Identification of the Cyclamate Interaction Site within the Transmembrane Domain of the Human Sweet Taste Receptor Subunit T1R3*

Peihua Jiang†, Meng Cui§, Baohua Zhao‡, Lenore A. Snyder§, Lumie M. J. Benard‡¶, Roman Osman‡, Marianna Max*, and Robert F. Margolskee*†‡¶§

From the Departments of *Neuroscience and §Physiology and Biophysics and the ‡Howard Hughes Medical Institute, Mount Sinai School of Medicine, New York, New York 10029

The artificial sweetener cyclamate tastes sweet to humans, but not to mice. When expressed in vitro, the human sweet receptor (a heterodimer of two taste receptor subunits: hT1R2 + hT1R3) responds to cyclamate, but the mouse receptor (mT1R2 + mT1R3) does not. Using mixed-species pairings of human and mouse sweet receptor subunits, we determined that responsiveness to cyclamate requires the human form of T1R3. Using chimeras, we determined that it is the transmembrane domain of hT1R3 that is required for the sweet receptor to respond to cyclamate. Using directed mutagenesis, we identified several amino acid residues within the transmembrane domain of T1R3 that determine differential responsiveness to cyclamate of the human versus mouse sweet receptors. Alanine-scanning mutagenesis of residues predicted to line a transmembrane domain binding pocket in hT1R3 identified six residues specifically involved in responsiveness to cyclamate. Using molecular modeling, we docked cyclamate within the transmembrane domain of T1R3. Our model predicts substantial overlap in the hT1R3 binding pockets for the agonist cyclamate and the inverse agonist lactisole. The transmembrane domain of T1R3 is likely to play a critical role in the interconversion of the sweet receptor from the ground state to the active state.

Taste is a primal sense that is essential for humans and other organisms to detect the nutritive quality of a potential food source while avoiding environmental toxins (1–3). Taste perception can be categorized into five distinct qualities: sweet, bitter, salty, sour, and umami (amino acid taste) (1–3). Sweet, bitter, and umami tastes are mediated in large part by G-protein-coupled receptors (GPCRs)2 and their linked signaling pathways. Sour and salty tastes are thought to be mediated by specialized ion channels (1–3). Sweet, bitter, and umami tastes are mediated in large part by G-protein-coupled receptors (GPCRs)2 and their linked signaling pathways. Sour and salty tastes are thought to be mediated by specialized ion channels (1–3).

How does the sweet receptor detect and respond to so many chemically diverse compounds? We have shown previously that the cysteine-rich domain of human (h) T1R3 is essential for sweet receptor responses to sweet proteins, suggesting that these proteins bind here on the receptor (14). It has also been shown that responses to aspartame depend on the canonical VFTM binding site within T1R2 and that lactisole (an inverse agonist) interacts with the TMD of hT1R3 (15, 16, 24). We show here that cyclamate also interacts with the TMD of hT1R3 within a potential binding pocket that overlaps with the proposed binding site of lactisole. Thus, there are at least three broadly defined potential binding domains on the heterodimeric sweet receptor, all of which appear capable of mediating its activation.

In this study, we show that activation of the sweet receptor by cyclamate requires the human form of T1R3. Using human/mouse chimeric receptors, we have determined that it is the TMD of hT1R3 that specifies responsiveness to cyclamate. From additional chimeras and mutants, we have identified several residues within the TMD of hT1R3 that account in large part for the species-specific response to cyclamate. From molecular models of the predicted binding pocket within the TMD and systematic alanine-scanning mutagenesis, we have identified additional residues involved in sweet receptor responses to cyclamate. Interestingly, certain of these mutations altered responsiveness to both...
cyclamate (agonist) and lactisole (inverse agonist). Our experimental results support our computationally derived molecular model of cyclamate docked into the TMD binding pocket of T1R3, suggesting that molecular models of the TMDs of T1R proteins and other family C receptors may be generally useful for probing active state conformations of these receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cyclamate and D-tryptophan were obtained from Sigma and, unless otherwise noted, used at a concentration of 10 mM in the cell-based assays.

**Chimeras and Mutants**—Most of the T1R3 chimeras and mutants used here have been reported previously (16). Site-directed mutagenesis or overlapping PCR was used to generate additional mutants of hT1R3. All mutants were confirmed by sequence analysis. Expression of wild-type hT1R3 and the alanine substitution mutants was examined by immunofluorescence confocal microscopy using an antibody generated against the N-terminal region of hT1R3 (6, 14). All mutants examined reached the cell surface; no significant differences in cell-surface expression were observed between these mutants and wild-type hT1R3 (supplemental Fig. 1).

**Cell Culture and Calcium Imaging**—Human embryonic kidney 293E (HEK293E) cells were cultured, maintained, and transfected as described previously (16). All transfections were done in triplicate, and all experiments were repeated at least twice. Cells were seeded in 6-well plates at a density of 8 × 10^6 cells/well. After 24 h, the cells were transiently transfected with plasmids encoding T1R proteins and Goargin-gust44 (a chimeric G protein and subunit containing the last 44 amino acids of gustducin). After an additional 24 h, the cells were trypsinized and resuspended onto polylysine-coated 96-well assay plates (Corning Inc.) at a density of 40,000 cells/plate in low glucose medium supplemented with 10% dialyzed fetal bovine serum (Invitrogen) and 1× GlutaMAX-I (Invitrogen). After another 24 h, the cells were washed once with Dulbecco’s phosphate-buffered saline and then loaded with 75 μl of 3 μM Fluo-4 (calcium-sensing dye; Molecular Probes, Inc.) in Dulbecco’s phosphate-buffered saline for 2 h. The cells were washed twice with Dulbecco’s phosphate-buffered saline and assayed using a FlexStation II (Molecular Devices Corp.). Fluorescence changes (excitation at 488 nm, emission at 525 nm, and cutoff at 515 nm) were monitored after addition of Dulbecco’s phosphate-buffered saline supplemented with 2× tutsants. For each response trace, the data were acquired at 2-s intervals; samples were added at 30 s; and scanning was continued for an additional 150 s.

**Data Analysis**—Calcium mobilization was quantified as the change in peak fluorescence (∆F) over the base-line level (F). As described previously (16), data are expressed as the mean ± S.E. of the ∆F/F value from three independent experiments. The analysis was done automatically using an in-house written SAS program. The data are presented as ∆F/F of three independent measurements. Curve-fitting and generation of bar graphs was done with the GraphPad Prism 3 program.

**Homology Modeling and Molecular Docking**—Homology modeling of hT1R3 based on sequence alignment to rhodopsin was as described (16). To dock cyclamate into the T1R3 TMD, the geometry of cyclamate (cyclohexyl sulfamate) was fully optimized by the ab initio quantum chemistry method at the HF/6-31G* level, followed by a single point calculation with the polarized continuum model solution method to obtain the electrostatic potentials using the Gaussian 98 package (25). The CHELPG charge-fitting scheme (26) was then used to calculate partial charges for cyclamate. The missing force field parameters for cyclamate were obtained from similar parameters taken from charm27 or QUANTA (Accelrys Software Inc., San Diego, CA).

Cyclamate was docked into the pocket of the TMD of hT1R3 using the automatic docking program AutoDock Version 3.0.5 (27). This program uses a powerful Lamarckian genetic algorithm method for conformational sampling and docking. The docked conformations of cyclamate were analyzed by the cluster analysis of AutoDock. The final docked conformation was selected from the total conformations based upon compatibility with results from mutagenesis, followed by some manual adjustments of the positions of cyclamate and the side chains of the hT1R3 TMD before employing model refinement by molecular dynamics simulations. The same protocol of molecular dynamics simulations was used as described previously (16). The α-carbon atoms of the helices were restricted by 1.0 kcal/mol/Å^2 harmonic restraint force.

**RESULTS**

The Sweet Taste of Cyclamate Requires the TMD of hT1R3—Although cyclamate tastes sweet to humans, mice do not detect it as sweet or show preference for it (28, 29). To determine whether this differential responsiveness to cyclamate’s sweetness is mediated by the human versus mouse forms of the sweet receptor, we expressed the human and mouse sweet receptors by transient transfection in HEK293E cells along with Goargin-gust44 and then monitored activation (calcium mobilization) by indicator dye using D-tryptophan as a positive control (Fig. 1). As expected, the human receptor (hT1R2 + hT1R3) responded to cyclamate, whereas the mouse (m) receptor (mT1R2 + mT1R3) did not; both receptors responded to the D-tryptophan control. To determine whether one or both human T1R subunits are required for responses to cyclamate, we expressed mismatched pairs of human and mouse T1R2 and T1R3 subunits and then tested for responses to cyclamate. One mismatched pair (hT1R2 + mT1R3) did not respond to cyclamate, but did respond to D-tryptophan (Fig. 1), indicating that hT1R3 is required for receptor sensitivity to cyclamate. The other mismatched pair (mT1R2 + hT1R3) is nonfunctional in this assay (14, 16) and did not respond to cyclamate or D-tryptophan (Fig. 1) or to several other sweet compounds (14, 16), precluding us from determining in this way whether hT1R2 is also required for sweet receptor sensitivity to cyclamate. Using human/mouse chimeras, we show below that hT1R2 is not required for receptor sensitivity to cyclamate (Fig. 2C).
The Cyclamate Interaction Site in Human T1R3 Transmembrane Domain

To identify the portion of hT1R3 required for sensitivity to cyclamate, we tested the responses to cyclamate of heterodimeric receptors composed of hT1R2 plus human/mouse chimeras of T1R3 in which varying portions of hT1R3 were substituted with the complementary portions of mT1R3 (Fig. 2A). As described above, receptor responses to D-tryptophan were used as a positive control for receptor activity. Heterodimers of hT1R2 plus T1R3 chimeras containing most or all of the extracellular region of hT1R3 coupled to the TMD and C-terminal tail of mT1R3 (i.e. h.1–547.mT1R3 and h.1–567.mT1R3) responded to D-tryptophan, but not to cyclamate. In contrast, heterodimers of hT1R2 plus T1R3 chimeras containing most or all of the extracellular domain of mT1R3 coupled to the TMD and C-terminal tail of hT1R3 (i.e. mT1R3.h.548–852 and mT1R3.h.568–852) responded to both D-tryptophan and cyclamate (Fig. 2B). These results indicate that sweet receptor responses to cyclamate require the TMD and/or C-terminal tail of hT1R3.

To determine whether the human form of T1R2 is required for sweet receptor responsiveness to cyclamate, we examined the responses of the heterodimeric receptor formed by mT1R2 and mT1R3.h.568–852 (Fig. 2C). Unlike the nonfunctional pairing of mT1R2 and hT1R3 (Fig. 1), mT1R2 can function in combination with the mT1R3.h.568–852 chimera (16). The heterodimer of mT1R2 and mT1R3.h.568–852 responded to D-tryptophan and cyclamate (as well as to several other sweeteners (16)). Thus, hT1R2 is not required for responsiveness to cyclamate, indicating that the hT1R3 component of the heterodimeric receptor is necessary and sufficient for responsiveness to cyclamate. The combined results of Fig. 2 (B and C) demonstrate that only the TMD and C-terminal tail portion of T1R3 need to be from human for the sweet receptor to respond to cyclamate.

**Extracellular Loop 3 and Transmembrane (TM) Helix 7 of hT1R3 Are Required for Responsiveness to Cyclamate**—To identify the portion(s) of the TMD and/or C-terminal tail of hT1R3 required for responses to cyclamate, we examined several additional human/mouse chimeras of T1R3 in combination with hT1R2. This set of chimeras contain the VFTM of hT1R3 along with varying portions of the TMD and C-terminal tail of hT1R3 coupled to the complementary portion of mT1R3 (Fig. 3A). All heterodimers formed by coexpressing hT1R2 plus these chimeras responded to D-tryptophan (Fig. 3B). However, only one heterodimer responded to cyclamate (i.e. hT1R2/h.1–812.mT1R3): this chimera contains the VFTM and entire TMD of hT1R3, but with the C-terminal tail of mT1R3. Heterodimers of hT1R2 with T1R3 chimeras in which mouse sequences replaced any portion of the TMD failed to respond to cyclamate. Thus, one or more differences between the human and mouse sequences within the TMD affect responsiveness to cyclamate. That hT1R2/h.1–812.mT1R3 responded to cyclamate but hT1R2/h.1–787.mT1R3 did not indicates that responsiveness to cyclamate requires human-specific residues between amino acids 787 and 812 of hT1R3, corresponding to extracellular loop 3 and TM helix 7. As noted below, additional residues within the TMD also contribute to sweet receptor responsiveness to cyclamate.

**Substitutions of Phe-730<sup>5.43</sup> in TM Helix 5 and Arg-790<sup>ex3</sup> in Extracellular Loop 3 Selectively Affect hT1R3 Responses to Cyclamate**—We had previously observed the importance of TM helices 5 and 7 and extracellular loop 3 of hT1R3 in determining human-specific sensitivity of the sweet receptor to the inverse agonist lactisole (16). For our study of the interaction of lactisole with the sweet receptor, we had generated a series of hT1R3 mutants in which the human-specific residues in these regions had been replaced with their mouse counterparts (16). We
The Cyclamate Interaction Site in Human T1R3 Transmembrane Domain

To investigate the physicochemical effects of these two residues of hT1R3 on receptor activity, we made various substitutions at these two positions and then tested the activity toward cyclamate and D-tryptophan of hT1R2 with these T1R3 mutants (Fig. 4). All mutant-containing heterodimers responded to D-tryptophan. Of the four human-to-mouse replacement mutants in TM helix 5, only the F7305.43L mutant showed diminished responsiveness to cyclamate (Fig. 4A). Of the eight replacement mutants in extracellular loop 3 and TM helix 7, only R790ex3Q showed an altered response to cyclamate: the response to cyclamate was completely absent in this mutant (Fig. 4B). Cyclamate and D-tryptophan dose-response curves were obtained for F7305.43L and R790ex3Q (Fig. 4C). In comparison with wild-type hT1R3, the F7305.43L mutant showed a pronounced right shift in its cyclamate dose-response curve (F7305.43L mutant EC50 = 1.5 × 10^{-3} M versus wild-type EC50 = 3.1 × 10^{-5} M), but was relatively unchanged in its D-tryptophan dose-response curve (F7305.43L mutant EC50 = 5.5 mM versus wild-type EC50 = 3.3 mM) (Fig. 4C). The R790ex3Q mutant showed an even more marked deficit: this mutant showed a complete loss of responsiveness to cyclamate (R790ex3Q mutant EC50 >> 4 × 10^{-2} M versus wild-type EC50 = 3.1 × 10^{-5} M), but a more moderate change in its D-tryptophan dose-response curve (R790ex3Q mutant EC50 = 12 mM versus wild-type EC50 = 3.3 mM) (Fig. 4C). Thus, substitution of leucine at position 7305.43 or of glutamine at position 790ex3 selectively affects receptor responses to cyclamate.

To test the responses to cyclamate and D-tryptophan of heterodimers of hT1R2 with these T1R3 mutants (Fig. 4). All mutant-containing heterodimers responded to D-tryptophan. Of the four human-to-mouse replacement mutants in TM helix 5, only the F7305.43L mutant showed diminished responsiveness to cyclamate (Fig. 4A). Of the eight replacement mutants in extracellular loop 3 and TM helix 7, only R790ex3Q showed an altered response to cyclamate: the response to cyclamate was completely absent in this mutant (Fig. 4B). Cyclamate and D-tryptophan dose-response curves were obtained for F7305.43L and R790ex3Q (Fig. 4C). In comparison with wild-type hT1R3, the F7305.43L mutant showed a pronounced right shift in its cyclamate dose-response curve (F7305.43L mutant EC50 = 1.5 × 10^{-3} M versus wild-type EC50 = 3.1 × 10^{-5} M), but was relatively unchanged in its D-tryptophan dose-response curve (F7305.43L mutant EC50 = 5.5 mM versus wild-type EC50 = 3.3 mM) (Fig. 4C). The R790ex3Q mutant showed an even more marked deficit: this mutant showed a complete loss of responsiveness to cyclamate (R790ex3Q mutant EC50 >> 4 × 10^{-2} M versus wild-type EC50 = 3.1 × 10^{-5} M), but a more moderate change in its D-tryptophan dose-response curve (R790ex3Q mutant EC50 = 12 mM versus wild-type EC50 = 3.3 mM) (Fig. 4C). Thus, substitution of leucine at position 7305.43 or of glutamine at position 790ex3 selectively affects receptor responses to cyclamate.

To test the responses to cyclamate and D-tryptophan of heterodimers of hT1R2 with these T1R3 mutants (Fig. 4). All mutant-containing heterodimers responded to D-tryptophan. Of the four human-to-mouse replacement mutants in TM helix 5, only the F7305.43L mutant showed diminished responsiveness to cyclamate (Fig. 4A). Of the eight replacement mutants in extracellular loop 3 and TM helix 7, only R790ex3Q showed an altered response to cyclamate: the response to cyclamate was completely absent in this mutant (Fig. 4B). Cyclamate and D-tryptophan dose-response curves were obtained for F7305.43L and R790ex3Q (Fig. 4C). In comparison with wild-type hT1R3, the F7305.43L mutant showed a pronounced right shift in its cyclamate dose-response curve (F7305.43L mutant EC50 = 1.5 × 10^{-3} M versus wild-type EC50 = 3.1 × 10^{-5} M), but was relatively unchanged in its D-tryptophan dose-response curve (F7305.43L mutant EC50 = 5.5 mM versus wild-type EC50 = 3.3 mM) (Fig. 4C). The R790ex3Q mutant showed an even more marked deficit: this mutant showed a complete loss of responsiveness to cyclamate (R790ex3Q mutant EC50 >> 4 × 10^{-2} M versus wild-type EC50 = 3.1 × 10^{-5} M), but a more moderate change in its D-tryptophan dose-response curve (R790ex3Q mutant EC50 = 12 mM versus wild-type EC50 = 3.3 mM) (Fig. 4C). Thus, substitution of leucine at position 7305.43 or of glutamine at position 790ex3 selectively affects receptor responses to cyclamate.

To investigate the physicochemical effects of these two residues of hT1R3 on receptor activity, we made various substitutions at these two positions and then tested the activity toward cyclamate and D-tryptophan of hT1R2 plus the T1R3 mutants (Fig. 4, D and E). The receptor activity toward cyclamate of Phe-7305.43 substitution mutants was abolished (alanine, cysteine, glutamine, serine, and threonine), slightly reduced (tryptophan), or slightly enhanced (tyrosine) (Fig. 4D). Receptor activity toward cyclamate was abolished by all substitutions of Arg-790ex3 (alanine, glutamate, histidine, lysine, and tyrosine). All Phe-7305.43 and Arg-790ex3 substitution mutants (with one exception, R790ex3H) responded to D-tryptophan. The R790ex3H mutant was completely unresponsive to D-tryptophan or cyclamate (Fig. 4E) and to other sweeteners (data not shown), indicating that it may be nonfunctional.

A Humanizing Substitution in Extracellular Loop 3 Confers Cyclamate Responsiveness on a Human/Mouse Chimera of T1R3—Given our observations in loss-of-function experiments (Fig. 4, B and E) that glutamate at position 790ex3 of hT1R3 is critical for human receptor responsiveness to cyclamate, we investigated whether a mouse-to-human substitution of the mouse version of this residue (Gln-795ex3) would confer responsiveness to cyclamate on a human/mouse chimera of T1R3. We tested responses to cyclamate and D-tryptophan of heterodimers of hT1R2 with "wild-type" and "humanized" forms of a human/mouse chimera of T1R3 (h.1–751.mT1R3) that contained human residues for the entire extracellular domain and TM helices 1–5 along with mouse residues for TM helices 6 and 7, extracellular loop 3, and the C-terminal domain. The unsubstituted chimera (h.1–751.mT1R3) responded well to D-tryptophan, but not at all to cyclamate (Fig. 4F). The humanized chimera (h.1–751.mT1R3(Q795ex3R)) responded well to D-tryptophan and cyclamate, displaying responses comparable with those of wild-type hT1R3 (Fig. 4F), confirming the importance of this residue in determining human-specific responsiveness to cyclamate. Humanizing substitutions of mT1R3 (e.g. Q795ex3R and/or L735ex3F) did not lead to responsiveness to cyclamate (data not shown), indicating that additional human-specific residues within the TMD may contribute to the human-specific responsiveness to cyclamate.

hT1R3 Sensitivity to Cyclamate Also Involves Residues in TM Helices 3 and 6 and Extracellular Loop 2—We had previously used the solved structure of rhodopsin as a template to model the structure of the TMD of hT1R3 and as a guide to identify potential ligand-binding sites of...
FIGURE 4. Responsiveness to cyclamate requires two human-specific residues in extracellular loop 3 and TM helix 5 of hT1R3. A, upper panel, the alignment of human and mouse T1R3 sequences shows that, in TM helix 5, only four residues differ between these two species. Lower panel, receptors composed of hT1R2 and T1R3 mutants in which species-specific positions within TM helix 5 were substituted with complementary mouse residues were coexpressed by transient transfection in HEK293E cells along with G_{o,x}
gust44. Only the receptor containing hT1R3 (F7305.43L) showed a diminished response to cyclamate. Receptors containing any of the other hT1R3 substitutions (A7335.46V, A7355.48I, 

The Cyclamate Interaction Site in Human T1R3 Transmembrane Domain
The Cyclamate Interaction Site in Human T1R3 Transmembrane Domain

hT1R3 for lactisole (16). In our earlier study, we used the alignment of rhodopsin with hT1R3 to identify 17 residues lining a potential binding pocket for lactisole within the hT1R3 TMD. By mutating each of these 17 residues to alanine, we identified four positions within the probable lactisole-binding pocket. Here, we tested these same 17 alanine substitution mutants for their responses to cyclamate and D-tryptophan (Fig. 5A).

Receptors with alanine substitution at Leu-6443.36, Thr-6453.37, Tyr-7716.44, Gln-7947.32, or Ile-8054.43 showed no responses to cyclamate or D-tryptophan (Fig. 5A) or to other sweeteners (e.g. aspartame) (data not shown). Receptors with alanine substitution at His-7347.47 or Trp-7754.48 showed no responses to cyclamate and severely diminished responses to D-tryptophan (Fig. 5A) and to other sweeteners (data not shown). All seven of these mutants were expressed at the cell surface at levels comparable with those of wild-type hT1R3 (supplemental Fig. 1). These seven positions might be required for generating the active state of the receptor or for structural integrity of the receptor. Given the global effects of these mutations, it is not possible to determine from this study if these residues contribute to a binding pocket for cyclamate. The other alanine substitution mutants (Q6363.32A, S6403.32A, H6413.33A, H7212-6A, R723ex2–51A, S7296.40A, V7766.40A, F7776.45A, and L7826.55A) showed normal or slightly reduced responses to D-tryptophan (Fig. 5A) and other sweeteners (data not shown). The responses of these 10 mutants to cyclamate varied from near wild type (S6403.32A, S7296.40A, V7766.40A, and V7776.45A), to diminished (H6413.33A, H7212–6A, R723ex2–51A, and F7776.45A), to absent (Q6363.32A and L7826.55A) (Fig. 5A). All 10 of these mutants were expressed at the cell surface at levels comparable with those of wild-type hT1R3 (supplemental Fig. 1).

Cyclamate and D-tryptophan dose-response curves were obtained for those mutants with selective decreases in response to cyclamate (i.e. Q6363.32A, H6413.33A, H7212–6A, R723ex2–51A, F7776.45A, and L7826.55A) (Fig. 5B). The response of the R723ex2–51A mutant to cyclamate was moderately reduced (EC50 = 1.9 × 10⁻² M) versus wild-type EC50 = 3.1 × 10⁻³ M). The responses of the other five mutants to cyclamate were more severely diminished (EC50 = 3.4 × 10⁻³ to >6.1 × 10⁻¹ M). Thus, these six positions are likely components of the cyclamate-binding pocket.

Docking Cyclamate into the Molecular Model of the hT1R3 TMD—As noted above, we have developed a model of the TMD of hT1R3 based on the structure of rhodopsin (16). We have used this model with docked lactisole to define the potential binding pocket for this inverse agonist within the TMD of hT1R3 (16). Here, we docked the agonist cyclamate into this homology-based model of the TMD of hT1R3. The energy-minimized structure of the hT1R3 TMD with cyclamate docked within the TMD shows that the sulfamate group of cyclamate is oriented toward the top part of the pocket formed by TM helix 3 and the C-terminal portion of extracellular loop 2 (Fig. 6A). Gln-6373.29Gln-6373.29, His-6413.33Gln-6373.29, His-7316.42Gln-6373.29, and Arg-7236.41Gln-6373.29 are in close proximity to the cyclamate sulfamate moiety (Fig. 6A). This orientation would permit ionic or hydrogen bond interactions between the sulfamate moiety of cyclamate and protonated or hydrophilic side chains of these residues of hT1R3. In support of this model, alanine substitutions at any of these positions diminished or abolished responses to cyclamate (Figs. 5 and 7). Phe-7786.44 and Leu-7826.55 are in close enough proximity to cyclamate that their side chains may make hydrophobic interactions with the cyclohexyl ring of cyclamate (Fig. 6A). In support of this model, alanine substitutions at either of these positions diminished or abolished responses to cyclamate (see Fig. 5).

To gain insight into differences between agonist and antagonist binding to the sweet receptor, we overlaid the structures of cyclamate and lactisole docked within their predicted binding pockets in the hT1R3 TMD. According to our model, cyclamate and lactisole reside in distinct but partially overlapping binding pockets. Cyclamate sits slightly higher in the pocket and more toward TM helix 7 than does lactisole. The position of cyclamate provides closer proximity to residues in extracellular loop 2, enabling hydrogen bonding to occur between the cyclamate sulfamate group and His-7316.42Gln-6373.29. In contrast, lactisole extends deeper into the pocket and appears to have no interaction with residues in extracellular loop 2.

Our model indicates that the side chain of Gln-6373.29 is close enough (3 Å) to the sulfamate group of cyclamate for hydrogen bonding between the two (Fig. 6A). Based on our model, we predict that introducing a negatively charged residue at position 6373.29 would interfere with the ability of the receptor to bind cyclamate. We made such a substitution (Q6373.29E) and observed that the receptor with this mutation gave no response to cyclamate even though its response to D-tryptophan was unaffected (Fig. 7), in agreement with our prediction.

DISCUSSION

The Sweetness of Cyclamate Requires the Human Form of the T1R3 TMD—Sodium cyclamate, a sulfamate salt, stimulates the perception of sweet taste in humans, but not in rodents (28, 29). In this study, we set out to molecularly identify the site of action of cyclamate and to determine mechanistically how cyclamate activates the human perception of sweet. We replicated in vitro the differential responsiveness of mouse and man to cyclamate: the heterologously expressed human sweet receptor (hT1R2 + hT1R3) responded to cyclamate, whereas the mouse sweet receptor (mT1R2 + mT1R3) did not. We used this in vitro correlate of sweet taste detection to determine that the human form of...
T1R3 is required for cyclamate’s sweetness. T1R2 does not contribute species-specific determinants to the detection of cyclamate’s sweet taste. Using human/mouse chimeras of T1R3 in combination with hT1R2, we determined that it is the TMD of hT1R3 that is required for the sweet receptor to respond to cyclamate: the VFTM and cysteine-rich domain from either mouse or human T1R3 support responses to cyclamate equally well. From additional human/mouse chimeras of T1R3, we determined that TM helix 5 and extracellular loop 3 must be from hT1R3 to obtain human-type responses to cyclamate. Substitution mutants with human-to-mouse changes at

FIGURE 5. Alanine scanning of candidate cyclamate-binding pocket residues in the hT1R3 TMD. A, residues of hT1R3 identified by homology to rhodopsin as potential contributors to a TMD binding pocket for cyclamate were replaced with alanine. hT1R2 and these hT1R3 mutants were coexpressed by transient transfection in HEK293E cells along with G_{i/o}-Gust44. The receptor-expressing cells were then assayed for their responses to cyclamate and o-tryptophan. Five of these mutants (L6443.36A, T6453.37A, Y7713.44A, Q7946.49A, and I8057.43A) were unresponsive to o-tryptophan and cyclamate. Two mutants (H7345.47A and W7756.48A) were unresponsive to cyclamate and had severely diminished responses to o-tryptophan. These seven mutants may have general loss of function. Seven mutants (Q6363.28A, H6413.33A, H7211.49A, R7232.51A, S7295.42A, F7786.51A, and L7826.55A) that retained responsiveness to o-tryptophan had reduced or absent sensitivity to cyclamate. Three mutants (S6403.32A, V7766.52A, and V7796.55A) responded well to o-tryptophan and cyclamate. All transfections were done in triplicate; each experiment was repeated two to three times. B, shown are o-tryptophan (■) and cyclamate (▲) dose-response curves for receptors containing hT1R2 plus selected alanine-scanning mutants. One mutant (R7232.51A) displayed moderately reduced responsiveness to cyclamate (EC_{50} = 9.8 \times 10^{-7} M versus wild-type WT EC_{50} = 3.1 \times 10^{-3} M). Five mutants (Q6363.28A, H6413.33A, H7211.49A, R7232.51A, S7295.42A, F7786.51A, and L7826.55A) displayed severely diminished responsiveness to cyclamate (EC_{50} = 3.4 \times 10^{-2} M versus hT1R3 WT EC_{50} = 6.1 \times 10^{-3} M). All of these receptors responded well to o-tryptophan.
His-6413.33 of TM helix 3 and His-721ex2–49 of extracellular loop 2 to form salt bridges. The sulfamate is close enough to Gln-6363.28 and Gln-6373.29 to permit hydrogen bonds. The cyclamate cyclohexyl ring is close enough to Phe-7786.51 of TM helix 6 and Leu-7826.55 for hydrophobic interactions. Our model predicts that cyclamate binds to hT1R3 within a pocket formed by TM helices 3, 5, and 6 and extracellular loop 2. This model has value for both explanatory and predictive purposes. 1) It explains the results from the alannine-scanning and human-to-mouse mutations, describing potential contacts between critical residues of hT1R3 and pharmacophores of cyclamate. 2) It predicts additional residues of hT1R3 that compose contacts for cyclamate (e.g. Gln-6373.29). 3) It predicts pharmacophores of cyclamate required for sweetness.

Within TM helix 6, substitution of Phe-7786.51 with alanine reduced receptor sensitivity to cyclamate, whereas substitution of Leu-7826.55 with alanine abolished responses to cyclamate. Our model predicts that the side chains of these residues are in close enough proximity to the negatively charged sulfamate group of cyclamate to form hydrogen bonds (Gln-6363.28 and Gln-6373.29) or salt bridges (His-6413.33). In addition, our model suggests that salt bridges could form between the sulfamate group and the side chains of two residues in extracellular loop 2: His-721ex2–49 and Arg-723ex2–51. Consistent with this suggestion is the result that alanine substitution at either of these positions greatly diminished responses to cyclamate. Within TM helix 6, substitution of Phe-7786.51 (conserved among class C GPCRs) with alanine reduced receptor sensitivity to cyclamate, whereas substitution of Leu-7826.55 with alanine abolished responses to cyclamate. Our model predicts that the side chains of Phe-7786.51 and Leu-7826.55 make hydrophobic interactions with the cyclohexyl ring of cyclamate. Phe-7305.43 within TM helix 5 may also contribute a hydrophobic interaction with the cyclamate cyclohexyl ring. The human-to-mouse substitution at this position (F7305.43L) or substitution with another small hydrophobic group (e.g. alanine) led to severely diminished responses to cyclamate, probably because these side chains are too short to interact with the cyclohexyl ring of cyclamate. Replacement of Phe-7305.43 with hydrophilic residues (e.g. serine and threonine) abolished the receptor response to cyclamate, presumably because these
side chains would have unfavorable interactions with the hydrophobic cyclohexyl group. Large hydrophobic groups (e.g. tryptophan and tyrosine) were well tolerated at position 730\textsuperscript{ex3}, indeed, by increasing the size of the side chain at position 730\textsuperscript{ex3} (i.e. the F730\textsuperscript{ex3}Y mutant) responsiveness to cyclamate was enhanced, presumably by achieving closer proximity between the side chain of this residue and the cyclamate cyclohexyl group.

What is the contribution to cyclamate binding of Arg-790\textsuperscript{ex3} within extracellular loop 3? In our model, Arg-790\textsuperscript{ex3} lies at the top of the TMD, outside of the plane of the binding pocket for cyclamate and thus is unlikely to contact cyclamate directly. However, all of our Arg-790\textsuperscript{ex3} substitution mutants (R790\textsuperscript{ex3}Q, R790\textsuperscript{ex3}K, R790\textsuperscript{ex3}V, R790\textsuperscript{ex3}A, and R790\textsuperscript{ex3}E) were completely unresponsive to cyclamate, suggesting that size, orientation, and charge at this position are all important. We speculate that Arg-790\textsuperscript{ex3} interacts with other residues at the top of the TMD, helping to set the dimensions of the pocket, thus determining which ligands can fit within the T1R3 TMD binding pocket.

**Overlapping but Non-identical Binding Pockets within the hT1R3 TMD for Cyclamate and Lactisole**—We have previously shown that the TMD of hT1R3 determines sweet receptor sensitivity to lactisole, a broad-acting inhibitor of sweet taste that affects humans and Old World primates, but not mice or other rodents (16). Using human/rat chimeras of T1R3, Xu et al. (24) and Winnig et al. (30) likewise concluded that the TMD of hT1R3 determines sensitivity to lactisole. In our earlier study, we identified seven critical residues within the TMD of hT1R3 that define the ligand-binding pocket for lactisole (16). In the present study, we determined that four of these seven residues (His-641\textsuperscript{ex3}, Phe-778\textsuperscript{ex5}, Leu-782\textsuperscript{ex5}, and Arg-790\textsuperscript{ex6}) are also important for human sweet receptor responsiveness to cyclamate. For example, the F778\textsuperscript{ex5}A mutant of hT1R3 has reduced responsiveness to cyclamate and reduced sensitivity to lactisole, whereas the H641\textsuperscript{ex3}A mutant has reduced responsiveness to cyclamate and completely lacks sensitivity to lactisole. In our models, TM helices 3, 5, and 6 contribute to the binding pockets for both cyclamate and lactisole. Phe-778\textsuperscript{ex5}A of TM helix 6 is in close proximity to the aromatic ring of lactisole and the cyclohexyl ring of cyclamate; this side chain is predicted to have a hydrophobic interaction with cyclamate and a π–π interaction with lactisole. The F778\textsuperscript{ex5}A replacement results in the loss of both of these interactions, rendering the mutant receptor insensitive to both lactisole and cyclamate. Histidine-641\textsuperscript{ex3} of TM helix 3 lies close to negatively charged pharmacophores of each ligand, the carboxyl group of lactisole and the sulfamate group of cyclamate. Under physiological conditions, His-641\textsuperscript{ex3} is likely protonated and could make a salt bridge with both of these two negatively charged groups.

Although these two mutations had concordant effects on responses to cyclamate and lactisole, several other mutations had divergent effects on responses to these two ligands. For example, the Q637\textsuperscript{ex3} A, to cyclamate and lactisole, several other mutations had divergent effects on charged groups.

The Cyclamate Interaction Site in Human T1R3 Transmembrane Domain

The Cyclamate Interaction Site in Human T1R3 Transmembrane Domain

The Cyclamate Interaction Site in Human T1R3 Transmembrane Domain

Acknowledgments—We thank all the members of the Margolskee laboratory for stimulating discussions and Jeanne Margolskee for editing.

REFERENCES

1. Gilbertson, T. A., Damak, S., and Margolskee, R. F. (2000) Curr. Opin. Neurobiol. 10, 519–527
2. Lindemann, B. (2001) Nature 413, 219–225
3. Gilbertson, T. A., and Boughter, J. D., Jr. (2003) Neuroreport 14, 905–911
4. Hoon, M. A., Adler, E., Lindemeyer, J., Battey, J. F., Ryba, N. J., and Zuker, C. S. (1999) Cell 96, 541–551
5. Kitagawa, M., Kubakabe, Y., Miura, H., Ninomiya, Y., and Hino, A. (2001) Biochem. Biophys. Res. Commun. 283, 236–242
6. Max, M., Shanker, Y. G., Huang, L., Rong, M., Liu, Z., Campagne, F., Weinstein, H., Damak, S., and Margolskee, R. F. (2001) Nat. Genet. 28, 58–63
7. Montmayeur, J. P., Liberles, S. D., Matsumani, H., and Buck, L. B. (2001) Nat. Neurosci. 4, 492–498
8. Sainz, E., Korley, J. N., Battey, J. F., and Sullivan, S. L. (2001) J. Neurochem. 77, 896–903
9. Bachmanov, A. A., Li, X., Reed, D. R., Ohmen, J. D., Li, S., Chen, Z., Tordoff, M. G., de Jong, P. J., Wu, C., West, D. B., Chatterjee, A., Ross, D. A., and Beauchamp, G. K. (2001) Chem. Sens. 26, 925–933
10. Nelson, G., Hoon, M. A., Chandrashekar, J., Zhang, Y., Ryba, N. J., and Zuker, C. S. (2001) Cell 106, 381–390
11. Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M., and Adler, E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4692–4696
12. Damak, S., Rong, M., Yasumatsu, K., Kokrashvili, Z., Varamazhan, V., Zou, S., Jiang, P., Ninomiya, Y., and Margolskee, R. F. (2003) Science 301, 850–853
13. Zhao, G. Q., Zhang, Y., Hoon, M. A., Chandrashekar, J., Erenbach, I., Ryba, N. J., and Zuker, C. S. (2003) Cell 115, 255–266
Jiang, P., Ji, Q., Liu, Z., Snyder, L. A., Benard, L. M., Margolskee, R. F., and Max, M. (2004) J. Biol. Chem. 279, 45068–45075
15. Jiang, P., Cui, M., Ji, Q., Snyder, L., Liu, Z., Benard, L., Margolskee, R. F., Osman, R., and Max, M. (2005) Chem. Senses 30, Suppl. 1, i17–i18
16. Jiang, P., Cui, M., Zhao, B., Liu, Z., Snyder, L. A., Benard, L. M., Osman, R., Margolskee, R. F., and Max, M. (2005) J. Biol. Chem. 280, 15238–15246
17. Pin, J. P., Galvez, T., and Prezeau, L. (2003) Pharmacol. Ther. 98, 325–354
18. Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakashima, S., Jingami, H., and Morikawa, K. (2000) Nature 407, 971–977
19. Binet, V., Brajon, C., Le Corre, L., Acher, F., Pin, J. P., and Prezeau, L. (2004) J. Biol. Chem. 279, 29085–29091
20. Malherbe, P., Kratochwil, N., Knoflach, F., Zener, M. T., Kew, J. N., Kratzeisen, C., Maerki, H. P., Adam, G., and Mutel, V. (2003) J. Biol. Chem. 278, 8340–8347
21. Pagano, A., Ruegg, D., Litschig, S., Stoehr, N., Stierlin, C., Heinrich, M., Hoersheim, P., Prezeau, L., Carroll, F., Pin, J. P., Cambria, A., Vranesic, I., Flor, P. J., Gasparini, F., and Kuhn, R. (2000) J. Biol. Chem. 275, 33750–33758
22. Petrel, C., Kessler, A., Kwasnik, P., Dauban, P., Dodd, R. H., Rognan, D., and Ruat, M. (2003) J. Biol. Chem. 278, 49487–49494
23. Petrel, C., Kessler, A., Masalah, F., Dauban, P., Dodd, R. H., Rognan, D., and Ruat, M. (2004) J. Biol. Chem. 279, 18990–18997
24. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Zakrzewski, V. G., Montgomery, J. A., Stratmann, R. E., Burant, J. C., Dapprich, S., Millam, J. M., Daniels, A. D., Kudin, K. N., Strain, M. C., Farkas, O., Tomasi, J., Barone, V., Cossi, M., Cammi, R., Mennucci, B., Pomelli, C., Adamo, C., Clifford, S., Ochterski, J., Petersson, G. A., Ayala, P. Y., Cui, Q., Morokuma, K., Malick, D. K., Rabuck, A. D., Raghavachari, K., Al-Laham, A. M., Peng, C. Y., Nanayakkara, A., Gonzalez, C., Challacombe, M., Gill, P. M. W., Johnson, B. G., Chen, W., Wong, M. W., Andres, J. L., Head-Gordon, M., Replogle, E. S., and Pople, J. A. (1998) Gaussian 98 Revision A.1, Gaussian Inc., Pittsburgh, PA
26. Breneman, C. M., and Wiberg, K. B. (1990) J. Comput. Chem. 11, 361–373
27. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) J. Comput. Chem. 19, 1639–1662
28. Richards, R. K., Taylor, J. D., O’Brien, J. L., and Duescher, H. O. (1951) J. Am. Pharm. Assoc. 40, 1–6
29. Bachmanov, A. A., Tordoff, M. G., and Beauchamp, G. K. (2001) Chem. Senses 26, 905–913
30. Winnig, M., Bufe, B., and Meyerhof, W. (2005) BMC Neuroscience 6, 22
31. Farrens, D., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Science 274, 768–770
32. Ward, S. D., Hamdan, F. F., Bloodworth, I. M., and Wess, J. (2002) J. Biol. Chem. 277, 2247–2257
33. Ghanouni, P., Steenhuis, J. J., Farrens, D. L., and Kobilka, B. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5997–6002