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Amino Acids and Peptides Activate at Least Five Members of the Human Bitter Taste Receptor Family

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ABSTRACT: Amino acids and peptides represent important flavor molecules eliciting various taste sensations. Here, we present a comprehensive assessment of the interaction of various peptides and all proteinogenic amino acids with the 25 human TAS2Rs expressed in cell lines. L-Phenylalanine and L-tryptophan activate TAS2R1 and TAS2R4, respectively, whereas TAS2R4 and TAS2R39 responded to D-tryptophan. Structure−function analysis uncovered the basis for the lack of stereoselectivity of TAS2R4. The same three TAS2Rs or subsets thereof were also sensitive to various dipeptides containing L-tryptophan, L-phenylalanine, or L-leucine and to Trp-Trp-Trp, whereas Leu-Leu-Leu specifically activated TAS2R4. Trp-Trp-Trp also activated TAS2R46 and TAS2R14. Two key bitter peptides from Gouda cheese, namely, Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser and Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn, both activated TAS2R1 and TAS2R39. Thus, the data demonstrate that the bitterness of amino acids and peptides is not mediated by specifically tuned TAS2Rs but rather is brought about by an unexpectedly complex pattern of sensitive TAS2Rs.

KEYWORDS: *calcium imaging, peptides, cheese, bitter, receptor*

INTRODUCTION

Taste is a decisive determinant of flavor in food that comes in the five basic qualities sweet, umami, bitter, sour, and salty. Whereas sweet-, umami-, salty-, and at least mildly sour-tasting chemicals contribute to the appreciation of food, the presence of bitter compounds is often subject to opposite assessments.[1] On one hand, bitterness is often associated with toxins and elicits rejection to prevent intoxication.[2] Therefore, certain bitter chemicals often produce unwanted off-flavors, for instance, in fruits, vegetables, soybean, and milk products.[3] On the other hand, in other food and beverages such as beer, chocolate, black or green tea, red wine, and coffee, bitter is a desired flavor note.[4,5] The molecules eliciting bitterness are countless and include natural chemicals of plant and animal origin as well as synthetic compounds or molecules generated during aging, spoiling, and fermentation of food.[3,4,6] Among the latter compounds are amino acids and peptides, which are important food constituents released from proteins that provide calories and flavor alike.[3,7−9] The flavor contributed by amino acids and peptides involves sweet, umami, kokumi, sour, and bitter sensations.[10−12] The bitterness of many peptides and amino acids has been determined in recent sensory studies[3,13−17] and explained by the hydrophobicity of the amino acid side chains and the proportion of hydrophobic amino acids in the peptides.[3,7,12−16,18] Whereas a clear correlation of bitterness and hydrophobicity has been disputed,[3,7,13−16,18,19] other structural parameters contributing to the bitterness of peptides and amino acids have been described, such as the acetylation or esterification of the amino and/or carboxy groups.[18] Based on the structural parameters of various bitter peptides and analogues that elicit bitter taste, a hypothetical bitter receptor site has been proposed that consists of a "binding subunit" and a "stimulating unit" spaced by 4.1 Å.[14] However, neither the biological significance of this receptor site nor its relation to the recently identified bitter receptors has been established.

Nevertheless, the identification of the receptive biological mechanisms, including special receptor molecules and their mode of action, stimulated by distinct flavor molecules emerges as an important principle in modern food science. Recent progress has shown that, whereas sour and salty stimuli are transmitted by ion channels, sweet, umami, and bitter tastes are mediated through special G protein-coupled receptors (GPCRs).[20−24] The flavor of peptides is mediated through different receptors. Three members of the taste 1 receptor (TAS1R) family assemble into the heterodimer TAS1R2−TAS1R3, which mediates the sweetness of sugars and all other tested sweet compounds including glycine and various D-amino acids,[25,26] or into the heterodimer TAS1R1−TAS1R3, which recognizes several L-amino acids in rodents and mostly L-glutamate in humans.[25,26] The amino-acid-induced responses of TAS1R1−TAS1R3 are enhanced by various ribonucleotides, a hallmark of umami taste.[26] Kokumi perception is less well understood, but peptides eliciting the kokumi sensation appear to bind to and activate the calcium-sensing receptor.[11] This receptor functions as a homodimer of a GPCR with sequence similarities to TAS1Rs.[27] In marked contrast, bitterness is elicited by GPCRs of the taste 2 receptor (TAS2R) family, which comprises ∼25 members in humans.[23,28] Previous

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Table 1. Compounds and Their Concentrations of Use

compound	concentration (mM)	compound	concentration (mM)	compound	concentration (mM)
		Amino Acids			
L-Ala	100.0	L-His	1.0	L-Thr	100.0
L-Arg	0.3	L-Ile	60.0	L-Trp	21.7
L-Asn	1.0	L-Leu	60.0	L-Tyr	0.67
L-Asp	0.03	L-Lys	300.0	L-Val	100.0
L-Cys	100	L-Met	113.3	D-Phe	53.3[a]
L-Glu	1.0	L-Phe	53.3[a]	D-Trp	21.7
L-Gln	60.0	L-Pro	100.0		
Gly	100.0	L-Ser	10.0		
		Peptides			
Leu-Leu	10.0	Ile-Phe	10.0	Leu-Leu-Leu	3.0
Leu-Trp	30.0	Phe-Ile	10.0	Trp-Trp-Trp	0.3
Phe-Trp	10.0				
Trp-Leu	1.0	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser			1.0[b]
Trp-Phe	3.0	Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn			1.0[b]
Trp-Pro	3.0				
Trp-Trp	3.0				
		Indole Derivatives			
3-acetylindole	1.0	indole-3-acetic acid	1.0	serotonin	3.0
3-(2-hydroxyethyl)-indole	1.0	indole-3-carbinol	0.3	skatole	0.3
5-hydroxy-L-tryptophan	0.3	indole-3-carboxaldehyde	0.03	tryptamine	0.03
indole	1.0	indole-3-carboxylic acid	1.0		
indole-3-acetamide	1.0	melatonin	0.3		

[a]Maximum compound concentrations reported here did not result in artificial responses in mock-transfected HEK 293T-cells, based on limited solubility. [b]Maximum compound concentrations reported here did not result in artificial responses in mock-transfected HEK 293T-cells, based on limited availability.

research revealed how TAS2Rs mediate the bitterness of numerous structurally diverse bitter compounds. Three "generalist" receptors, namely, TAS2R10, TAS2R14, and TAS2R46, with extensively overlapping agonist spectra appear to recognize about half of the chemically distinct bitter chemicals that humans perceive.[29] Several less broadly tuned receptors including TAS2R1, TAS2R4, TAS2R7, TAS2R31, TAS2R39, TAS2R40, TAS2R43, and TAS2R47 with unique but partially overlapping agonist profiles detect many additional substances.[29] In addition, TAS2R16 and TAS2R38 are sensitive to classes of bitter chemicals with common structural motifs mediating the bitterness of a wide range of β-glycopyranosides or thioamides, carbamides, and isothiocyanates.[28,30] Finally, some narrowly tuned "specialist" receptors such as TAS2R3, TAS2R5, TAS2R8, TAS2R9, TAS2R13, TAS2R20, and TAS2R50 that detect only a few compounds contribute to human bitter perception.[29] Thus, whereas numerous bitter chemicals activate several TAS2Rs, although with different potencies, every receptor displays its own specific spectrum of agonists. Thus, the tuning breadth and some additional properties of TAS2Rs, including genetic variability and/or receptor oligomerization, likely account for the ability of humans to perceive an incredible number of bitter chemicals.[31,32]

In the mentioned studies, mostly synthetic drugs and plant toxins or minor metabolites have been used to identify the cognate TAS2Rs, whereas studies with food-derived and nutritionally relevant bitter chemicals are rare.[33] They identified TAS2Rs for the bitter off-taste of the sugar surrogates saccharin, acesulfame K, and steviol glycosides; the bitter principles in beer and hops; and soy isoflavones.[34−37]

Three other recent studies demonstrated that mainly TAS2R1, TAS2R8, and TAS2R39 are sensitive to a few selected di- and tripeptides and one tetrapeptide.[8,38,39] However, these studies are incomplete, as only very few of the ∼25 TAS2Rs have been examined for their sensitivity to bitter peptides. Thus, it remains unknown whether other peptide-responsive TAS2Rs exist aside for the three mentioned receptors. Moreover, complex peptides that are bitter principles in real food and proteinogenic amino acids have not been examined.

In this report, we present a comprehensive analysis of amino acids and peptides as activators for the human bitter taste receptors. It is well described that protein-rich food induces the production of numerous of bitter peptides and amino acids.[3,7−9] Milk products such as cheese and soybean products are generated by the hydrolysis of proteins, which is often involved in the development of bitter taste.[40,41] Several amino acids and synthetic peptides or peptides from food have been tested in sensory studies.[3,13−16] Bitter peptides and amino acids are ideal candidates for structure−function analysis, because of their structural and sequence diversity. We used functional expression of TAS2Rs in engineered cell lines to identify receptors for amino acids and model peptides. We also analyzed the relevance of structural groups in these molecules for their ability to activate TAS2Rs. Furthermore, we identified the taste receptors for intensely bitter oligopeptides present in matured Gouda cheese.

MATERIALS AND METHODS

Reagents. Compounds were purchased from Sigma-Aldrich (Taufkirchen, Germany), if not mentioned otherwise. The peptides Ile-Phe, Phe-Ile, Phe-Trp, Leu-Leu, Trp-Leu, Trp-Phe, Trp-Pro, Trp-Trp, and Trp-Trp-Trp were purchased from Bachem (Weil am Rhein, Germany), and the amino acids Gly and L-Thr were obtained from Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany), respectively. The peptides Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser

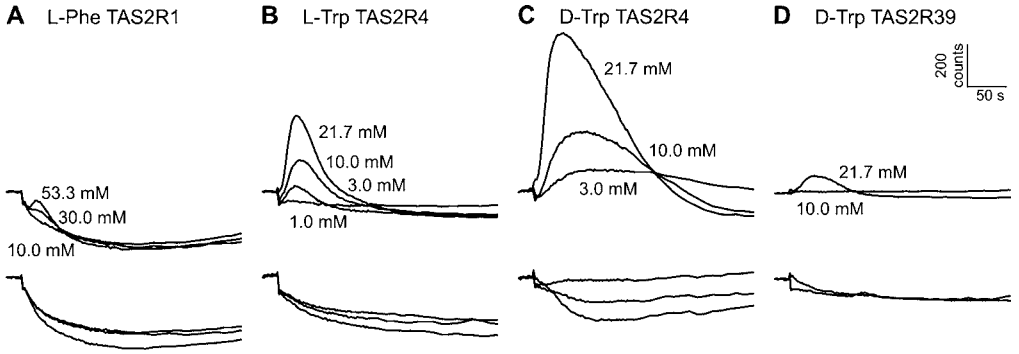

Figure 1. Amino acids activating TAS2R1-, TAS2R4-, and TAS2R39-transfected cells. (A) Detailed view of the calcium responses of TAS2R1-transfected cells stimulated with 53.3, 30.0, and 10.0 mM L-Phe. Recorded calcium traces of TAS2R4-transfected cells treated with (B) 21.7, 10.0, 3.0, and 1.0 mM L-Trp and (C) 21.7, 10.0, and 3.0 mM D-Trp. (D) Changes in intracellular calcium levels of TAS2R39-expressing cells recorded after bath application of 21.7 and 10.0 mM D-Trp. Mock-transfected cells were treated with the same compounds and used as controls for specificity of receptor activation (calcium traces below according to compound).

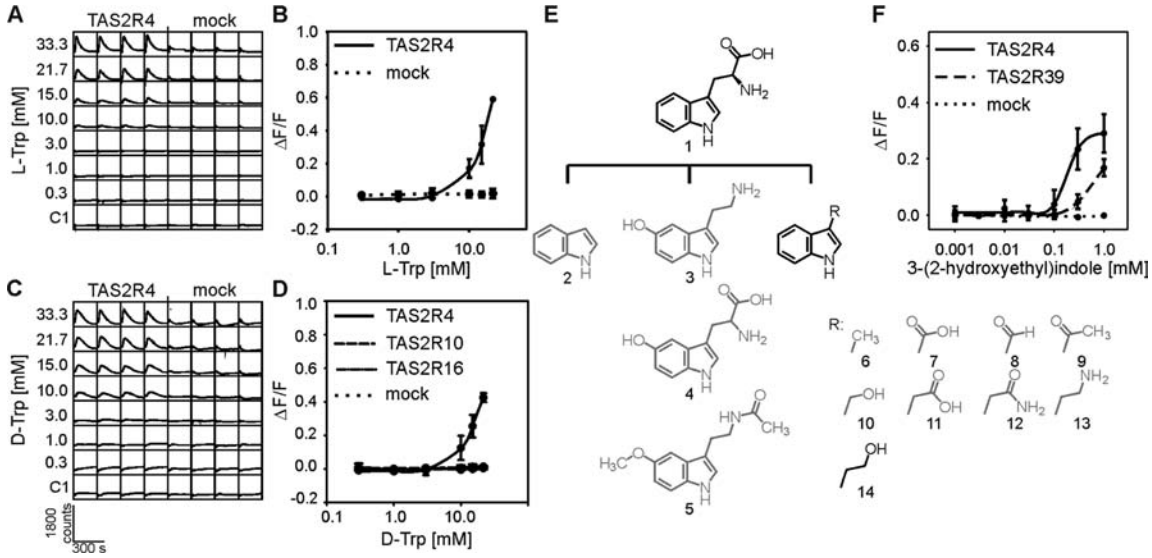

Figure 2. Trp activating TAS2R4 and detailed analysis of the structural elements required for a TAS2R response. HEK293T-Gα16gust44 cells were transfected with TAS2Rs or empty vector (mock) and then subjected to bath application of diverse concentrations of compounds. Changes in intracellular calcium levels were recorded using a fluorescence imaging plate reader. For dose−response relations, changes in fluorescence ($\Delta F/F$) were plotted versus the logarithm of the agonist concentration. (A) Calcium traces of TAS2R4- and mock-transfected cells were recorded after bath application of increasing concentrations of L-Trp. (B) Dose-dependent activation was observed for TAS2R4 by L-Trp. (C) Calcium traces of TAS2R4- and mock-transfected cells were recorded after bath application of increasing concentrations of D-Trp. (D) Dose-dependent activation was observed for TAS2R4 by D-Trp. Note that, for additional specificity controls, cDNAs of the nonresponsive receptors TAS2R10 and TAS2R16 were tested along with the empty vector negative control. With the exception of 33.3 mM L-Trp and D-Trp, mock-transfected cells showed no artificial signals. Receptor-independent calcium responses for 33.3 mM L-Trp and D-Trp were not accepted for dose−response calculations. (E) For the analysis of a structurally relevant element of Trp for the activation of TAS2R4 and TAS2R39, Trp-related compounds were used in calcium imaging experiments: (1) L-Trp, (2) indole, (3) serotonin, (4) 5-hydroxy-L-tryptophan, (5) melatonin, (6) skatole, (7) indole-3-carboxylic acid, (8) indole-3-carboxaldehyde, (9) 3-acetylindole, (10) indole-3-carbinol, (11) indole-3-acetic acid, (12) indole-3-acetamide, (13) tryptamine, and (14) 3-(2-hydroxyethyl)indole. Gray colored compounds did not result in activation of TAS2R4 and TAS2R39. (F) 3-(2-Hydroxyethyl)Indole (14) led to increases in intracellular calcium levels for TAS2R4- and TAS2R39-expressing cells in a dose-dependent manner. Threshold and EC_{50} values are listed in Table 2 and were calculated from at least three independent experiments.

and Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn were obtained from EZBiolab (Carmel, IN).

Functional Analysis. To examine the function of TAS2R cDNAs, we used a heterologous expression system as described before.[42] The expression plasmids were based on pcDNA5/FRT (Invitrogen, San Diego, CA) or pEAK10 (Edge BioSystems, Gaithersburg, MD). The constructs contain a plasma-membrane-targeting sequence of the rat somatostatin (SST) receptor type 3 at their amino terminus and a herpes simplex virus (HSV) glycoprotein D epitope at their carboxyl terminus.[28,43] Human embryonic kidney cells stably expressing the chimeric G-protein α-subunit Gα16-Gust44 (HEK293T-Gα16gust44)[44] were seeded into 96-well plates (Greiner Bio-One, Frickenhausen, Germany). After 24−26 h, the cells were transiently transfected with 150 ng of plasmid DNA using 300 ng of Lipofectamin 2000 (Invitrogen, San Diego, CA). The empty pcDNA5/FRT vector was used as a negative control (mock). Cells were loaded with the calcium-sensitive dye Fluo-4-AM (2 μM, Molecular Probes, Karlsruhe, Germany) in the presence of 2.5 mM probenecid 24 h after transfection. Calcium responses of the cells were recorded at 510 nm following excitation at 488 nm with a fluorometric imaging plate reader (FLIPR^{tetra}, Molecular Devices, Munich, Germany). The taste compounds were dissolved and applied in C1 solution {130 mM NaCl, 5 mM KCl, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (Hepes, pH 7.4), 2 mM $CaCl_2$, and 10 mM

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Table 2. Threshold (mM) and EC_{50} (mM) Values of TAS2Rs Activated by Amino Acids and Peptides

compound	1	4	14	39	46
L-Phe	53.3	–	–	–	–
L-Trp	–	10.0	–	–	–
D-Trp	–	10.0	–	21.7	–
Ile-Phe	10.0	10.0	–	10.0	–
Leu-Trp	3.0	3.0	–	3.0	–
Phe-Ile	10.0		–		–
Phe-Trp	1.0	1.0	–	–	–
Trp-Leu	–	3.0	–	–	–
Trp-Phe	–	0.3	–	–	–
Trp-Pro	–	3.0	–	–	–
Trp-Trp	0.3	1.0[a]	–	1.0	–
Leu-Leu-Leu	–	3.0	–	–	–
Trp-Trp-Trp	0.1	0.01[b]	0.1	0.1	0.1
3-(2-hydroxyethyl)-indole		0.3[c]		0.3	
Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser	0.3	–	–	1.0	–
Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn	0.3	–	–	1.0	–

[a] EC_{50} = 0.66 ± 0.03. [b] EC_{50} = 0.03 ± 0.005. [c] EC_{50} = 0.19 ± 0.02.

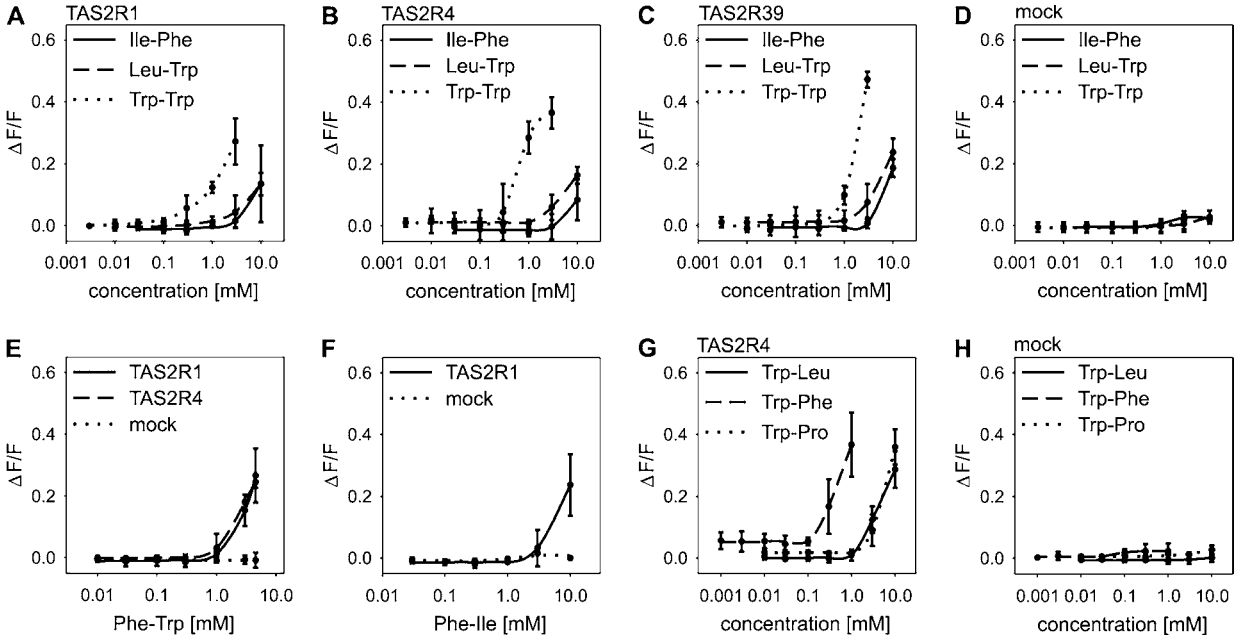

Figure 3. Dipeptides activated TAS2R1-, TAS2R4-, and TAS2R39-expressing cells in dose–response relations. After TAS2R transfection of the HEK293T-Gα16gust44 cells and loading with calcium-sensitive dye, the cells were treated with different concentrations of the mentioned compounds. Changes in fluorescence ($\Delta F/F$) were recorded by fluorescence imaging plate reader and plotted against the logarithm of the agonist concentrations. Ile-Phe, Leu-Trp, and Trp-Trp activated (A) TAS2R1-, (B) TAS2R4-, and (C) TAS2R39-expressing cells. (D) Dose–response curves of mock-transfected cells are illustrated separately. (E) Phe-Trp activated TAS2R1- and TAS2R4-expressing cells in a dose-dependent manner. (F) Phe-Ile activated TAS2R1-transfected cells. Dose–response relations for mock-transfected cells are illustrated by dotted lines. (G) For TAS2R4-expressing cells, dose-dependent activation was observed for Trp-Leu, Trp-Phe, and Trp-Pro. (H) Dose–response curves of mock-transfected cells are illustrated separately. Threshold and EC_{50} values are listed in Table 2 and were calculated from at least three independent experiments.

glucose}. Data were collected from at least two independent experiments carried out in duplicate for screening of the 25 TAS2Rs with agonists and in triplicate for establishing dose–response functions. In a pilot experiment, all compounds were tested at various concentrations for their ability to elicit nonspecific calcium signals in mock-transfected HEK293T-Gα16gust44 cells. Based on these experiments, we used a maximum compound concentration not leading to artificial response in mock-transfected cells (Table 1).

Calculation of Dose–Response Curves, EC_{50} Values, and Threshold Values. For the calculation of dose–response functions, the fluorescence changes of mock-transfected cells were subtracted from those of receptor-transfected cells and further normalized to the background fluorescence ($\Delta F/F$). Amplitudes were averaged from three wells and three independent experiments. For determination of dose–response curves, the calculated average signal amplitudes were plotted against the logarithm of the agonist concentration. By nonlinear regression using the function $f(x) = \{(a - d)/[1 + (x/EC_{50})^{nH}] + d\}$ (where x is the agonist concentration, nH is the Hill coefficient, a is the maximum, and d is the minimum), we calculated the half-maximum effective concentration, EC_{50}.[45] Illustration of the dose–response curves and calculation of the EC_{50} values and standard deviation were done using SigmaPlot 11.0 (Systat Software Inc.)

The threshold value of activation was defined as the lowest substance concentration that evoked calcium signals in receptor-

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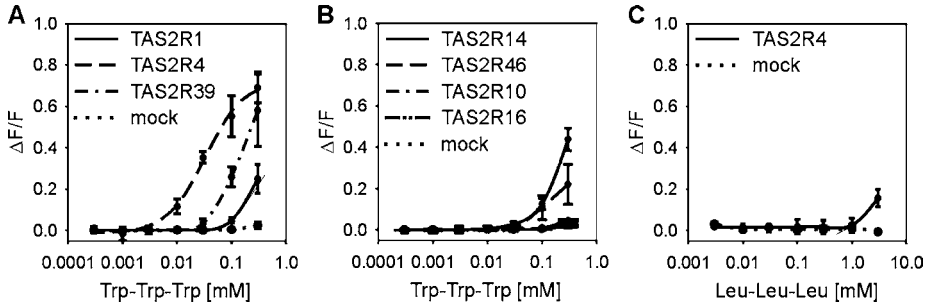

Figure 4. Tripeptides activated TAS2R1, TAS2R4, TAS2R14, TAS2R39, and TAS2R46. HEK293T-Gα16gust44 cells were transfected by the indicated TAS2Rs. After bath application of tripeptides, increases in intracellular calcium levels were recorded using a fluorescence imaging plate reader. (A) The recorded fluorescence changes ($\Delta F/F$) of TAS2R1-, TAS2R4-, and TAS2R39-transfected cells are plotted versus the logarithm of 0.3−0.0003 mM Trp-Trp-Trp. (B) TAS2R14- and TAS2R46-expressing cells were activated by Trp-Trp-Trp. Note that, for additional specificity controls, cDNAs of the nonresponsive receptors TAS2R10 and TAS2R16 were tested along with the empty vector negative control. (C) For Leu-Leu-Leu, a dose-dependent activation was observed for TAS2R4-expressing cells. Dose−response curves of mock-transfected cells are illustrated by dotted lines. Threshold and EC_{50} values are listed in Table 2 and were calculated from at least three independent experiments.

transfected compared to mock-transfected cells. The statistical significances of the identified threshold values were confirmed using Student's t test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Amino Acids as Activators of TAS2Rs. To identify TAS2Rs for bitter-tasting amino acids,[17,46,47] we transfected the cDNAs of the 25 TAS2Rs in human embryonic kidney (HEK) 293T cells stably expressing the chimeric G protein α-subunit Gα16gust44. This G protein effectively couples active TAS2Rs to phospholipase C activity and calcium release from internal stores.[44] Transfected cells expressing the 25 TAS2Rs individually were loaded with calcium indicator dye and challenged with different concentrations of the proteinogenic amino acids ranging from moderate to the highest possible concentrations determined in pilot experiments. We chose these high concentrations to match the reported high bitter threshold values of L-amino acids in the range of 0.063−75.0 mM[17,46,47] (Table 1). Figure 1 shows that L-Phe induced tiny but highly reproducible calcium responses specifically in TAS2R1-expressing cells, whereas L-Trp elicited robust responses in TAS2R4-expressing cells. These responses were dose-dependent (Figures 1 and 2A−D). However, the fact that, for solubility problems, the dose−response functions did not saturate prevented us from determining EC_{50} values for activation (Figure 2A−D). The threshold values obtained in sensory studies ranging from 5.4 to 45.0 mM for L-Phe and from 2.29 to 6.0 mM for L-Trp[10,17,46] compare well with our data from the receptor assays showing higher potency for L-Trp at TAS2R4 than for L-Phe at TAS2R1 (threshold values are 53.3 mM for L-Phe and 10.0 mM for L-Trp). Mock-transfected cells were insensitive to all stimuli, demonstrating the specificity of the signals (Figure 1, data not shown). All other proteinogenic amino acids including those that are known to taste bitter, namely, L-Arg, L-Ile, L-Leu, L-Tyr, and L-Val,[10,17] did not stimulate any other TAS2R-expressing cells at the concentrations tested. The major reason likely is that we could not always employ in our assays the required high amino acid concentrations that have been used in sensory studies. Even though the reported bitter thresholds in sensory studies for L-Ile, L-Leu, and L-Val[17] were below the concentrations used for these compounds in our heterologous assay system, receptor activation was not observed. Nevertheless, it appears possible that use of even higher concentrations would identify more amino-acid-responsive TAS2Rs or more L-amino acid activators for TAS2R1 and TAS2R4.

Interestingly, the concentration ranges in which the individual amino acids elicited TAS2R-independent cellular responses deviated considerably. Whereas such responses occurring at rather high millimolar concentrations might reflect osmotic and related effects, some responses elicited by low- or submillimolar concentrations could result from the presence of

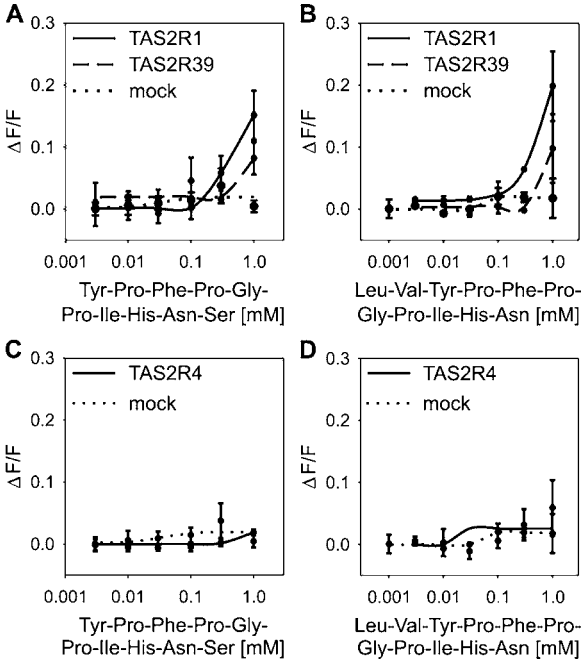

Figure 5. Activation of TAS2R1- and TAS2R39-expressing cells induced by two isolated oligopeptides from Gouda cheese[16] in a dose-dependent relation. HEK293T-Gα16gust44 cells were transfected by the indicated TAS2Rs. Changes in fluorescence ($\Delta F/F$) were recorded using a fluorescence imaging plate reader and plotted against the logarithm of the agonist concentrations. TAS2R1- and TAS2R39-transfected cells were activated in a dose-dependent manner by (A) Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser and (B) Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn. (C,D) For TAS2R4-expressing cells, no activation by either of the two olipeptides was observed. Dose−response curves of mock-transfected cells are illustrated by dotted lines. Threshold values are listed in Table 2 and were calculated from at least two independent experiments.

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endogenously expressed receptors, transporters, or pathways specifically interacting with individual L-amino acids in HEK 293T cells.

We also included D-amino acids in our analysis because they are sweeteners and activators of the TAS1R2−TAS1R3 sweet taste receptor but are also associated with a weak bitter off-taste.[25,26,46−48] We found that TAS2R4- and TAS2R39-expressing cells were modestly and selectively sensitive to D-Trp (Figure 1C,D), with TAS2R4 being more sensitive than TAS2R39. Together the data suggest that the two TAS2Rs TAS2R4 and TAS2R39 mediate the bitter off-taste of D-tryptophan.[45]

It is interesting to note that the three TAS2Rs differ regarding their stereoselectivity of activation. TAS2R1 and TAS2R39 both discriminate between the stereoisomers of Phe and Trp. The former is selective for the L configuration and the latter for the D configuration. In contrast, TAS2R4 lacks stereoselectivity and shows similar dose−response functions for both isomers, although the D-isomer induces higher response amplitudes than the L-isomer (Figure 2B,D). Stereoselective bitter taste receptor activation has already been observed in the case of TAS2R16, which is sensitive to β- but not α-glucopyranosides.[28]

Structural Properties of Amino Acids Required for TAS2R Activation. The finding that the three TAS2Rs were highly selective for Phe or Trp indicates that the α-amino acid group alone is not responsible for receptor activation. In that case, numerous amino acids would serve as activators. Moreover, the observation that TAS2R4 is sensitive to both Trp stereoisomers whereas TAS2R39 is selective for the D isomer suggests that activation of TAS2R4 requires only the side chain and not the amino acid function whereas activation of TAS2R39 probably involves both. To test this conjecture, we challenged cells expressing TAS2R4 or TAS2R39 with a series of chemicals related to Trp (**1**−**14**, Table 1, Figure 2E). Figure 2F demonstrates that only 3-(2-hydroxyethyl)indole[14] stimulated dose-dependent calcium responses in both TAS2R4- and TAS2R39-expressing cells. Mock-transfected cells showed no responses (Figure 2F, dotted line). The threshold concentration of **14** was ∼0.3 mM for both receptors. The compound was more potent than the Trp isomers on TAS2R4, exhibiting an EC_{50} value of 0.19 ± 0.02 mM, but it had lower efficacy. At TAS2R39, it was less potent, resembling D-Trp and again preventing us from determining an EC_{50} value of activation.

Both receptors were highly sensitive to modifications of the indole ring. Several indole derivatives have been found in cruciferous vegetables, such as broccoli, cabbage, and brussels sprouts, that are assumed to have anticarcinogenic activity.[49−52] Hydroxylation in position 5 (serotonin and melatonin, **3** and **5**) abolished agonist activity. Both receptors were also highly sensitive to modifications of the alanyl residue. Presence of the amino or carboxyl group alone or replacement of the carboxyl function by a keto group is insufficient for receptor activation (compounds **11**−**13**). Chain length also appears to be a crucial factor (compare compounds **10** and **14**), and more severe modifications also shut down receptor activation (compounds **2** and **5**−**9**). Thus, in contrast to our expectation, structural properties in both the ring system and the alanyl residue were crucial for activation of TAS2R4 and TAS2R39 by Trp. The data also suggest that the amino and carbonyl group are probably not important for activating TAS2R4, explaining its lack of stereoselectivity. This appears to be different in TAS2R39, where both groups, if present in the L configuration, prevent activation of the receptor by Trp. Thus, although TAS2R1, TAS2R4, and TAS2R39 generally show low potency for their agonists, subtle structural differences can have detrimental effects on receptor−agonist interactions, confirming observations made recently with TAS2R16, TAS2R31, TAS2R38, and TAS2R46.[28,42,45,53]

Peptides as TAS2R Activators. Next, we investigated the responses of the 25 TAS2Rs in the cell-based assay to dipeptides. Table 2 and Figure 3 show that different combinations of the three amino-acid-responsive receptors TAS2R1, TAS2R4, and TAS2R39 were sensitive to different sets of dipeptides in a dose-dependent manner. Similarly to the amino acids, dipeptides stimulated the three TAS2Rs only weakly or moderately with threshold values above 1.0 mM. The exception was TAS2R4 and Trp-Trp, for which we could establish a dose−response curve with an EC_{50} value of 0.66 ± 0.03 mM (Figure 3B). In general, TAS2R4 also demonstrated the most robust responses to other dipeptides. Human TAS2R4 was found to be sensitive to all dipeptides but Phe-Ile. This is astonishing because Ile-Phe was effective on this receptor. Apparently, complex structure−function relations govern the ability of peptides to activate TAS2R4, and the mere presence of amino acids that, by themselves, activate this receptor does not predict agonist activity. The responses of the other TAS2Rs were more restricted. Human TAS2R1, which is sensitive to only one amino acid, L-Phe, recognized the dipeptides Ile-Phe, Phe-Ile, and Phe-Trp (Table 2, Figure 3A,E,F). However, the presence of a Phe per se in a dipeptide appears insufficient for the activation of TAS2R1 because Trp-Phe did not elicit responses (Table 2). The receptor was also sensitive to Trp-Trp and Leu-Trp, suggesting that the alanyl residue, which is common to Trp and Phe, could contribute to TAS2R1 activation. Trp-Trp and Leu-Trp also activated TAS2R39. However, the fact that Trp-Leu and other Trp-containing dipeptides failed to stimulate responses from TAS2R39 within the applicable concentration range again argues that neither the presence nor the position of a "stimulatory" amino acid in a dipeptide predicts agonist activity. None of the other 25 TAS2Rs responded to dipeptides (data not shown).

Next, we investigated two tripeptides, namely, Trp-Trp-Trp and Leu-Leu-Leu, for their ability to activate TAS2Rs. Both were robust stimulators of TAS2R4, with Trp-Trp-Trp being more potent than Leu-Leu-Leu (Table 2, Figure 4). Trp-Trp-Trp best activated TAS2R4, with an EC_{50} value of 0.03 ± 0.005 mM. A comparison of the data in Figures 1−4 indicates that both the potency and efficacy of TAS2R4 agonists depend on the number of amino acid residues, paralleling the observation that bitterness of peptides increases with the number of amino acids present in the peptide chain.[7,13,15] Human TAS2R1 and TAS2R39 were less sensitive than TAS2R4 but showed robust responses. Intriguingly, Trp-Trp-Trp also activated TAS2R14 and TAS2R46 (Figure 4B). Whereas the peptide was a potent activator of TAS2R46, it stimulated TAS2R14 only weakly. This finding indicates that other TAS2Rs aside from TAS2R1, TAS2R4, and TAS2R39 contribute to the bitterness of peptides and supports the existence of further peptide-responsive TAS2Rs. In general, the threshold value of receptor activation compared reasonably well with the sensory data obtained in humans.

Aside from the short-chain di- and tripeptides investigated as just described, larger oligopeptides were found to be released as key bitter stimuli by proteolysis during maturation of cheese

such as Gouda.[16] In a previous study, Toelstede and Hofmann identified the casein-derived decapeptide Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser and the undecapeptide Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn as the most intense bitter peptides in Gouda cheese, with human bitter taste recognition thresholds of 0.05 and 0.08 mM, respectively.[16] We used them for calcium imaging experiments with cells expressing TAS2R1, TAS2R4, or TAS2R39. Because of the limiting amount of the oligopeptides, no full analysis of all 25 TAS2Rs was possible. Both peptides activated TAS2R1 and TAS2R39 in a dose-dependent manner, with thresholds of 0.3 and 1.0 mM, respectively (Table 2, Figure 5A,B). The threshold concentrations determined in the cell-based assay are about 4-fold higher than those measured in sensory experiments but are in the same concentration range. A similarly greater sensitivity in sensory experiments relative to receptor assay was previously observed for hop bitter compounds and attributed to interaction of the bitter principles with the oral mucosa.[34] Cells transfected with TAS2R4-DNA and mock-transfected cells did not respond (Figure 5C,D). Together, the data show that bitter detection of amino acids and peptides is more complex than anticipated, involving not only TAS2R1, TAS2R8, and TAS2R39[7,8,38,39] but at least six different TAS2Rs with different subsets of them being responsive to individual stimuli.

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Notes

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