The Cysteine-rich Region of T1R3 Determines Responses to Intensely Sweet Proteins

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A wide variety of chemically diverse compounds taste sweet, including natural sugars such as glucose, fructose, sucrose, and sugar alcohols, small molecule artificial sweeteners such as saccharin and ascesulfame K, and proteins such as monellin and thaumatin. Brazzein, like monellin and thaumatin, is a naturally occurring plant protein that humans, apes, and Old World monkeys perceive as tasting sweet but that is not perceived as sweet by other species including New World monkeys, mouse, and rat. It has been shown that heterologous expression of T1R2 plus T1R3 together yields a receptor responsive to many of the above-mentioned sweet tasting ligands. We have determined that the molecular basis for species-specific sensitivity to brazzein sweetness depends on a site within the cysteine-rich region of human T1R3. Other mutations in this region of T1R3 affected receptor activity toward monellin, and in some cases, overall efficacy to multiple sweet compounds, implicating this region as a previously unrecognized important determinant of sweet receptor function.

Obesity and diabetes have reached epidemic proportions in developed societies. Although in part this is because of a more sedentary lifestyle, our strong preference for sweet tasting foods and their abundance is a major factor. Replacing sugar with low- or non-caloric sweeteners may be of benefit. To design more effective sweeteners it is important to understand at the molecular level how the sweet taste receptor functions. It has been demonstrated that the combination of T1R2 + T1R3 recognizes and responds to many sweet ligands, including sugars, small molecule artificial sweeteners, and protein sweeteners (1, 2).

T1R2 and T1R3 are subclass 3 G-protein-coupled receptors (1–7). Other members of this subclass are metabotropic glutamate receptors (mGluRs),1 calcium-sensing receptors, pheromone receptors, and other taste/olfactory receptors (T1R1, 5, 24).

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1 The abbreviations used are: mGluR, metabotropic glutamate receptor; BRET, bioluminescence resonance energy transfer; DPBS, Dulbecco’s phosphate-buffered saline; ATD, amino-terminal domain; GFP, green fluorescent protein.

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required for a human-like response to monellin lie within the ATD of T1R2. hT1R2 + hT1R3 responds to brazzein, but hT1R2 + mT1R3 does not, indicating that residues in hT1R3 are required for receptor activity toward brazzein. We have located these human-specific residues within a small area in the cysteine-rich region of T1R3 (amino acids 536–545); this is the first case in which the cysteine-rich region of a G-protein-coupled receptor has been implicated in receptor function. Replacement of the cysteine-rich region of mouse T1R3 with the corresponding human segment allows hT1R2 + humanized mT1R3 to respond to brazzein. This same segment of T1R3 influences receptor activity toward monellin as well.

**EXPERIMENTAL PROCEDURES**

**Preparation of Chimeras and Point Mutations—**Human T1R expression constructs were generated in the pEGAK12 vector (Edge Biosystems) by genomic DNA-based methods. To subclone each gene into pEGAK12 vector, an EcoRI Kozak cassette was introduced at the 5’ end before the start codon, and a NotI site was introduced at the 3’ end after the stop codon. Mouse T1R2 and T1R3 were cloned as reported (1, 4). Go6 chimeras were generated by polymerase chain reaction (PCR) with primers and cloned into pcDNA3. The five-residue carboxyl-terminal tail of Go6 was replaced by its counterpart from Gougust(DCGLF) or Go3(ECGLY).

Construction of human/mouse chimeras of T1Rs was performed by PCR using overlapping primers (19). The integrity of all DNA constructs was confirmed by automated DNA sequencing. Point mutations in genes were made using the same overlapping PCR strategy.

To construct prLuc-hT1R2 and pGFP-hT1R3, hT1R2 and hT1R3 were amplified by PCR using primers that removed the stop codons and introduced a novel NruI restriction site. The restriction fragments containing the hT1R2 or hT1R3 coding portions were inserted into the GFP- or Rluc-BRET plasmids between the EcoRI and EcoRV sites. This placed either GFP or Rluc at the COOH terminus of the designated mutant hT1R3 sequence.

**Functional Expression—**HEK293 EBNA (HEK293E) cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For calcium imaging experiments, cells were seeded onto 6-well plates, and plasmid DNAs were transiently transfected at 0.1 μg of plasmid, Go6-16-i3 or Go3-16-i3. After 24 h, transfected HEK293E cells were washed with DPBS and then fixed for 5 min with 4% paraformaldehyde. After three washes, the fixed cells were blocked in 2% horse serum for 20 min and then incubated with anti-T1R3 polyclonal antibody (see below) for 30 min. Alexa fluoro-488-conjugated anti-rabbit secondary antibodies were used to visualize the staining.

Polyclonal antibodies were made against epitopes within the extracellular domain of mT1R3 (HEGLLVFQHDTSGQQLKG) and hT1R3 (EEAGLRSRTRPSSP) and affinity purified using peptide columns (12). No immunostaining was seen in mock transfected cells (supplemental Fig. 2). Using these antibodies we observed specific staining of mouse (12) and human taste receptor cells (data not shown). Images were taken using an Olympus Fluoview confocal laser scanning microscope.

**Bioluminescence Resonance Energy Transfer Analysis of Receptor Heteromerization—**HEK293E cells in 12-well plates were transiently transfected using Mirus TransIT-293 with pairs of Rluc- and GFP fusion constructs (e.g. 0.3 μg of DNA of each pRluc-hT1R2 and pGFP-hT1R3). After 24 h, transfected cells were rinsed with 1× phosphate-buffered saline and resuspended (by titration) into 300 μl of phosphate-buffered saline. 90 μl of cells (~50,000) were transferred into triplicate wells of a 96-well microplate (white Optiwell plates from Packard). To start the assay, 10 μl of Deep Blue Coelenterazine was added to each well to a final concentration of 5 μM. Bioluminescence resonance energy transfer (BRET) readings were performed using a FUSION plate reader (Packard). The BRET ratio was defined as ([emission at 500–530] − [emission at 370–450]) × CF/(emission at 370–450), where CF corresponds to emission at 500–530/370–450, with receptors-Rluc construct expressed alone in the same experiment.

**RESULTS**

**Activation of Human Sweet Receptors by Brazzein—**Heterologously expressed hT1R2 + hT1R3 has been shown to respond to many sweeteners, including monellin and thiamin (1, 2). To determine whether hT1R2 + hT1R3 responds as well to brazzein, we expressed these receptors together and singly in HEK293E cells along with a chimeric G-protein, Go16-i3. Consistent with earlier characterizations of the sweet receptor, HEK293E cells expressing hT1R2 + hT1R3 responded to all tested sweet compounds, including D-tryptophan, N-saccharin, sucrose, aspartame, and monellin (Fig. 1A). As expected, HEK293E cells expressing hT1R2 + hT1R3 responded to brazzein with a strong calcium response (Fig. 1, A and B).

**Responses to Brazzein Require hT1R3—**To determine whether responsiveness to brazzein depends on hT1R2, we compared the responses to brazzein, as well as five other sweeteners, in cells transfected with same species pairs of T1R2 + T1R3 (i.e. hT1R2 + hT1R3, mT1R2 + mT1R3) or species-mismatched pairs (i.e. hT1R2 + mT1R3, mT1R2 + hT1R3) (Fig. 1A). As expected, the mT1R2 + mT1R3 receptor did not respond to brazzein or the other human-specific sweet ligands (aspartame and monellin). Although monellin and aspartame activated the hT1R2 + mT1R3 receptor, brazzein did not. This indicates that hT1R3 is required for brazzein-induced activity. Cells transfected with hT1R2 + mT1R3 displayed robust responses to the small molecule sweeteners but a reduced response to monellin, suggesting that hT1R3 also contributes to responsiveness of the receptor to monellin. The response of hT1R2-containing receptors to the small molecule sweeteners was either unaffected or somewhat enhanced by the presence of mT1R3 in place of hT1R3 (Fig. 1A). We saw no response to any of the tested sweeteners in the mT1R2 + hT1R3 pairing. Although mT1R2 + hT1R3 heterodimers formed (data not
shown) and this receptor was located at the membrane (Fig. 1C), the resulting receptor was not stimulated by any of the ligands tested. Lack of activity could result from an inability to bind ligand or from an inability to be stabilized by ligand in the active conformation.

**Human Residues in the ATD of T1R2 Are Required for Responses to Monellin**—To determine whether the extracellular or transmembrane/intracellular portions of hT1R2 were required for the response of the sweet receptor to monellin, we made a human/mouse chimera joining the extracellular domain of hT1R2 (residues 1–564) to the mouse transmembrane/intracellular domain of mT1R2 (mouse residues 569–843, corresponding to human 565–839). Chimera designations follow a scheme where the residues from hT1R2 or mT1R3 are specified in the name of the construct and the end residues come from mT1R2.) The human/mouse chimera (h.1–564.mT1R2) was coexpressed with mT1R3 and G-protein and tested for responses to the panel of sweeteners (Fig. 1D). The response profile of mT1R3 + T1R2 chimera was indistinguishable from that of mT1R3 + fully human hT1R2, indicating that the human-specific residues of hT1R2 required for the sweet receptor to respond to monellin all reside in the extracellular portion.

**Human Residues in the Cysteine-rich Region of T1R3 Are Required for Responses to Brazzein**—That hT1R2 + mT1R3 did not respond to brazzein suggests that human-specific residues in hT1R3 are required for this response. This contrasts with the other human-specific sweeteners that activated hT1R2 + mT1R3 (1, 2, 10). We reasoned that the human-specific residues in T1R3 required for responsiveness to brazzein would reside in the ATD. To map the portion of hT1R3 essential for brazzein-induced activity, we generated several human/mouse chimeras encompassing the entire extracellular domain containing the presumptive ligand-binding domain and the cysteine-rich region (Fig. 2, A and B). The T1R3 chimeras, in combination with hT1R2, were tested for responses to brazzein and the panel of sweeteners. Only two chimeras, h.1–567.mT1R3 and h.1–545.mT1R3, responded to brazzein (Fig. 2B). h.1–567.mT1R3 displayed enhanced responses to brazzein, monellin, and aspartame, whereas h.1–545.mT1R3 had diminished responses to all sweeteners. The other constructs with less extensive 5’ hT1R3 sequences (e.g. h.1–535.mT1R3) did not respond to brazzein but did respond to all other sweeteners in the panel and in some cases showed large increases in responsiveness to the small molecule sweeteners. These results indicate that human-specific residues of hT1R3 between 536 and 545 are required for responses to brazzein.

To determine whether hT1R3 residues upstream of amino acid 536 are required for responses to brazzein we constructed a series of chimeras where the amino-terminal residues of hT1R3 were replaced by the corresponding residues from...
Brazzein sensitivity of the sweet taste receptor is determined by the cysteine-rich domain of human T1R3. A, schematic diagram showing the ATD, cysteine-rich region (C in oval), and transmembrane domain (rectangles 1–7) of human (black) and mouse (gray) T1R3. Human/mouse and mouse/human chimeras of T1R3 are indicated by color-coded segments. By our naming convention h.1–150.mT1R3 comprises residues 1–150 from hT1R3 and the remainder from mT1R3; mT1R3.h.568–852 contains residues 568–852 from hT1R3 and the remainder from mT1R3 etc. B, HEK293E cells transiently transfected with the indicated human/mouse T1R3 chimera, hT1R2, and Goα16-i3 were assayed for sweetener responses. Only chimeras h.1–545.mT1R3 and h.1–567.mT1R3 responded to brazzein. Although the other chimeras (e.g. h.1–150.mT1R3) did not respond to brazzein, they did retain responsiveness to the other sweeteners. Experimental details were as in Fig. 1A. C, HEK293E cells transiently transfected with the indicated human/mouse T1R3 chimera, hT1R2, and Goα16-i3 were assayed for sweetener responses. Chimeras mT1R3.h.477–852, mT1R3.h.525–852, and mT1R3.h.536–852 all responded to brazzein, whereas chimeras mT1R3.h.548–552 and mT1R3.h.568–852 did not respond to brazzein, although they did retain responsiveness to the other sweeteners. Experimental details were as in Fig. 1A. D, HEK293E cells transiently transfected with “humanized” mT1R3, hT1R2, and Goα16-i3 were assayed for sweetener responses. The humanized form of mT1R3 (mT1R3(h536–545)) contains human residues 536–545 in place of the corresponding mouse residues. mT1R3(h536–545) + hT1R2 was similar to native hT1R3 + hT1R2 in its response to brazzein and the other sweeteners. Experimental details were as in Fig. 1A.
mT1R3. (Fig. 2, A and C). Chimeras that retained 3' hT1R3 residues from 536 onward (e.g. mT1R3.h.536–852) responded to brazzein. Chimeras with less extensive 3' ends from hT1R3 (e.g. mT1R3.h.546–852) did not respond to brazzein. These results confirm that hT1R3 residues between 536 and 545 are required for responses to brazzein. To confirm directly that only this restricted portion of T1R3 need be of human origin to elicit a response to brazzein, we replaced the corresponding mT1R3 residues in this region with human residues 536–545 and then tested the activity of the resulting “humanized” construct (named mT1R3(h536–545)) in the presence of hT1R2.

The response of the mT1R3(h536–545) construct to brazzein and the other sweeteners in our panel was similar to that of hT1R3 (Fig. 2D), confirming the importance of this region in determining responsiveness to brazzein.

Ala-537 and Phe-540 Are Important for hT1R3 Responses to Brazzein—Within region 536–545 of hT1R3 there are five amino acids that differ from mT1R3 (Fig. 3A). We mutated singly each of these five amino acids of hT1R3 to their mouse counterpart and then tested for “loss of function” (Fig. 3B). Significantly diminished responses to brazzein were seen with the A537T and F540P mutations. hT1R3(A537T) lost all responsiveness to brazzein but retained responses to the small molecule sweeteners and to monellin. hT1R3(F540P) had greatly diminished responses to brazzein, normal responses to monellin, and greatly elevated responses to the small molecule sweeteners (Fig. 3B). The selective and severe loss of responsiveness to brazzein by hT1R3(A537T) demonstrates that Ala-537 is an essential determinant of the response of hT1R3 to brazzein.

The hT1R3(G542N) mutation had no effect on T1R3 responses to brazzein or other sweeteners (Fig. 3B). The hT1R3(E545Q) mutant had decreased responsiveness to brazzein, and similarly decreased responses to the other sweeteners (Fig. 3B) that may be due to reduced efficacy of the receptor (surface expression and BRET activity for E545Q was normal (supplemental Figs. 1A and 2)). The responses of hT1R3(I536F) to all sweeteners were reduced in comparison with those of wild-type hT1R3, with a relative increase in responses to monellin over brazzein and to the protein sweeteners over the small molecule sweeteners (Fig. 3B). Thus, it appears that only A537T and F540P differentiate hT1R3 from mT1R3 in determining responsiveness to brazzein.

To determine whether substitution of any of these amino acids conferred brazzein responsiveness on mT1R3 we mutated singly each of these five amino acids of mT1R3 (Fig. 3A). Only the T542A mutation conferred brazzein responsiveness upon mT1R3, confirming the importance of Ala-537 for the responses of hT1R3 to brazzein. The other mouse to human mutations in this segment did not rescue brazzein activity but did have effects on the relative responses to other sweeteners in the test panel, implicating this region in determining, at least in part, responses to monellin and the small molecule sweeteners (see “Discussion”).

T542A and P545F Confer Brazzein Responsiveness on mT1R3—In comparison with hT1R3, the mT1R3(T542A) mu-
tant displayed reduced responsiveness to brazzein, suggesting that other residues might also contribute to the ability of hT1R3 to respond to brazzein. (Fig. 4A). Double and triple mutations were made by replacing mouse residues in mT1R3 with their corresponding human residues. The mutants tested were mT1R3(AF) (substituted by T542A and P545F), mT1R3(IA)...

**Fig. 4. Humanized mT1R3 responds to brazzein.** A, double and triple mouse-to-human substitutions within the cysteine-rich region of mT1R3 (top line) were made. The human residues are indicated in **bold black type**. Mutants are designated by their mouse-to-human substitutions (e.g. mT1R3(IF) has F541I and P545F substitutions). B, HEK293E cells transiently transfected with humanized versions of mT1R3, hT1R2, and Gα16-i3 were assayed for sweetener responses. Only mutants containing the T542A substitution (mT1R3(AF), mT1R3(IA), mT1R3(IAF), and mT1R3(h536–545)) responded to brazzein. Mutants that also contained the P545F substitution along with the T542A substitution showed a greater response to brazzein (e.g. mT1R3(IA) versus mT1R3(IAF)). C, dose-response relationships of selected T1R3 mutants toward brazzein. HEK293E cells transiently transfected with mT1R3 mutants, hT1R2, and Gα16-i3 were assayed for responses to a series of brazzein concentrations. Data were fitted with sigmoid dose-response curves using Graphpad Prism software. EC_{50} values for the mutants are: hT1R2 + mT1R3(AF), 6.10 \times 10^{-5}; hT1R2 + mT1R3(IAF), 1.20 \times 10^{-5}; hT1R2 + mT1R3(h536–545), 2.12 \times 10^{-5}; hT1R2 + mT1R3(T542A), 1.11 \times 10^{-3}; hT1R2 + hT1R3, 3.66 \times 10^{-5}. Note that EC_{50}s of mT1R3(AF) and wild-type hT1R3 toward brazzein are comparable. Values represent the mean ± S.E. of two independent experiments. D, dose-response relationships shown by selected T1R3 mutants toward monellin. HEK293E cells transiently transfected with mT1R3 mutants, hT1R2, and Gα16-i3 were assayed for responses to a series of monellin concentrations. Data were fitted with sigmoid dose-response curves using Graphpad Prism software. EC_{50} values for the mutants are: hT1R2 + hT1R3 1.13 \times 10^{-5}; hT1R2 + mT1R3(IAFGE), 2.05 \times 10^{-5}; hT1R2 + mT1R3(AF), 4.06 \times 10^{-6}; hT1R2 + mT1R3(P545F), 9.39 \times 10^{-6}; and hT1R2 + mT1R3 6.90 \times 10^{-5}. Note that EC_{50} values of mT1R3(P545F) and hT1R3 toward monellin are comparable. Values represent the mean ± S.E. of three independent experiments.
mT1R3(IF) (substituted by F541I and P545F), and mT1R3(IAF) (substituted by F541I, T542A, and P545F) (Fig. 4, A and B). As expected, only those mutants containing the T542A substitution responded to brazzein, but those that also contained the P545F substitution showed a greater response than did mT1R3(IA) or mT1R3(T542A) (Fig. 4, B and C).

To more fully characterize the effects of humanizing mT1R3 we determined brazzein dose-response curves for several mT1R3 mutants (paired with hT1R2) (Fig. 4C). mT1R3(AF) was nearly identical to hT1R3 in its response to brazzein (EC_{50} = 6.10 \times 10^{-5} for mT1R3(AF) and EC_{50} = 8.66 \times 10^{-5} for hT1R3). In comparison with hT1R3, mT1R3(IAF) had increased apparent affinity for brazzein, as measured by receptor activity (EC_{50} = 1.20 \times 10^{-5}) but an equivalent maximal response. mT1R3(IAFGE) had increased apparent affinity (EC_{50} = 2.12 \times 10^{-5}) and a greater maximal response indicative of increased efficacy of the receptor. mT1R3(T542A) had greatly reduced apparent affinity for brazzein (EC_{50} \approx 2.05 \times 10^{-5}) was slightly less sensitive than hT1R3.

Ala-537 and Phe-540 Substitutions Affect T1R3 Responses to Many Sweeteners—To investigate the physicochemical effects of residues Ala-537 and Phe-540 on receptor activity toward brazzein, monellin, and small molecule sweeteners, we made various substitutions at these two positions (Fig. 5, A and C). As with the A537T mutant, substitution of Ala-537 by serine, glutamine, or valine abolished responses to brazzein without affecting the responses to monellin or the other sweeteners (Fig. 5B). hT1R3(A537G) had a markedly diminished response to brazzein and diminished responses to monellin and the other sweeteners (Fig. 5B). hT1R3(A537P) lost responsiveness to all of the sweeteners in our test panel. The surface expression of these mutants was comparable with that of hT1R3, and the BRET activities of hT1R2 mutants were indistinguishable from those of hT1R2/hT1R3 (supplemental Figs. 1B and 2).

Ala-537 and Phe-540 Substitutions Affect T1R3 Responses to Many Sweeteners. A, HEK293E cells transiently transfected with hT1R3 substitution mutants, hT1R2, and G16-i3 were assayed for sweetener responses. Of the mutants tested only hT1R3(A537G) responded to brazzein; this mutant showed reduced responses to all sweeteners tested. hT1R3(A537P) lost responses to all sweeteners. B, HEK293E cells transiently transfected with hT1R3 substitution mutants, hT1R2, and G16-i3 were assayed for sweetener responses. All Phe-540 substitution mutants of hT1R3 retained responsiveness to the sweeteners in our test panel, although their response profiles were altered versus wild-type hT1R3. C, summary of effects of substitutions of hT1R3 residues Ala-537 and Phe-540 on responses to sweeteners. A537G reduced responses to all sweeteners. A537P abolished responses to all sweeteners. Substitution of Ala-537 by Gln, Ser, or Val led to selective loss of responses to brazzein. Substitution of Phe-540 by Ala, His, Leu, or Tyr altered responses to only some sweeteners (see B).

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Although mT1R3 paired with hT1R2 responds to monellin (EC_{50} of 6.90 \times 10^{-5}), it is with much lower apparent affinity than that shown by hT1R3 + hT1R2 (EC_{50} of 1.13 \times 10^{-5}) (Fig. 4D). Responses to monellin were also altered by mutations in this region. The dose-response curves (Fig. 4D) showed that several of these mutants left-shifted the responses to monellin to varying degrees; mT1R3(P545F) (EC_{50} of 9.39 \times 10^{-5}) was comparable with hT1R3, mT1R3(AF) (EC_{50} of 4.06 \times 10^{-5}) was more sensitive than hT1R3, and mT1R3(IAFGE) (EC_{50} of 2.05 \times 10^{-5}) was slightly less sensitive than hT1R3.

Substitution of hT1R3 Phe-540 with alanine, histidine, leucine, or tryptophan retained responses to all sweeteners but altered responses to some sweeteners depending on the particular substitution (Fig. 5, B and C). As with the A537T mutant, substitution of Ala-537 by serine, glutamine, or valine abolished responses to brazzein without affecting the responses to monellin or the other sweeteners (Fig. 5B). hT1R3(A537G) had a markedly diminished response to brazzein and diminished responses to monellin and the other sweeteners (Fig. 5B). hT1R3(A537P) lost responsiveness to all of the sweeteners in our test panel. The surface expression of these mutants was comparable with that of hT1R3, and the BRET activities of hT1R2 + mutants were indistinguishable from those of hT1R2 + hT1R3 (supplemental Figs. 1B and 2).
higher than those of wild-type hT1R3. A similar BRET activity was observed for hT1R2 + mutants or + hT1R3 (supplemental Fig. 1c).

**DISCUSSION**

**The Cysteine-rich Region of T1R3 Determines Sweet Receptor Responsiveness to Brazzein**—That hT1R2 + hT1R3 responds to brazzein but hT1R2 + mT1R3 does not demonstrates that brazzein requires the human form of T1R3. This was a surprising result in that other human-specific sweeteners only required T1R2 to be from human (1, 2, 10). Using human/mouse chimeras of T1R3 paired with hT1R2, we determined that T1R3 residues 536–545 within the cysteine-rich region were required for responsiveness to brazzein. Ala-537 and Phe-540 within this region of hT1R3 were shown to be critical for responsiveness to brazzein. hT1R2 + humanized mT1R3 with human substitutions at these two positions fully supported the human-like response to brazzein.

**Effects of Substitutions at Ala-537 and Phe-540 of hT1R3 on Receptor Responses**—Substitution of hT1R3 Ala-537 with threonine, serine, glutamine, or valine abolished responses to brazzein. Only the A537G mutant retained partial activity toward brazzein; however, this mutation also suppressed responses to the other sweeteners tested, suggesting that sweetener signal transmission in general may be altered in this mutant. The inability of valine, a small hydrophobic residue, to substitute for A537, and the partial activity seen with A537G suggest that it is the small size of the side chain of alanine that is important at this position. Presumably, steric hindrance by the larger side chains of threonine, serine, glutamine, or valine led to the lack of responses shown by these mutants. Because the majority of Ala-537 substitutions selectively affected responses to brazzein, it seems likely that this is steric interference acting on the binding site of brazzein, either directly or indirectly.

The A537P substitution of hT1R3 resulted in a receptor that was unresponsive to all sweeteners tested. Ala-537 is in a segment predicted to have a $\beta$ strand conformation (20). The neighboring C538 residue is predicted to be buried and may be in a disulfide bond with another conserved cysteine in this region (20). A change in backbone flexibility (either more flexible (A537G) or less flexible and kinked (A537P)) might alter the formation of the predicted $\beta$ strand and thereby alter the conformation of this region in a way that makes it less able to transmit the signal through the receptor. This suggests that the cysteine-rich region may couple ligand binding effects in the ATD to receptor output in the transmembrane/cytoplasmic domain to modulate the strength of coupling. It is particularly surprising to find such an effect for the cysteine-rich region of T1R3 in light of T1R2 appearing to be the ligand-binding monomer for most ligands (1, 2, 10). Thus, mutations at Ala-537 (and at F540, see below) may exert two separate effects: 1) altered access to the brazzein binding site and 2) altered signal transmission for all sweeteners in our test panel.

Specific mutations at position Phe-540 reduced the response to brazzein (F540Y, F540P), to monellin (F540L), or to brazzein and monellin (F540A and F540H). In addition, hT1R2 (F540P) and hT1R2 (F540P) also enhanced responses to the small molecule sweeteners. Substitution of hT1R3 by proline, native to mT1R3 at this position, makes the response profile of hT1R2 + hT1R3 (F540P) nearly identical to that of hT1R2 + mT1R3, indicating that this substitution plays a key role in differentiating responses of the wild-type human receptor (hT1R2 + hT1R3) from those of the mixed species receptor (hT1R2 + mT1R3) (see Fig. 1B). Brazzein-induced activity of T1R3 benefits from large nonpolar side groups at position 540, although there is not a strict requirement for a hydrophobic residue here, because tyrosine at this position supported near normal activity. Monellin-induced activity was best supported by Phe-540 itself or F540Y, suggesting that an aromatic residue is important for this response.

The selective alteration of responses to brazzein by some T1R3 mutants at Ala-537 and Phe-540 argues that at least some of the essential interactions of brazzein with the receptor occur at a site distinct from the small molecule binding site, i.e. the brazzein and small molecule binding site (presumed to be in the cleft of T1R2) may not overlap in part or in entirety.

**The Effect of T1R2 on Brazzein-induced Activity**—It is tempting to assume that brazzein, unlike the other human-specific sweet ligands, interacts primarily with T1R3 because it is the species of T1R3 monomer that determines responsiveness to brazzein. In preliminary studies, we have determined that hT1R2 is better able than mT1R2, when paired with the humanized mT1R3, to support brazzein activity, suggesting that there are human T1R2 residues that interact favorably with brazzein (data not shown). This suggests that brazzein may interact with both T1R2 and T1R3. We and others have modeled the structure of brazzein docked to hT1R3 (21) or hT1R2 (data not shown); such models may prove useful for generating testable hypotheses regarding the structure of receptor-sweetener complexes.

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