Biochimica et Biophysica Acta 1798 (2010) 82–86

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Interactions between the human sweet-sensing T1R2–T1R3 receptor and sweeteners detected by saturation transfer difference NMR spectroscopy

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ARTICLE INFO

Article history:
Received 24 February 2009
Received in revised form 23 June 2009
Accepted 17 July 2009
Available online 4 August 2009

Keywords:
Ligand binding
Sweet receptor
G-protein coupled receptor (GPCR)
Saturation transfer difference (STD)
Nuclear magnetic resonance (NMR) spectroscopy
Brazzein

ABSTRACT

The sweet receptor is a member of the G-protein coupled receptor family C that detects a wide variety of chemically and structurally diverse sweet-tasting molecules. We recently used saturation transfer difference spectroscopy (STD) to monitor the direct binding of a set of sweet agonists and antagonists to the human taste receptor in membranes prepared from human embryonic kidney (HEK293) cells transfected with and expressing the sweet receptor [F.M. Assadi-Porter, M. Tonelli, E. Maillet, K. Hallenga, O. Benard, M. Max, J.L. Markley, J. Am. Chem. Soc. 130 (2008) 7212–7213]. Here we review this work and related studies, discuss the procedures involved, and expand on their potential for identifying specific binding interactions of ligands to the membrane spanning and extracellular regions of the full heterodimeric sweet taste receptor. Whereas activity assays are unable to distinguish mutations that alter ligand-binding sites from those that alter signal transduction downstream of the binding site, STD NMR now allows us to make this distinction.

1. Introduction

1.1. Human heterodimeric sweet receptor

The primary human sweet taste receptor has been identified as a heterodimer of two G-protein coupled receptor (GPCR) subunits, hT1R2 and hT1R3 (***h***uman ***t***aste type ***1*** ***r***eceptor ***2*** and ***3***) [1–4]. T1R receptors are variously called T1R, T1r and Tas1R. At the amino acid level, hT1Rs have substantial sequence similarity with metabotropic glutamate receptors (mGluRs), making them members of GPCR family C [5].

Family C GPCRs are hetero- or homodimeric receptors with a large extracellular region composed of two domains: the Venus flytrap module (VFTM) and the cysteine-rich domain (CRD) [6]. The VFTM is a two-lobed clamshell-like structure (Fig. 1). The CRD lies between the VFTM and the heptahelical transmembrane domain (TMD). The structure of the CRD and the extracellular regions of the group II/III metabotropic glutamate receptors was solved recently [7], but its exact function is still unknown. A disulfide bond between one of the nine highly conserved cysteines in the CRD, located within its span of ~70 residues, and a conserved cysteine in the VFTM have been shown to be important for signal transduction from a bound ligand [8]. As shown by an X-ray crystal structure, the homodimeric mGluR1 VFTM consists of two asymmetrical similar subunits oriented 180° to one another, each with two lobes that together form a clamshell. Lobe 1 and lobe 2 of each subunit approach each other, and their intersection forms the cleft that binds ligand. The amino acid sequences of the VFTMs of T1R and mGluR have ~25% identity and ~41% similarity, and the predicted secondary structural features (helices and beta strands) of the extracellular domains of T1Rs match those found in the crystal structure of mGluR1. Thus, T1Rs and other family C GPCRs are expected to dimerize and to bind ligand in a manner similar to mGluR1. The conformational change in family C GPCRs that takes place upon ligand binding appears to be transmitted from the VFTM and CRD to the TMD helical bundle, then onward to the cytoplasmic surface, where it contacts and activates the G-protein. In the heterodimeric sweet receptor the conformational change is apparently transduced from the VFTM of T1R2 to the TMD helical bundle of T1R3 (Fig. 1). This specialization of subunits is reminiscent of GABAB receptors, in which one subunit binds the ligand while the other transmits the signal to the coupled G-protein [9,10].

T1R2 + T1R3 are the primary receptors for a diverse range of sweet ligands [1–3]. Calcium imaging assays of HEK cells transfected with hT1R2 + hT1R3 respond to all sweet taste stimuli tested, including sugars, amino acids, sweet proteins, and synthetic sweeteners [1,3,11–13]. All of these responses are inhibited by the sweet taste inhibitor lactisole, which acts on the transmembrane domain (TMD) of T1R3 [12,14,15].

1.2. Sweet proteins

Over the last three decades a number of high potency, naturally occurring, sweet-tasting proteins have been isolated from a variety of African and South Asian fruits. The first sweet protein to be identified

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0005-2736/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.bbamem.2009.07.021

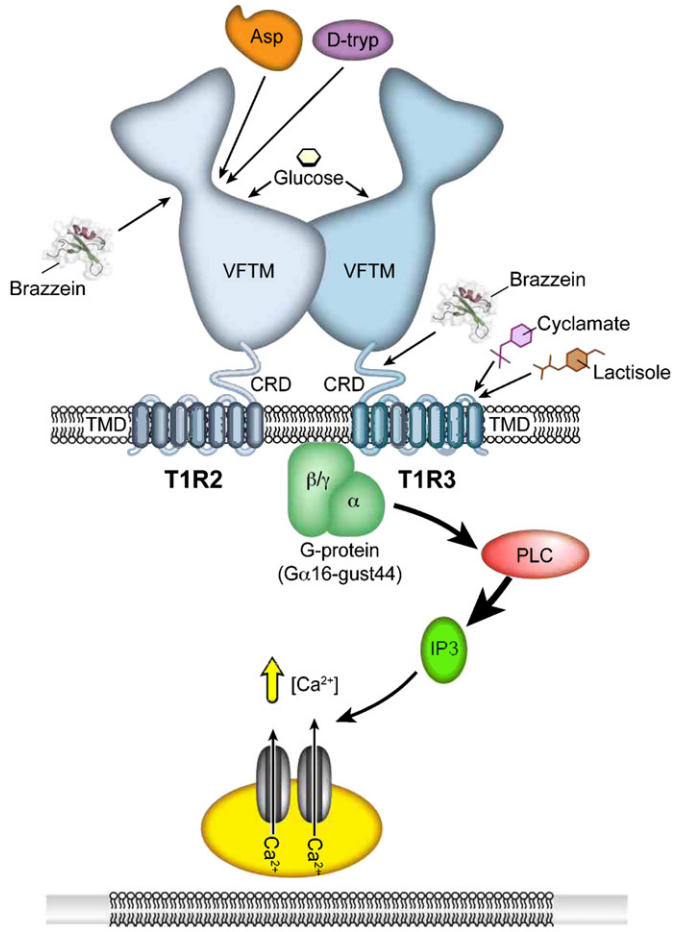

Fig. 1. Scheme illustrating interactions between the heterodimeric sweet receptor and sweet ligands in our heterologous expression assay. In the calcium-sensitive dye-based assay, ligand binding to the membrane-bound sweet receptor is detected as an increase in cytoplasmic calcium due to the action of receptor-induced G-protein activity. The sweet receptor undergoes a conformational change upon ligand binding that leads to subsequent activation of a modified promiscuous Gα protein (G16) that has the receptor-interacting domain substituted with the taste cell G-protein gustducin's C-terminal 44 amino acids (Gα16-gust44). This creates a G-protein with specificity for the sweet receptor, but which activates an endogenous calcium mobilization pathway in the host HEK cells. The activated reporter G-protein stimulates release of calcium stores into the cytoplasm via its βγ subunits which activate the PLC/IP3 pathway. The resulting increase in Ca^{2+} concentration is monitored by cell permeable calcium-sensitive fluorescent dyes.

was thaumatin (22,206 Da) [16], closely followed by monellin (11,086 Da) [17], mabinlin (12,441 Da) [18], brazzein (6,473 Da) [19] and neoculin [20]. Sweet proteins have a sweetness ranging from 300 to 3000 times that of sucrose on a weight basis.

Although sweet proteins exhibit no sequence or structural homology, they all require charged residues (specifically positively charged residues) on the protein surface over a non-continuous area for sweet activity. Thus, it may be that protein sweeteners share a yet-to-be identified receptor binding motif and interact with the sweet receptor in a similar, but not identical, manner.

Interestingly, human-specific sweet proteins, such as brazzein, interact with human T1R2 + T1R3 receptors but not rat [2] or mouse [3] T1R2 + T1R3 receptors. Rodent and human T1Rs exhibit 70% sequence identity [1]. These findings opened up an avenue for elucidating the mechanisms of human sweetener–receptor interactions and the resulting signal transduction.

1.3. Brazzein

Brazzein is the sweet-tasting protein that occurs naturally in the fruit of the African plant *Pentadiplandra brazzeana* Baillon. Brazzein is ~2000 times sweeter than sucrose on a weight basis (17,000 times sweeter than sucrose on a per-molecule basis) [19,21,22]. The protein contains no carbohydrate and bears no structural resemblance to sucrose. The species difference in brazzein's perceived sweetness makes it an attractive compound with which to define the sweet taste receptor ligand-binding sites. Our current hypothesis is that brazzein binds to a non-continuous, multisite, multidomain surface that includes the "hinge" region of T1R2 and the CRD of T1R3. The brazzein binding site is distinct from the sucrose binding site, but, nevertheless, both sweeteners trigger a conformational change on the pathway leading to the sweet taste response (Fig. 2) [3,22,23].

1.4. Brazzein stimulation of human T1R2 + T1R3

Recently, a cell-based calcium mobilization assay for sweetener–receptor activity was developed that employs human T1R2 + T1R3 sweet receptors expressed heterologously in HEK293 cells [3,12,13]. HEK293 cells are co-transfected with plasmids containing the cDNA for the human versions of T1R2 and T1R3 and the promiscuous chimeric G-protein reporter G16gus44 (Fig. 1). The cells contain, in addition, a plasmid with cDNA encoding red fluorescent protein as a means for identifying transfected cells. Responses to sweet ligand activation of receptors are monitored by calcium mobilization imaging using the calcium indicator dye fluo-4 acetoxymethyl ester. This approach allows quantitative measurement of the activity of the sweet receptor in response to an array of sweet ligands. The activity assay has been useful for analyzing the functional properties of wild type and mutant sweet receptors in response to sweeteners, but it does not provide information about the status of ligand binding in those receptor mutants that are defective in their response to multiple sweet ligands.

1.5. Mutagenesis studies of brazzein interaction with the CRD of T1R3

Jiang et al. [3] showed that the CRD of hT1R3 contains residues critical for its interaction with the sweet protein brazzein. Because brazzein's sweetness is specific to humans and old world primates (but not to mice), the heterologous expression in HEK cells of various combinations of human and mouse T1R receptors was used, along with a reporter G-protein, to determine whether responses to brazzein require that the T1R3 monomer be of the human type.

To locate the residues of hT1R3 required for responsiveness to brazzein, several human/mouse chimeras were constructed and tested in combination with hT1R2 [3]. Within the critical region of the CRD, only five amino acids differ between mT1R3 and hT1R3. A single-site human-to-mouse mutant (A537T) was found to be unresponsive to brazzein. In addition, a nearby mutant (D535Q) was found to abolish the ability of brazzein to activate the receptor, but spared responsiveness toward cyclamate (which binds in the TMD of T1R3) and toward other small molecule sweeteners that bind within the VFTM [24]. These results identified a critical contact site on the CRD of T1R3 for brazzein and likely for other protein sweeteners (the response to monellin also is reduced by mutations in this region of the receptor). Protein modeling studies have shown that residues critical for brazzein activity all lie on the same face of the CRD [24,25].

1.6. Saturation transfer difference (STD) investigations of the interaction of sweeteners with the sweet taste receptor

The work described above set the stage for the possibility of carrying out NMR experiments aimed at the direct detection of interactions between sweeteners and the human sweet receptor expressed in HEK cells [23]. We describe these experiments here and their implications for future experiments of this kind between receptors and small molecules that alter their function.

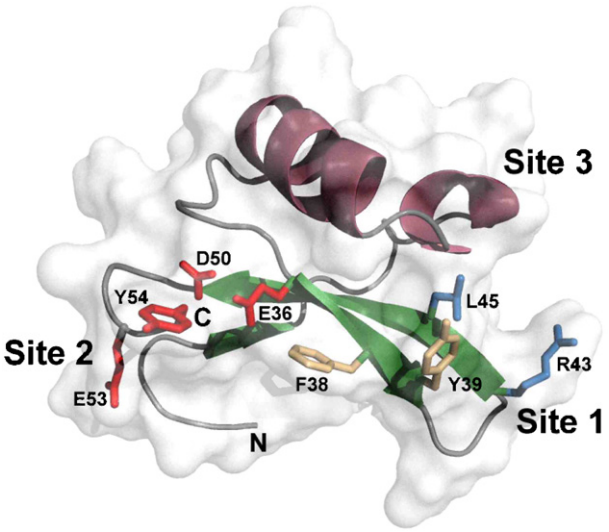

Fig. 2. Surface representation of brazzein showing the three major putative receptor binding sites (side chains shown in color are from residues found to be important for sweetness). Site 1: includes Loop43 at end of an antiparallel β-hairpin and contains mostly charged and aromatic residues. Site 2 includes the N- and C-termini and contains the cluster of acidic residues shown in red [22]. Site 3: includes Loop19 and is located near Loop43 (site 1).

2. Materials and methods

2.1. Preparation of membranes containing the sweet receptor

Wild-type hT1R2 + hT1R3 and mutant hT1R2 + hT1R3(D535Q) were co-expressed in HEK293 EBNA (HEK293E) cells. The level of protein production was determined by immunostaining against the flag epitope tag inserted at the C-terminus of the T1R2 construct and against an antigenic peptide located the VFTM of T1R3. Membranes used in NMR experiments were isolated from parental cells, from stable cell lines expressing wild-type hT1R2 + hT1R3/Gα16-gust44 (generated by clonal dilution under hygromycin (500 g/ml) and Zeocine (100 g/ml) selection), or from HEK cells transfected with other T1R# plasmid(s) of interest. The parental cells were HEK 293 cells subjected to mock procedures.

Lipofectamine™-2000 (Invitrogen, Carlsbad, CA) was used to transfect 293 E cells according the manufacturer's protocol. After 48 h, the cells were harvested from the flask and spun at 800×g for 10 min in phosphate buffered saline containing 0.5 mM EDTA to pellet the cells. The cells were transferred into homogenization buffer containing 20 mM Tris–HCl (pH 7.4), 10% glycerol, and Complete™ Protease Inhibitor Cocktail (Roche, Nutley, NJ) and dispersed in a Polytron homogenizer (Glen Mills, Clifton, NJ). Particulate matter was removed by centrifuging at 1500×g for 15 min. The supernatant was then centrifuged at 100,000×g for 1 h at 4 °C. The pellet was washed with homogenization buffer lacking protease inhibitor and centrifuged at 100,000×g for 30 min at 4 °C. 200 μl of homogenization buffer lacking protease inhibitor was added to the pellet, and the membrane was resuspended by 20 passages through a 25 gauge needle. The membrane suspension was stored at −80 °C until used.

2.2. Preparation of brazzein

The construct used to produce brazzein in *E. coli* cells was an artificial gene coding for des-pGlu1-brazzein joined to the gene coding for SUMO followed by a His-tag [26]. The brazzein-SUMO fusion protein was produced under the control of a T7 promoter from BL21(pLysS)-PRIL-CodonPlus cells with induction by 0.5 mM IPTG. Following induction, cells were grown for 24 h at 25 °C. Cells were harvested by centrifugation and disrupted by sonication, and the soluble fraction was applied to a Ni^{+}-NTA column to isolate the fusion protein. SUMO protease (1 U/50 μg fusion) was added to cleave brazzein from the rest of the protein. The SUMO protease (a small ubiquitin-like modifier) recognizes only the tertiary sequence of SUMO and efficiently cleaves at the junction between SUMO protein and brazzein. The cleavage yield was >90% in the presence of 0.1 mM DTT. Brazzein was purified from the cut and uncut fusion protein by reversed-phase HPLC [26]. The concentration of brazzein was determined by measuring the absorbance at 205/280 nm [26].

2.3. NMR sample

The 150 μl NMR sample contained 2 mg brazzein and 50–75 μg membrane. The receptor concentration was estimated to be in the nM range. Thus the ligand to receptor ratio was ~500:1. Although this ratio is higher than those previously reported in the literature for STD NMR experiments, the membrane-bound receptor has a very long correlation time, which yields a much higher STD amplification than a protein in solution.

2.4. NMR data collection and analysis

Two-dimensional STD ^{1}H–^{15}N HSQC data were collected on a 800 MHz Varian VNMRS spectrometer equipped with a cryogenic probe. Data were collected at 298 K with 96 accumulations per FID and 1024×32 complex points in the direct (^{1}H) and indirect (^{15}N) dimension, respectively, for a total acquisition time of 9 h for each 2D experiment. The receptor-saturated spectrum was obtained by applying a 3-s continuous-wave pulse with a 50 Hz field strength at −1.0 ppm (where brazzein has no ^{1}H signal) prior to the first pulse of the HSQC sequence. On alternate scans, the reference spectrum was collected with the saturation pulse shifted to 50 ppm (well beyond the ^{1}H spectral region of both brazzein and receptor). The STD spectrum was obtained by subtracting the two signals. This procedure was used to collect STD spectra from a sample containing membranes from HEK cells expressing the wild-type sweet receptor (hT1R2 + hT1R3), membrane from cells expressing the mutant (hT1R2 + hT1R3 (D535Q)), and membrane from parental cells (cells lacking the expression plasmids). As a means for removing signals arising from non-specific binding, the STD spectrum from parental cell membranes was subtracted from the STD spectrum of each receptor-containing membrane preparation to yield the final saturation transfer double difference (STDD) spectra.

3. Results and discussion

3.1. Monitoring ligand binding to T1R2 + T1R3 by STD NMR spectroscopy

Previous studies of ligand binding to sweet receptors were hindered by the low affinity of the receptor for sweeteners [27,28]. Our novel assay for sweet receptor/ligand-binding interactions based on saturation transfer difference (STD) and saturation transfer double difference (STDD) NMR spectroscopy was found to be responsive even for weakly binding sweeteners [23].

STD NMR is well established as a sensitive and powerful tool for monitoring direct binding interactions of single or multiple ligands in a complex system. Because the spectrum of the ligand is monitored, the approach is not limited by the apparent molecular weight of the receptor. Thus, we postulated that it could be used to study interactions between receptors expressed and displayed on the cell surface and soluble sweeteners. Although STD signals could be detected with intact cells, we found that the sensitivity of the experiment could be increased by using receptor–containing membrane isolated from the cells [23]. This general approach can be used to obtain important information about ligand-receptor interactions. 1) It provides information about ligand affinity and the nature of the

binding site at a detailed atomic level. 2) It allows study of the ligand interaction in competitive or noncompetitive circumstances based on equilibrium constants (the association constant K_a or dissociation constant K_d) and on- and off-rates (k_{on} and k_{off}). 3) The method can be used to examine the properties of truncated or full-length hetero- or homodimeric receptors in cells (*in vivo*) under physiological conditions or in a membrane environment. 4) Only a very small amount of receptor (pmol–μmol) is required for this assay.

Previously, it was shown that STD NMR could be applied to platelets [29]. Thus, cells displaying receptors on their surface at normal heterologous expression densities are ideal substrates for monitoring ligand-receptor interactions by STD NMR. As explained in more detail below, binding is detected from changes in the signal of free ligand which, depending on K_d, is adjusted to have a concentration 20–1000-fold greater than that of the receptor protein [30]. The preferred receptor concentration for STD NMR experiments is nM–μM. From our histocytochemical assays for cell surface localization of receptors we estimate that the membranes contain 1–10 nM receptor [29]. We adjusted the ligand and receptor concentrations for maximal complex formation according to,

$$[L] + [P] \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} [C]$$

where L stands for ligand, P for protein receptor, C for the ligand-receptor complex, and k_{on} and k_{off} are the association and dissociation rate constants. The STD NMR technique relies on spin diffusion to transfer saturation from the receptor to the bound ligand [30,31]. The *saturation spectrum* is obtained by applying a saturating pulse to a region of the receptor spectrum that is far from any ligand resonance (here we irradiated at −1 ppm). For large proteins, such as the heterodimeric sweet receptor, efficient spin diffusion rapidly produces a state of saturation throughout the protein [30,31]. Spin diffusion also causes signals from the ligand to become saturated during transient binding to the receptor. As the saturated ligand molecules are released in solution, new molecules are bound and saturated, causing the pool of free ligand to become gradually saturated. A *control spectrum* is then taken with the saturating pulse applied outside the spectral regions of the receptor and ligand (here we used 50 ppm). The *control spectrum* is then subtracted from the *saturation spectrum* to yield the *STD spectrum*, which reveals the saturation transferred to the pool of free ligand.

The degree of saturation depends on the ratios of the concentrations of L and P and on the dissociation constant. We were able to achieve suitable conditions by using membrane protein concentrations of 7.5–10 μg/μl (roughly in the 1–10 nM concentration range). The ratio of ligand to receptor for known K_d values for lactisole, cyclamate, alitame, and brazzein indicated that we could achieve the right concentration range simply by adjusting the membrane concentration [23]. To account for STD signals arising from non-specific interactions between brazzein and cell membranes, we ran additional STD experiments using membranes derived from parental HEK cells that do not express the sweet receptor. Subtraction of this STD spectrum from those of receptor-containing membranes yielded STDD spectra containing signals only from specific binding.

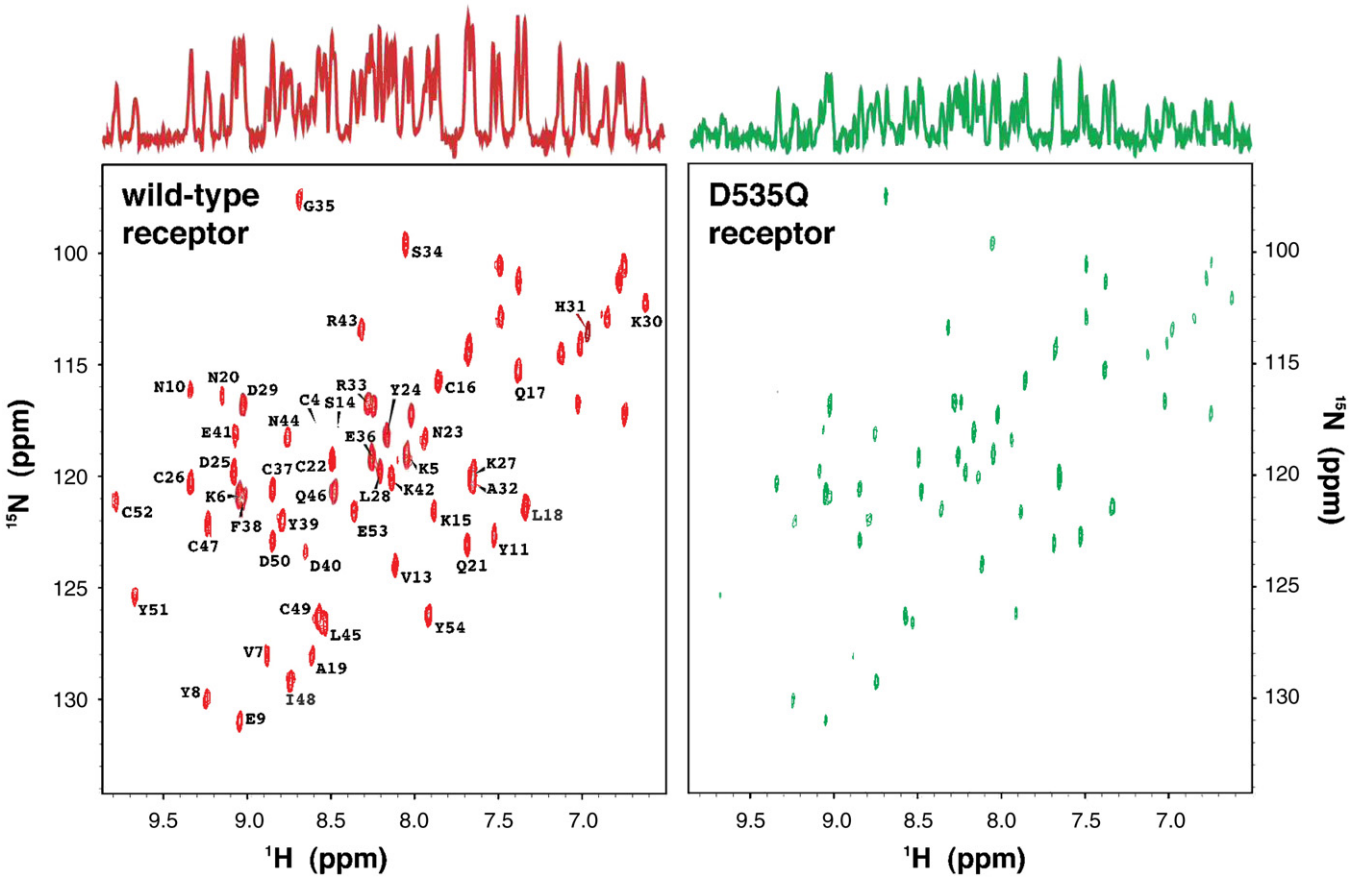

Fig. 3. Two-dimensional ^{1}H–^{15}N HSQC saturation transfer difference (STDD) results reporting on the interaction of wild-type brazzein with (A) wild-type hT1R2 + hT1R3 and (B) brazzein-defective hT1R2 + hT1R3(D535Q) mutant receptor in membranes isolated from HEK293 cells expressing and displaying the sweet receptor (HEK + r). Each STDD spectrum was obtained by subtracting the STD signals of HEK cells without expressed receptor (to remove effects from non-specific binding) from the STD spectrum of HEK + r. At the top of each panel is a projection of the 2D spectrum along the ^{1}H NMR dimension to allow for a better comparison of signal intensity between the two spectra. In the 2D spectrum, each peak comes from individual backbone $^{1}H^{N}$–^{15}N. The decreased peak intensity in 2D spectrum B compared to A indicates a significant loss of binding affinity.

3.2. Role of cysteine-rich domain of T1R3 in binding brazzein

Previous mutagenesis studies had identified a mutant of T1R3 with a "brazzein-specific binding deficit", hT1R3(D535Q). Because this mutation was found to impair activation of the receptor by brazzein but not by cyclamate or other sweeteners that bind within the Venus flytrap module (VFTM) [31], it appears to identify a critical contact site for brazzein on the CRD of T1R3. Thus, to investigate the role of this mutation on its ability to alter brazzein binding, we designed STD NMR experiments to test the binding between the wild type or the hT1R2 + hT1R3(D535Q) mutant form of the sweet receptor with wild-type brazzein. Because signals from larger ligands such as brazzein are more complex to analyze, we developed and utilized a two-dimensional version of the STD experiment (2D-STD ^{1}H–^{15}N-HSQC NMR). The 2D-STD NMR experiments (Fig. 3) clearly showed that mutant human receptor (T1R2 + T1R3(D535Q)) binds brazzein much less tightly than wild-type receptor (T1R2 + T1R3).

4. Conclusions and future prospects

The sweet receptor is involved in sensation of a wide variety of chemically and structurally diverse sweet molecules (>50). We have monitored the direct ligand binding of a set of sweeteners to the human taste receptor in membranes prepared from HEK293 cells. Our results show that the saturation transfer NMR binding assay can be used to monitor specific ligand–receptor binding interactions with both small ligands (lactisole, cyclamate, alitame) and a sweet protein (brazzein) [23]. In addition, the results of our binding assay confirm earlier mutagenesis and modeling data that suggested that the loss of activity of the CRD of T1R3(D535Q) mutant is due to disruption of the brazzein binding site. The STD NMR method provides a powerful way of identifying and mapping ligand and receptor binding sites, and it should prove to be valuable for determining the molecular mechanisms for ligand binding and consequent signaling events.

Conflict of interest statement

Dr. Marianna Max is a named inventor on a number of patents and patent applications relating to the area of taste receptors and taste signaling. The patents have been assigned by the inventors to Mount Sinai School of Medicine (MSSM). MSSM has received compensation in return for a license to certain technology, the value of which may be affected by the outcome of this study. As a co-inventor of the licensed technology, Dr. Max is entitled to a share of any compensation that MSSM receives.

Acknowledgements

This research was supported by NIH grants P41 RR02301, which funds the National Magnetic Resonance Facility at Madison, R01 DC009018, R01 DC006696, R01 DC008301, and R21 DC008805-02.

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