oligomerize. Hence, we used BRET, a method suitable for high-throughput screening (Boute et al. 2002), to confirm our previous results and to test all TAS2R combinations for oligomerization in living cells under physiological conditions (Pfleger and Eidne 2005). To make use of the BRET technique, fusion proteins of the proteins of interest and Rluc or a modified GFP^2 are generated and coexpressed in a heterologous cell system. If Rluc and GFP^2 are brought into close proximity by a protein–protein interaction of the proteins investigated, energy can be transferred from Rluc (BRET donor) to GFP^2 (BRET acceptor) after substrate oxidation and be detected by measuring donor and acceptor light emission in a luminometer (Xu et al. 1999; Ramsay et al. 2002).

Because the BRET signal depends on distance and orientation of the BRET partners as well as on their molar ratios (Milligan and Bouvier 2005), we first optimized the BRET assay for the detection of TAS2R oligomerization using 1 receptor combination, TAS2R44 and TAS2R46, for which we had obtained positive coimmunoprecipitation results, as well as the sweet taste receptor heteromer TAS1R2/TAS1R3 and the β_2-adrenergic receptor as 2 unrelated positive controls for which receptor oligomerization has been shown previously by BRET (Mercier et al. 2002; Jiang et al. 2004). These experiments led to the detection of BRET signals that were robustly above those obtained for the receptor-Rluc fusion proteins alone and in the same range as those published for the adrenergic and sweet taste receptor (Mercier et al. 2002; Jiang et al. 2004), with a cotransfection ratio of receptor-GFP^2:receptor-Rluc of 3:1 providing both high BRET signals compared with the negative control as well as sufficient total light emission (Supplementary Figure S3). Noteworthy, in these assays, the BRET signal obtained for the TAS2Rs exceeded somewhat those of the positive controls (Supplementary Figure S3).

We then generated fusion constructs of all TAS2Rs and Rluc and GFP^2, respectively, and examined their expression in HEK293T Gα16gust44 cells by either luciferase activity or GFP^2 fluorescence (Supplementary Figure S4). The data confirm that all TAS2R fusion proteins are expressed in HEK293T Gα16gust44 and show Rluc activity (Supplementary Figure S4a) or GFP^2 fluorescence (Supplementary Figure S4b,c). The data also reveal that the apparent expression levels differ between TAS2Rs. Although the majority of receptors are expressed at similar levels as the positive controls TAS1R2, TAS1R3, and the β_2-adrenergic receptor, some receptors, including TAS2R4, TAS2R8, TAS2R9, TAS2R13, and TAS2R45, are expressed at lower levels because they are expressed either at high levels in only few cells (TAS2R9, TAS2R13, and TAS2R45) or at low levels in many cells (TAS2R4 and TAS2R8). This appears to be an intrinsic property of these TAS2Rs as the results obtained with Rluc- or GFP^2-modified receptors are generally in reasonable agreement. We then additionally tested the BRET fusion proteins of 4 TAS2Rs, TAS2R10, TAS2R14, TAS2R16, and TAS2R38, for their abilities to respond to agonist activation in calcium-imaging experiments. All receptors were functional in these assays (Supplementary Figure S5). The modifications had little to moderate impact on most receptors, with only TAS2R16 being clearly impaired though still functional (Supplementary Figure S5).

Finally, we transfected all possible pairs of Rluc and GFP^2 receptor fusion constructs in HEK293T Gα16gust44 cells and recorded their BRET signals. Figure 2 shows the results exemplarily for all combinations with TAS2R44. Almost all combinations of TAS2R44-Rluc with TAS2Rx-GFP^2 caused BRET signals that were significantly ($P < 0.05$; Student's t-test) above the level of the negative control, that is, the signal obtained from the TAS2R44-Rluc expressed alone (Figure 2a) and were therefore positive for oligomerization. Many BRET signals were in the range of that of the positive control, the sweet taste receptor heteromer TAS1R2/TAS1R3, and some were even higher. Only 2 combinations did not result in BRET signals that were significantly above those of the negative control, that is, TAS2R44-Rluc with

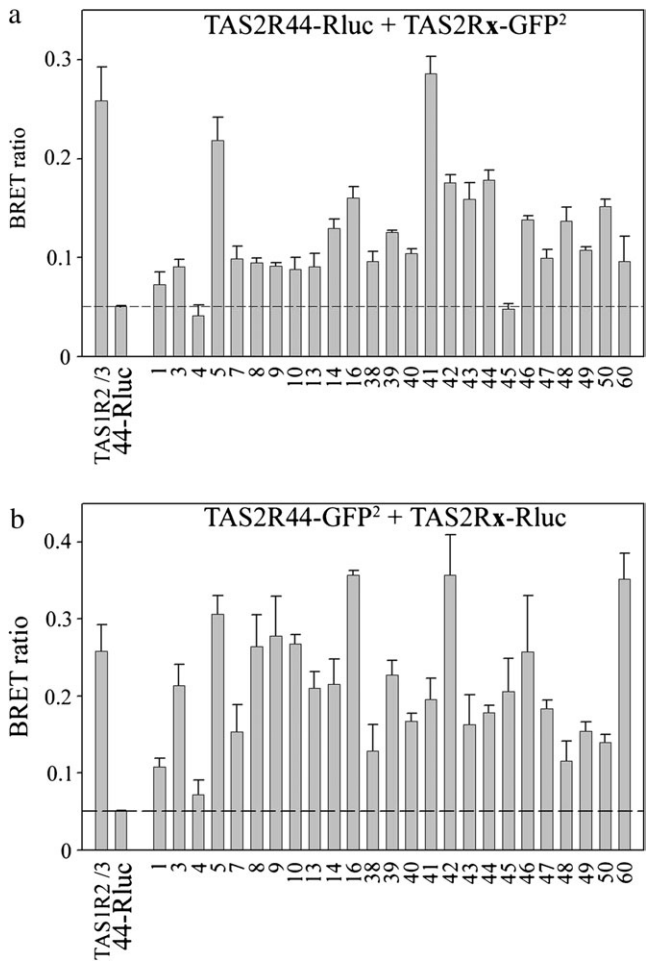

Figure 2 Detection of TAS2R44 homo- and hetero-oligomers by BRET. HEK293T Gα16gust44 cells were transiently transfected with plasmids for **(a)** TAS2R-Rluc as BRET donor and 1 TAS2R-GFP^2 as BRET acceptor or **(b)** TAS2R44-GFP^2 as BRET acceptor together with 1 TAS2R-Rluc as BRET donor. Twenty-two to 28 h later, Rluc and GFP^2 light emissions were recorded after application of the substrate DeepBlueC, and BRET signals were calculated as light emission at 500–530 nm (GFP^2) over light emission at 370–450 nm (Rluc). The sweet taste receptor heteromer TAS1R2/TAS1R3 served as positive control. Negative controls were (a) TAS2R44-Rluc, (b) the respective BRET donors alone with TAS2R44-Rluc displayed as 1 representative. Data represent means ± standard deviation from 2 experiments carried out in duplicate.

either TAS2R4-GFP^2 or TAS2R45-GFP^2 (Figure 2a). An oligomerization of these 2 receptor pairs could therefore not be demonstrated. But as these combinations included receptors that were expressed at low levels (Supplementary Figure S2), the data suggest that poor protein expression most likely accounts for the small BRET signals and probably not the inability of these receptors to form oligomers. When we investigated the reciprocal combinations, that is, TAS2R44-GFP^2 with TAS2Rx-Rluc, we obtained similar results (Figure 2b), although BRET signals appear to be larger if TAS2R44 is tagged with GFP^2, that is, fused to the BRET acceptor, than when TAS2R44 is tagged with Rluc, that is, fused to the BRET donor. Again, almost all combinations caused BRET signals that were significantly above the level of the negative controls, that is, the signal obtained from the respective TAS2R-Rluc expressed alone. We observed only one combination, TAS2R44-GFP^2 with TAS2R4-Rluc, with a BRET signal not significantly above the level of the respective negative control. Therefore, we could show oligomerization for all but one combination. A comparison of the experiments with the reciprocal combinations (Figure 2a,b) shows that one case, the combination of TAS2R44/TAS2R45, gave a negative result regarding its oligomerization (Figure 2a) and in the other case the result was positive (Figure 2b). It is known that slightly increased distances or an unfavorable orientation of the BRET partners can diminish BRET signal intensities (Kroeger et al. 2003; Milligan and Bouvier 2005). Also, excessive Rluc fusion protein in relation to GFP^2 fusion protein can reduce the BRET signal (Pfleger and Eidne 2006). We therefore considered the positive result to be more reliable if we obtained opposite results from reciprocal pairs of TAS2Rs. Hence, if we take all data for TAS2R44 together, all receptor combinations except the one with TAS2R4 showed oligomerization in HEK293T Gα16gust44 cells.

Table 2 summarizes our results of BRET assays with all combinations of TAS2Rs. Of a total of 325 possible combinations, the vast majority (293 or 90%) of receptor pairs showed positive results in BRET assays. Four receptors, TAS2R16, TAS2R46, TAS2R48, and TAS2R50, had positive results in combination with all other receptors; all TAS2Rs except TAS2R45 were positively tested when analyzed in combination with themselves. Thus, the data clearly demonstrate that almost all TAS2Rs form both homo- and hetero-oligomers in living cells.

Functional analysis of TAS2R heteromers

After we had demonstrated with 2 different and independent methods that bitter taste receptors indeed form oligomers when expressed in HEK293T cells, we were next interested in possible functional consequences of TAS2R hetero-oligomerization. Previous studies with other GPCRs found effects of receptor hetero-oligomerization on protein maturation and transport to the plasma membrane, signal transduction pathways, internalization, and pharmacological properties including ligand binding affinity, positive or negative binding cooperativity, as well as specific agonists for heteromers (Hansen and Sheikh 2004; Terrillon and Bouvier 2004; Waldhoer et al. 2005).

We first investigated if receptor hetero-oligomerization improves plasma membrane localization of TAS2Rs as hetero-oligomerization of, for example, $GABA_B$ receptors is necessary for their plasma membrane transport and consequently for formation of functional receptors in vitro and in vivo (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998; Kuner et al. 1999). This is a particularly

Table 2 Overview of homo- and hetero-oligomers of TAS2Rs as detected by BRET assays

TAS2R	1	3	4	5	7	8	9	10	13	14	16	38	39	40	41	42	43	44	45	46	47	48	49	50	60
1	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
3		+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4			+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	o	−	+	−	+	−	−
5				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7					+	−	−	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
8						+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9							+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10								+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
13									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14										+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16											+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
38												+	+	+	+	+	+	+	+	+	+	+	+	+	+
39													+	+	+	+	+	+	+	+	+	+	+	+	+
40														+	+	+	+	+	o	+	+	+	+	+	+
41															+	+	+	+	+	+	+	+	+	+	+
42																+	+	+	+	+	+	+	+	+	+
43																	+	+	+	+	+	+	+	+	+
44																		+	+	+	+	+	+	+	+
45																			−	+	−	+	+	+	+
46																				+	+	+	+	+	+
47																					+	+	+	+	+
48																						+	+	+	+
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60																									+

Data were obtained in duplicate and from at least 2 independent experiments. +, At least 1 signal of a reciprocal combination was significantly higher ($P < 0.05$, Student's *t*-test) than signal obtained from respective BRET donor fusion proteins alone; −, both reciprocal combinations gave signals that were not significantly higher ($P > 0.05$, Student's *t*-test) than signals obtained from respective BRET donor fusion proteins alone; o, no data.

interesting question as TAS2Rs are, like many chemosensory receptors, often poorly localized to the plasma membrane, which leads to their malfunction in heterologous assay systems and necessitates the use of membrane export tags or auxiliary factors (Chandrashekar et al. 2000; Bufe et al. 2002; Behrens et al. 2006; Reichling et al. 2008). We therefore coexpressed various N-terminally unmodified TAS2Rs, that is, without sst export tag, in HEK293T Gα16gust44 cells and analyzed their plasma membrane expression both indirectly in functional calcium-imaging experiments as well as directly with immunocytochemistry. However, both experiments did not reveal any influence of receptor hetero-oligomerization on plasma membrane expression of TAS2Rs (data not shown).

We next examined if the pharmacological properties of heteromeric TAS2Rs differed from those of the monomeric/homo-oligomeric receptors. A variety of recent studies with GPCRs found considerable differences in signal amplitudes and dose–response curves obtained with known agonists as well as positive or negative binding cooperativity of simultaneously applied ligands when receptors were coexpressed (Jordan and Devi 1999; Maggio et al. 1999; Gomes et al. 2000; Xu et al. 2003). We therefore expressed selected TAS2Rs either alone or in combinations in HEK293T Gα16gust44 cells and compared the dose–response functions of their respective agonists (Figure 3). However, these experiments did not reveal any differences of the dose–response relationships of the receptors when they were coexpressed with other TAS2Rs (Figure 3a,b). The EC_{50} value of TAS2R46 activation by strychnine (1.41 ± 0.04 μM) did not change by more than the value of the standard deviation if the receptor was coexpressed with TAS216, TAS2R43, or TAS2R44 (Figure 3a). When we coexpressed TAS2R43 and TAS2R44 and stimulated them with their common agonist aristolochic acid, the dose–response curve matched that of the more sensitive receptor TAS2R43 (Figure 3b). We also tested TAS2R heteromers for the occurrence of positive or negative binding cooperativity by simultaneously stimulating 2 coexpressed receptors with 2 of their respective agonists (Figure 3c,d). We found that the presence of the TAS2R46 agonist strychnine did not influence the dose response curve of the TAS2R44 agonist aristolochic acid recorded from cells coexpressing the receptors (Figure 3c), as both EC_{50} values and Hill coefficients were not altered. The observed amplitudes of the fluorescence signals were additive (Figure 3d). Thus, also these experiments did not show any functional consequences of TAS2R receptor hetero-oligomerization.

Finally, we investigated the possibility that receptor heteromers form functionally new and distinct receptors, which would lead to a greatly expanded bitter taste receptor repertoire and help explain how the enormous number and variety of bitter compounds can be detected by so few receptors. In order to identify heteromer-specific agonists, we performed calcium-imaging experiments with HEK293T Gα16gust44 cells transfected with different mixtures of DNAs for TAS2Rs (containing 5 to all 25 receptors) and challenged with various compounds. We tested 104 known bitter compounds we also used in another study (Meyerhof et al. 2010). These included 58 natural and 46 synthetic bitter substances of various chemical classes, for example, alkaloids, terpenoids, phenolic glycosides, flavonoids, sulfonamides, and heterocyclics. Whereas we could identify many new agonists for single TAS2Rs (Meyerhof et al. 2010), we did not find a compound that activated only cells transfected with a receptor mix but not cells transfected with any of the 25 TAS2Rs alone (Supplementary Table 1 and data not shown); that

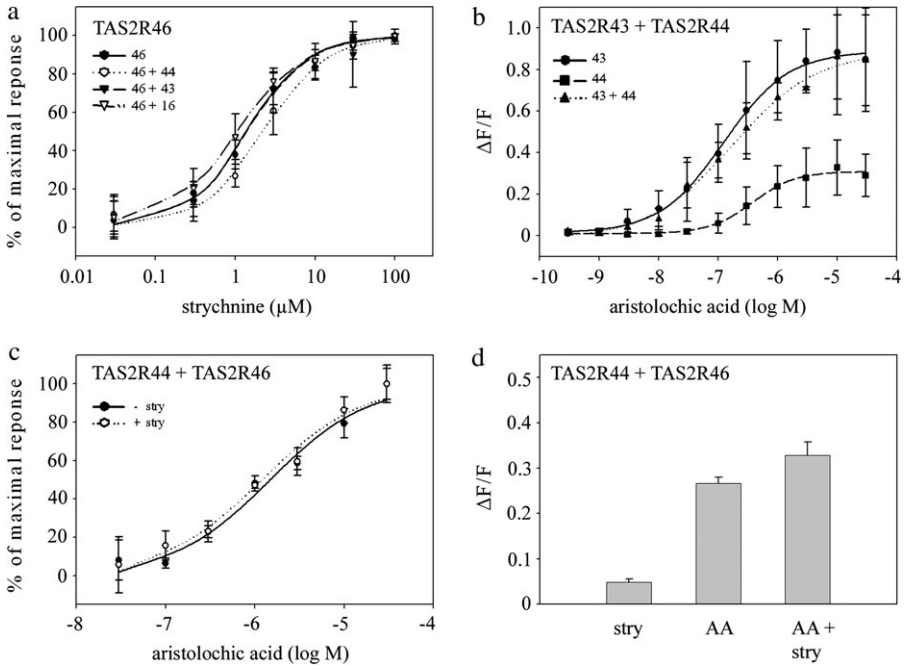

Figure 3 Hetero-oligomerization of TAS2Rs does not alter the pharmacological properties of receptor activation by agonists. HEK293T Gα16gust44 cells were transiently transfected with equal amounts of DNA encoding for **(a)** TAS2R46 and TAS2R16, TAS2R43, TAS2R44, or empty plasmid, **(b)** TAS2R43 and empty plasmid, TAS2R44 and empty plasmid, or TAS2R43 and TAS2R44, and **(c, d)** TAS2R44 and TAS2R46. Calcium-imaging experiments were carried out 22–26 h later; cells were stimulated with (a) strychnine, (b) aristolochic acid, and (c, d) aristolochic acid in the absence or presence of 0.3 μM strychnine. (d) Compares the signal amplitudes from (c) for 0.3 μM strychnine (stry), 30 nM aristolochic acid (AA), and 30 nM aristolochic acid + 0.3 μM strychnine (AA + stry). Data represent means ± standard deviation from 2–4 experiments (a, b) or from 1 representative experiment (c, d) carried out in duplicates.

means we were not able to find a heteromer-specific agonist in our studies.

Discussion

Oligomerization is a widespread phenomenon among membrane proteins (Woolf and Linderman 2003) and has recently also been discussed and demonstrated for a variety of GPCRs (Devi 2001; Milligan 2004; Maggio et al. 2005). Here, we provided clear evidence that also the human bitter taste receptors form oligomers in vitro.

We first demonstrated TAS2R oligomers in coimmunoprecipitation experiments. Differently, epitope-tagged receptors were expressed in HEK293T cells, precipitated by antibodies against one of the epitopes, and subsequently analyzed by western blots for their presence in the immunoprecipitates. This experimental approach has the advantage that also proteins, for which specific antibodies are not available, like TAS2Rs, can be analyzed (Milligan and Bouvier 2005). Furthermore, the use of different epitopes enables the investigation of receptor homo-oligomerization (Hebert et al. 1996). Using coimmunoprecipitation experiments, we could thus show both homo-oligomerization (for 5 receptors) as well as hetero-oligomerization of 16 selected TAS2R pairs including 12 structurally divergent receptors (Figure 1, Table 1). It should also be pointed out here that all the analyzed receptor combinations gave positive results. We did, however, undertake several controls to minimize the possibility of obtaining false-positive results. First, we confirmed that the antibodies against the different epitopes were specific and did not cross-react (Figure 1). We also attempted to rule out that unspecific interactions between the receptors and the epitope tags or unspecific aggregates of the receptor proteins arising during experimental manipulations accounted for our positive coimmunoprecipitation results, a general major criticism of biochemical methods (Devi 2001; James and Davis 2007). Therefore, we always included mix controls (Figure 1, Supplementary Figure S1), where we expressed both receptors separately and mixed the protein extracts prior to the immunoprecipitation, as has been suggested (Jordan and Devi 1999; Kroeger and Eidne 2004). We furthermore subjected the lysed cell extracts to ultracentrifugation before the immunoprecipitation to remove any remaining intact membrane fragments that might contain both coexpressed receptors individually without them being in physical interaction (Milligan and Bouvier 2005).

Supporting evidence for TAS2R oligomerization came from data obtained with a second independent technique, BRET. These data not only confirmed our previous results from the coimmunoprecipitation experiments but also further established that the oligomerization occurred in living cells and under physiological conditions (Kroeger et al. 2003;

Pfleger and Eidne 2006). Previous studies that used BRET assays to demonstrate GPCR oligomerization also assessed the possibility that BRET results could be false positive due to random collision events of highly expressed receptor proteins in the plasma membrane by performing experiments at physiological expression levels as well as with titrated expression levels of both BRET fusion proteins. These experiments confirmed that the conclusions drawn from BRET assays are valid (Mercier et al. 2002; Ramsay et al. 2002). They illustrated that random collisions of BRET fusion proteins will cause the BRET signal to rise linearly with an increased ratio of GFP to Rluc fusion protein, whereas real protein–protein interactions will result in the BRET signal to saturate. We also performed titration experiments with 1 TAS2R pair, TAS2R44 with TAS2R46, as well as with the sweet taste receptor heteromer TAS1R2/TAS1R3 and the β_2-adrenergic receptor and found that in all these cases the BRET signal saturated with increasing transfection ratios of GFP to Rluc fusion protein (Supplementary Figure S3). Therefore, false-positive BRET results because of random protein collision events in the membrane are unlikely. Whether receptor oligomerization occurred in the plasma membrane or in intracellular membrane compartments cannot be established as BRET assays are not sensitive enough to provide subcellular resolution (Ayoub et al. 2002). Fluorescence images of TAS2R-GFP^2 expressing HEK293T cells illustrated plasma membrane localization of at least several receptors (Supplementary Figure S4c). TAS2R oligomerization in the plasma membrane is therefore likely, but proof will require its direct demonstration using other methods, for example, time-resolved fluorescence resonance energy transfer (Maurel et al. 2004).

In our studies, we found that the ability to oligomerize seems to be a general property of members of the TAS2R bitter taste receptor family (Table 2). Ninety percent of all possible TAS2R combinations gave positive results; restricted specificity was not observed. The remaining 10% of combinations, for which we could not show oligomerization, usually included 1 receptor that was poorly expressed (Table 2, Supplementary Figure S4). It is therefore not unlikely that only technical limitations prevented us from substantiating oligomerization for all TAS2Rs in all combinations. To clarify if these receptor complexes consist of only the 2 demonstrated partners, that is, are really receptor dimers, or include more receptor subunits, a modified FRET assay (3-FRET), which uses 3 instead of the commonly used 2 FRET partners, might be applied (Galperin et al. 2004). A recent study successfully used this approach to demonstrate oligomeric and not just dimeric complexes of α_{1b}-adrenergic receptors (Lopez-Gimenez et al. 2007). Alternatively, a combination of time-resolved FRET and snap tag technologies might be used to resolve this question. Using this approach, Maurel et al. (2008) could recently demonstrate that metabotropic glutamate receptors assemble strictly into dimmers, whereas $GABA_B$ receptors form dimers of $GABA_B$R1 + 2 heterodimers at the cell surface.

These data taken together, we could clearly establish the oligomerization of TAS2Rs in a heterologous expression system. But is bitter taste receptor oligomerization also biologically significant? A first step in answering this question would be to demonstrate that these oligomers also exist in vivo (Kroeger and Eidne 2004). Although this is technically more demanding (Bouvier 2001), in vivo oligomers could recently be shown for several GPCRs, including $GABA_B$ receptor heteromers as shown by coimmunoprecipitations as well as rhodopsin homo-oligomers as shown by atomic force microscopy (Kaupmann et al. 1998; Fotiadis et al. 2003). Due to the lack of specific antibodies for TAS2Rs and the very limited availability of human tissue samples, in vivo investigations of receptor oligomerization could not be carried out. However, given the fact that the coexpression of TAS2Rs in taste receptor cells in vivo has already been established (Adler et al. 2000; Zhang et al. 2003; Mueller et al. 2005; Behrens et al. 2007), it is quite likely that bitter taste receptors also form oligomers in vivo.

For GPCRs in general and for TAS2Rs in particular, the possibility of receptor diversification through receptor hetero-oligomerization has been considered (Zhao et al. 2003; Andres-Barquin and Conte 2004; Park and Palczewski 2005). We therefore searched for heteromer-specific agonists in functional calcium-imaging experiments. Although we screened more than 100 known bitter substances of various chemical classes, we could not identify a single functionally distinct TAS2R heteromer. Thus, we find it unlikely that heteromer-specific agonists are prevalent, and we conclude that functional diversity of bitter taste receptor cells and people's ability to perceive the huge number and variety of bitter substances mostly depends on the molecular receptive ranges of TAS2Rs (Behrens et al. 2004; Brockhoff et al. 2007; Sainz et al. 2007; Meyerhof et al. 2010).

Additionally, we also investigated other possible functional consequences of TAS2R hetero-oligomerization, which had recently been described for other GPCRs (Hansen and Sheikh 2004; Terrillon and Bouvier 2004; Waldhoer et al. 2005): facilitation of plasma membrane export, *trans*-complementation of nonfunctional TAS2R variants with functional receptors, changes of the pharmacological properties of receptor–ligand binding including cooperativity of simultaneously applied agonists, as well as differences in G protein coupling or desensitization and internalization. However, our studies did not reveal any functional effects of receptor hetero-oligomerization (Figure 3 and data not shown).

Although some presume that GPCR oligomerization does not take place in vivo or is without functional significance (Chabre and le Maire 2005), it is now commonly believed that GPCRs usually form oligomers and that this is of general importance for the function of these receptors (Kroeger and Eidne 2004; Maggio et al. 2005). Oligomerization could be important for the organization of receptors in the plasma membrane when receptors are present in high copy numbers

and density (Kroeger and Eidne 2004). Rhodopsin, for example, was shown by atomic force microscopy to exist in oligomeric rows of dimers in native disc membranes (Fotiadis et al. 2003). Additionally or alternatively, oligomerization of GPCRs could be necessary for efficient and fast activation of G proteins (Herrick-Davis et al. 2005; Jastrzebska et al. 2006). Thus, oligomerization might possibly be essential for the function of GPCRs including TAS2Rs, whereas it might not be relevant if this is accomplished through homo- or hetero-oligomerization.

To summarize, we could conclusively show with 2 different and independent experimental approaches that most, if not all, TAS2R bitter taste receptors form oligomers in vitro. Although a physiological significance of the receptor hetero-oligomerization was not obvious, receptor oligomerization might in general be necessary for receptor function.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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