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(54) Title: NUCLEIC ACID, POLYPEPTIDE AND ITS USE

(57) Abstract: Novel chimeric proteins functional to screen for sweet taste modulators, the corresponding nucleic acid sequences, expression vectors, transfected host cells, and screening methods for modulators and enhancers of the sweet taste response employing the aforementioned are provided.

NUCLEIC ACID, POLYPEPTIDE AND ITS USE

Sweetness modulators and in particular sweetness enhancers are of great interest to the food and flavor industry, for example, to allow reduction of sweeteners including sugars or artificial sweeteners. The use of sweetness enhancers can reduce calories, prevent teeth from damage by sugars, and avoid or reduce the bitter/metallic off- and aftertastes associated with many artificial sweeteners.

To screen for sweetness modifiers or enhancers, known screens employing the T1R2/T1R3 heterodimeric sweet receptor can be used.

To identify or characterise a sweetness modifier/enhancer, usually the results of samples with and without potential enhancer/modifier, both samples additionally containing a sweetener, are compared. However, sweeteners and in particular sugars have a great effect on osmolarity, and/or are viscous. Due to changes in properties of the samples such as viscosity and osmolarity, artifacts may occur that cause incorrect results when using standard screening methods.

Another disadvantage of known screens is that the wildtype T1R2/T1R3 receptor comprises several binding domains, in particular the extracellular amino terminal domains including the venus flytrap ("VFT") domain that bind to carbohydrate sweeteners such as sucrose, glucose, fructose as well as the artificial sweeteners aspartame and sucralose. Therefore, a screen for specific modulators of specific ligands, and in particular for ligands of the transmembrane domains ("TMD(s)") and cystein-rich domains of T1R2 and/or T1R3, excluding the VFT ligands, is not possible with known screening methods.

Agonists that bind in the TMD of T1R3 are cyclamate and Neohesperidin Dihydrochalcone (NDHC). Sucrose and sucralose bind in the VFTs of T1R2 and T1R3, asparatame binds in the VFT of T1R2.

In order to prevent identification of agents that may compete with sugars for binding to the receptor, a screen that allows identification of sweet receptor modulators that bind at a site physically distinct from the VFT domains, and in particular in the TMD and/or cysteine-rich domains, would be desirable.

SUMMARY

The screening methods and binding assays that are provided avoid the above problems and allow for improved results by using CSR::T1R chimeric proteins.

In a first aspect, provided is a CSR::T1R chimeric protein able to bind to at least one sweetener or sweetness enhancer, comprising one or more CSR::T1R selected from the group consisting of a CSR::T1R2 polypeptide substantially homologous to SEQ ID NO:2 (CSR::T1R2-a) or SEQ ID NO:20 (CSR::T1R2-b)with a sequence identity of at least 90%, a CSR:: T1R3 polypeptide substantially homologous to SEQ ID NO:4 (CSR::T1R3-a) or SEQ ID NO: 22 (CSR::T1R3-b) with a sequence identity of at least 90%.

In another aspect, provided is a CSR::T1R chimeric protein as defined herein-above comprising two polypeptide subunits in form of a heterodimeric protein selected from the group consisting of a CSR::T1R2/CSR::T1R3 heterodimeric chimeric protein, a CSR::T1R2/ T1R3 heterodimeric chimeric protein, and a T1R2/CSR::T1R3 heterodimeric chimeric protein, wherein the T1R2 subunit of the heterodimer comprises a polypeptide a polypeptide essentially homologous to SEQ ID NO:8 with a sequence identity of at least 90%; and wherein the T1R3 subunit of the heterodimer comprises a polypeptide essentially homologous to SEQ ID NO:10 with a sequence identity of at least 90%.

In another aspect, provided is a CSR::T1R chimeric protein comprising two polypeptide subunits as defined herein-above which is a CSR::T1R2/CSR::T1R3 heterodimeric chimeric protein including but not limited to a CSR::T1R2-a/CSR::T1R3-a heterodimeric protein, a CSR::T1R2-b/CSR::T1R3-b heterodimeric protein, a CSR::T1R2-a/CSR::T1R3-b heterodimeric protein, a CSR::T1R2-b/CSR::T1R3-a heterodimeric protein, or a heterodimeric protein substantially homologous thereto as herein defined, wherein CSR::T1R2-a corresponds to SEQ ID NO: 2, CSR::T1R2-b corresponds to SEQ ID NO:20, CSR::T1R3-a corresponds to SEQ ID NO:4 and CSR::T1R3-b corresponds to SEQ ID NO:22.

In another aspect, provided is a nucleic acid encoding a CSR::T1R chimeric protein able to bind at least one sweetener or sweetness enhancer comprising one or more of a nucleic acid substantially homologous to a nucleotide sequence selected from

the group consisting of SEQ ID NO:1 (CSR::T1R2-a), SEQ ID NO: 19 (CSR::T1R2-b); SEQ ID NO:3 (CSR::T1R3-a), and SEQ ID NO: 21 (CSR::T1R3-b), as determined by sequence identity, a nucleic acid substantially homologous to a nucleotide sequence selected from the group consisting of SEQ ID NO:1 (CSR::T1R2-a), SEQ ID NO: 19 (CSR::T1R2-b); SEQ ID NO:3 (CSR::T1R3-a), and SEQ ID NO: 21 (CSR::T1R3-b), as determined by hybridisation, a nucleic acid substantially homologous to a nucleotide sequence encoding the CSR::T1R chimeric protein as defined in claim 1, wherein the substantially homologous nucleic acid as determined by sequence identity has a sequence identity of at least 90%; wherein the substantially homologous nucleic acid as determined by hybridisation hybridises under stringent hybridization conditions at a temperature of 42° C in a solution consisting of 50% formamide, 5×SSC, and 1% SDS, and washing at 65° C in a solution consisting of 0.2×SSC and 0.1% SDS; wherein the nucleic acid optionally comprises SEQ ID NO:6 (HSV tag) at or near its end to form the C-terminus in the corresponding protein.

In another aspect, provided is an expression vector comprising the nucleic acid as defined herein-above.

In another aspect, provided is a host cell transfected with an expression vector as defined in herein-above.

In another aspect, provided is a host cell as described herein-above stably expressing a CSR::T1R chimeric protein as defined herein-above and a G-Protein, optionally a G-Protein substantially homologous to Gaq-Gustducin.

In another aspect, provided is a host cell as described herein-above, transiently expressing a CSR::T1R chimeric protein as described herein-above and a G-Protein, optionally a G-Protein substantially homologous to Gaq-Gustducin.

In another aspect, provided is a method of producing a CSR::T1R chimeric protein as defined herein-above, comprising the step of culturing host cells comprising an expression vector encoding for the CSR::T1R chimeric protein, under conditions sufficient for expression, thereby forming the CSR::T1R chimeric protein and optionally recovering it from the cells.

In another aspect, provided is a method to identify an agent that modulates sweet taste signaling in taste cells, the method comprising:
(i) contacting cells that express a CSR::T1R chimeric protein that responds to stimuli selected from sweet taste stimuli and calcium stimuli with an agent thereby providing a functional response, optionally in presence of another agent; and
(ii) determining whether at least one agent affects the functional response of said CSR::T1R chimeric protein in said cells by at least one functional response in said cells;
wherein said CSR::T1R chimeric protein is as defined herein-above.
In another aspect, provided is a method as defined herein-above wherein the cells also express a G-Protein.

In another aspect, provided is a method as defined herein-above wherein the G-Protein is a chimeric G-protein substantially homologous to Gaq-Gustducin.

In another aspect, provided is a method as defined herein-above wherein the G-Protein is the chimeric G-protein G alpha 16-gustducin 44.

In another aspect, provided is a method as defined herein-above wherein step (ii) is performed by measuring a change in or caused by intracellular messengers.

In another aspect, provided is a method as defined herein-above wherein the functional response is determined by measuring a change in an intracellular messenger selected from IP3 and calcium^{2+}.

In another aspect, provided is a method as defined herein-above wherein said cells are selected from the group consisting of bacterial cells, eucaryotic cells, yeast cells, insect cells, mammalian cells, amphibian cells, and worm cells.

In another aspect, provided is a method as described herein-above, wherein the cell is a mammalian cell.

In another aspect, provided is a method as described herein-above wherein the cell is a mammalian cell selected from the group consisting of CHO, COS, HeLa and HEK-293 cells.

In another aspect, provided is a method as described herein-above, wherein step (i) further comprises contacting the CSR::T1R chimeric protein with a test agent in presence of calcium.

In another aspect, provided is a method as described herein-above, wherein the calcium is provided in the form of calcium chloride.

In another aspect, provided is a kit comprising
(i) recombinant cells that express a CSR::T1R chimeric protein as defined herein-above, and
(ii) an agonist of the CSR::T1R chimeric protein,
for combined use to identify test agents as modulators of the CSR::T1R chimeric protein.

In another aspect, provided is a method of using the kit as defined herein-above comprising:
(i) growing recombinant cells that express a CSR::T1R chimeric protein as defined herein-above;
(ii) adding test agents in the presence of an agonist in a suitable concentration;
(iii) determining a change in a functional response of the cells by comparing the response in presence and absence of the test agent, and the test agent is thereby identified as a modulator of the CSR::T1R chimeric protein as defined herein-above.

In another aspect, provided is a method to identify an agent that modulates the CSR::T1R chimeric protein as defined herein-above, the method comprising:
(i) measuring a parameter that changes in response to a ligand binding to the CSR::T1R chimeric protein, and
(ii) determining a change of the parameter in response to a test agent, optionally in presence of a ligand, in comparison to a negative control and thereby identifying a modulator or ligand.

In another aspect, provided is a method as defined herein-above wherein the ligand is selected from the group consisting of calcium, calcium ions and calcium chloride.

In another aspect, provided is a method as defined herein-above, wherein step (i) is performed by a method selected from the group consisting of fluorescence spectroscopy, NMR spectroscopy, measuring of one or more of absorbance,

refractive index, hydrodynamic methods, chromatography, measuring solubility, biochemical methods, wherein the methods measure the properties of the CSR::T1R chimeric protein in a suitable environment selected form the group consisting of solution, bilayer membrane, attached to a solid phase, in a lipid monolayer, bound on a membrane, and in vesicles.

DETAILED DESCRIPTION

CSR::T1R chimeric proteins include but are not limited to a CSR::T1R2 monomer, a CSR::T1R3 monomer, a CSR::T1R2/CSR::T1R3 heterodimer, a CSR::T1R2/T1R3 heterodimer (chimeric T1R2 subunit with wildtype T1R3), and a T1R2/CSR::T1R3 heterodimer (chimeric T1R3 subunit with wildtype T1R2).

CSR::T1R2 includes but is not limited to CSR::T1R2-a and CSR::T1R2-b.
CSR::T1R3 includes but is not limited to CSR::T1R3-a or CSR::T1R3-b.
Each -a variant differs from the relevant –b variant in the exact location where the two parts of different origin (CSR and T1R, respectively) are joined to give the chimeric CSR::T1R protein. Variants-a are joined just before the cysteine-rich domain (CRD), variants-b are joined just after the CRD, as is apparent from their sequences (SEQ ID NO: 1 + 2: CSR::T1R2-a nucleic acid + protein; SEQ ID NO: 3 + 4: CSR::T1R3-a nucleic acid + protein; SEQ ID NO: 19 + 20: CSR::T1R2-b nucleic acid + protein; SEQ ID NO: 21 + 22: CSR::T1R3-b nucleic acid + protein).

Each chimeric subunits may be combined with each other chimeric subunit or the wildtype subunit. Accordingly, the CSR::T1R chimeric proteins include, in particular, a CSR::T1R2-a monomer, a CSR::T1R3-a monomer, a CSR::T1R2-a/CSR::T1R3-a heterodimer, a CSR::T1R2-a/T1R3 heterodimer (chimeric T1R2-a subunit with wildtype T1R3), and a T1R2/CSR::T1R3-a heterodimer (chimeric T1R3-a subunit with wildtype T1R2), as well as a CSR::T1R2-b monomer, a CSR::T1R3-b monomer, a CSR::T1R2-b/CSR::T1R3-b heterodimer, a CSR::T1R2-b/T1R3 heterodimer (chimeric T1R2-a subunit with wildtype T1R3), a T1R2/CSR::T1R3-b heterodimer (chimeric T1R3-a subunit with wildtype T1R2), a CSR::T1R2-a/CSR::T1R3-b heterodimer, and a CSR::T1R2-b/CSR::T1R3-a heterodimer.

The CSR:T1R chimeric protein does not possess the VFT domains of T1R2, T1R3, or T1R2 and T1R3, and therefore allows specifically identification of compounds that bind to the TMD domains and the cystein-rich domains of T1R2 and/or T1R3. These identified compounds are of particular interest as they would not be expected to compete with carbohydrates binding in the VFT site for binding to the sweet taste receptor in vivo and are therefore particularly interesting potential candidates for sweetness enhancers of carbohydrates.

Chimeric proteins are joined fragments of two or more original proteins that sometimes are able to combine desired properties or eliminate unwanted ones. As the folding of a protein in the three dimensional space is critical and the position of amino acids will influence the folding, not any two fragments can be joined. Even if critical domains and amino acids are known, the successful expression, correct folding and intact functionality of desired properties is very much unpredictable.

Applicant has found that the chimeric monomers, CSR::T1R2 and CSR::T1R3, are functional and are able to form a functional CSR::T1R2/CSR::T1R3 heterodimer (see examples for the CSR::T1R2-a/CSR::T1R3-a and CSR::T1R2-b/CSR::T1R3-b heterodimers; CSR::T1R2-a/CSR::T1R3-b and CSR::T1R2-b/CSR::T1R3-a heterodimers may also work). Experiments of the applicant indicate that the CSR::T1R2 monomeric subunit also functions as a functional sweet receptor on its own, without forming a heterodimer. Preliminary experiments indicate that the CSR::T1R3 may have difficulties in engaging and/or activating certain G-proteins; however, CSR::T1R3 is useful in binding assays that do not require the ability to activate a G-Protein.

Accordingly, CSR::T1R2 and CSR::T1R3 are also useful in their monomeric form in the methods described herein. Alternative heterodimers that can be used in these methods are chimeric subunit/wildtype subunit heterodimers (CSR::T1R2/T1R3 and T1R2/CSR::T1R3).

In the CSR::T1R2/CSR::T1R3 heterodimer, each of the CSR::T1R subunits of the heterodimeric complex consist of joined sequence fragments from two source proteins. The two source proteins are the human calcium-sensing receptor (hCaSR), and a T1R protein (T1R2 or T1R3). The hCaSR-derived fragment (CSR) common to both subunits comprises the extracellular domain (ECD) of hCaSR. The T1R-derived

fragments comprise the transmembrane domains (TMD) of the T1R sequences and differ, as they are derived from either T1R2 or T1R3.

The chimeric protein allows to use calcium as a ligand/agonist for receptor activation instead of sweeteners, so that any adverse effects due to the presence of sweeteners can be avoided.

The term CSR::T1R, as used herein, designates the CSR::T1R2 homomer; or the CSR::T1R3 homomer; or the heterodimeric complex of CSR::T1R2 with CSR::T1R3 or with the wildtype T1R3 (CSR::T1R2/CSR::T1R3 or CSR::T1R2/T1R3); or the heterodimeric complex of CSR::T1R3 with CSR::T1R2 or with the wildtype T1R2 (CSR::T1R2/CSR::T1R3 or T1R2/CSR::T1R3).

More generally, as the receptor is coupled to a G-Protein in vivo and in many in vitro methods, CSR::T1R is also referred to as "the GPCR".

The chimeric CSR::T1R constructs that are provided (DNA, vectors, transfected cells, proteins) are useful when screening, without limitation, for modulators of the sweet taste response, for example, without limitation, sweetness enhancers. Traditional screening methods and binding assays may be used to screen for modulators and enhancers. Such screening methodology is well-known in the art, and is outlined below.

Alternatively, as the CSR (part of the hCaSR) in the chimeric CSR::T1R constructs renders the resulting receptors responsive to calcium, when screening for a modulator in presence/absence of a ligand/agonist of the T1R receptor, the ligand is replaced with calcium (for example, without limitation, in the form of calcium chloride). This has the additional advantage of avoiding any negative effects of the actual ligand/agonist being present. For example, when screening for modulators of sugar ligands/agonists, the adverse effects of sugars on osmolarity etc. is avoided.

Cells used in the assays:

Transfected or endogenous T1R3 and T1R2 can negatively interfere with methods that determine agonist responses of CSR::T1R2 and/or CSR::T1R3, respectively, or the change of said responses dependent on another modulator. The absence of T1R3 and T1R2 provides a null background for the determination of CSR::T1R2

and/or CSR::T1R3 activation, so that observed signals can be directly attributed to CSR::T1R2 and/or CSR::T1R3 activity. This allows the identification of agents that specifically modulate CSR::T1R2 and/or CSR::T1R3, and excludes agents that activates the wildtype T1R2 and T1R3, which could in the case of T1R3 also include umami tastants, as T1R3 is part of both the sweet and the umami heterodimers.

The presence of the endogenous wildtype T1R2 and/or T1R3 will cause some backgrounds signals, which are undesirable. While cells with endogenous T1R2 and/or T1R3 can still be useful to obtain results with sufficiently low background, a better choice are cells that do not contain the endogenous T1R2 and T1R3 receptors. An exception occurs when using a CSR::T1R2/T1R3 chimeric protein, which may contain wildtype T1R3 without adverse effect on the background, or a T1R2/CSR::T1R3 chimeric protein, which may contain wildtype T1R2 without adverse effects on the background.

The cells listed below are particularly useful as they do not contain endogenous/wildtype T1R3, or endogenous wildtype T1R2.

However, alternative cells are also useful in the methods described herein.

Suitable eucaryotic cells include eucaryotic cells, for example, without limitation, mammalian cells, yeast cells, or insect cells (including Sf9), amphibian cells (including melanophore cells), or worm cells including cells of Caenorhabditis (including Caenorhabditis elegans).
Suitable mammalian cells include, for example, without limitation, COS cells (including Cos-1 and Cos-7), CHO cells, HEK293 cells, HEK293T cells, HEK293 T-RexTM cells, or other transfectable eucaryotic cell lines.

Suitable bacterial cells include without limitation E. coli.

Cells may be transfected with a GPCR and a G-protein (which links the receptor to a phospholipase C signal transduction pathway) transiently or stably, as is well known in the art. An excellent heterologous expression system that employs the chimeric G-protein G alpha 16-gustducin 44 (also known as G.sub..alpha.16 gust(ducin)44, G.sub.alpha.16gust(ducin)44, Gα16gust(ducin)44, Ga16gust(ducin)44, Gα16-gustducin 44, or as used herein-below, "G16gust44") which provides for enhanced coupling to taste GPCRs, is described in detail in WO 2004/055048. Alternatively,

other chimeric G-proteins based on Gaq-Gustducin described in WO 2004/055048, or other G-Proteins, for example, G16 or G15, may also be used.

The CSR::T1R can be expressed in a cell with a G-protein that links the receptor to a signal transduction pathway, for example, the phospholipase C signal transduction pathway, or signal transduction pathways including, for example, the following: adenylate cyclase, guanylate cyclase, phospholipase C, IP3, GTPase/GTP binding, arachinoid acid, cAMP/cGMP, DAG, protein kinase c (PKC), MAP kinase tyrosine kinase, or ERK kinase.

Alternatively, any suitable reporter gene may be linked to a CSR::T1R-activation responsive promoter and used to determine CSR::T1R activity, as described in more detail herein-below.

Vector constructs used in cells described herein-above:

The vector constructs for expressing the GPCR and/or the G-protein in such cells may be produced in a manner known per se using Polymerase Chain Reactions. After verfication of the sequence, cDNA fragments may be sub-cloned into a suitable vector, for example pcDNA 3.1 mammalian expression vector for mammalian cells, and transiently transfected in a corresponding host cell to enable the correct expression of the gene.

After a post-transfection period, for example 48 hours, cell lysates may be prepared, analysed by a Western-Blot analysis in order to confirm the correct expression of the protein. Once correct protein expression is confirmed, suitable cells, for example mammalian cells including HEK293T cells and HEK T-Rex™, may be transfected to generate cells stably expressing the protein according to techniques well known in the art.

Alternatively, a variety of non-mammalian expression vector/host systems can be used to contain and express sequences encoding the CSR::T1R G-Protein coupled receptor (GPCR). These include, for example, microorganisms including bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems

infected with viral expression vectors (for example baculovirus), or with bacterial expression vectors (for example pBR322 plasmids).

Examples of specific vectors that may be used with the systems described herein-above are described in "G-protein coupled receptors (Signal Transduction Series)"; Editors: Tatsuya Haga and Gabriel Berstein, 1st ed., CRC Press - Boca Raton FL; September 1999.

In bacterial systems, a number of cloning and expression vectors may be selected depending upon the use intended for polynucleotide sequences encoding the GPCR. For example, routine cloning, subcloning, and propagation of polynucleotide sequences encoding a GPCR can be achieved using a multifunctional E. coli vector such as pBLUESCRIPT (Stratagene, La Jolla Calif.) or pSPORT1 plasmid (Life Technologies). Ligation of sequences encoding a GPCR into the vector's multiple cloning site disrupts the lacZ gene, allowing a colorimetric screening procedure for identification of transformed bacteria containing recombinant molecules. In addition, these vectors may be useful for in vitro transcription, dideoxy sequencing, single strand rescue with helper phage, and creation of nested deletions in the cloned sequence. When large quantities of a GPCR are needed, for example, for the production of antibodies, vectors which direct high level expression of a GPCR may be used. For example, vectors containing the strong, inducible SP6 or T7 bacteriophage promoter may be used.

Yeast expression systems may be used for production of a GPCR. A number of vectors containing constitutive or inducible promoters, such as alpha factor, alcohol oxidase, and PGH promoters, may be used in the yeast Saccharomyces cerevisiae or Pichia pastoris. In addition, such vectors direct either the secretion or intracellular retention of expressed proteins and enable integration of foreign sequences into the host genome for stable propagation.

For the expression of heterologous proteins in insect cell lines is, for example, derivatives of the Lepidopteran baculovirus, *Autographa californica* multicapsid nucleo-virus (*Ac*MNPV) can be used. In this system, foreign gene expression is directed by a very strong late viral promoter, either the polyhedrin or *p10* promoters, and a wide array of vectors is available that optimises expression and recovery of recombinant proteins. These vectors enable expression of both membrane-bound and secreted proteins at high levels, and also many post-translational modifications known to occur in mammalian systems, including N- and O-linked glycosylation,

phosphorylation, acylation, proteolysis and secreted vaccine components. A number of vectors are commercially available, for example the InsectSelect™ System from Invitrogen.

Expression systems:

In order to express cDNAs encoding the desired proteins (GPCR (CSR::T1R) and G-protein), one typically subclones the appropriate cDNA into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and a ribosome-binding site for translational initiation. Suitable bacterial promoters are well known in the art, for example, E. coli, Bacillus sp., and Salmonella, and kits for such expression systems are commercially available. Similarly, eukaryotic expression systems for mammalian cells, yeast, and insect cells are commercially available. The eukaryotic expression vector may be, for example, an adenoviral vector, an adeno-associated vector, or a retroviral vector.

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the protein-encoding nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding the protein and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding the protein may typically be linked to a membrane-targeting signal such as the N-terminal 45 amino acids of the rat Somatostatin-3 receptor sequence to promote efficient cell-surface expression of the recombinant protein, which is useful for cell-surface receptors. Additional elements may include, for example, enhancers.

An expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

For expression of the proteins, conventional vectors for expression in eucaryotic or procaryotic cells well known in the art may be used. Examples of vectors include bacterial expression vectors, for example, plasmids including pBR322-based

plasmids, pSKF, and pET23D, and fusion expression systems , for example, GST and LacZ.

Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, for example SV40 vectors, cytomegalovirus vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A$^+$, pMTO10/A$^+$, pMAMneo-5, baculovirus pDSVE, pcDNA3.1, pIRES and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, dihydrofolate reductase and the like.

The elements that are typically included in expression vectors may also include a replicon that functions in E. coli, a gene encoding drug resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in non-essential regions of the plasmid to allow insertion of eukaryotic sequences. The particular drug resistance gene chosen is not critical, any of the many drug resistance genes known in the art are suitable. The prokaryotic sequences are optionally chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

In bacterial systems the GPCR cDNA fragment may be expressed alone or as a fusion protein wherein the GPCR of interest is fused to the E. coli periplasmic maltose-binding protein (MBP) wherein the MBP, including its signal peptide, is linked to the amino terminus of the GPCR. The wild-type GPCR cDNA or the MBP:GPCR fusion cDNA is subcloned into a suitable plasmid, for example pBR322, where in E. coli, GPCR expression is driven by the *lac* wild-type promoter. Methods of expression of GPCRs in E. coli are described, for example, in "G-protein coupled receptors (Signal Transduction Series)"; Editors: Tatsuya Haga and Gabriel Berstein, 1st ed., pp. 265-280 CRC Press - Boca Raton FL; September 1999.

Genetically engineered yeast systems and insect cell systems which lack endogenous GPCRs provide the advantage of a null background for CSR::T1R activation screening.

Genetically engineered yeast systems substitute a human GPCR and Gα protein for the corresponding components of the endogenous yeast pheromone receptor pathway. Downstream signaling pathways are also modified so that the normal yeast response to the signal is converted to positive growth on selective media or to reporter gene expression (described by Broach, J. R. and J. Thorner (1996) Nature 384 (supp.):14-16).

Genetically engineered insect systems incorporate a human GPCR and Gα protein that enables receptor coupling the the phospholipase C signaling pathway (see for example Knight and Grigliatti, (2004) J Receptors and Signal Transduction 24: 241-256).
Amphibian cell systems, in particular melanophore cells, are described, for example, in WO 92/01810 that describes a GPCR expression system.

Overexpression of CSR::T1R:

CSR::T1R may be overexpressed by placing it under the control of a strong constitutive promoter, for example the CMV early promoter. Alternatively, certain mutations of conserved GPCR amino acids or amino acid domains can be introduced to render the employed GPCR constitutively active.

Transfection of CSR::T1R expression vector constructs into cells:

Standard transfection methods can be used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of the protein.

Any known method for introducing nucleotide sequences into host cells may be used. It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing the relevant genes into the host cell capable of expressing the proteins of interest. These methods may involve introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell and include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and the like.

For example, without limitation, the T-Rex™ expression system (Invitrogen Corp., Carlsbad, CA) may be used. The T-Rex™ System is a tetracycline-regulated mammalian expression system that uses regulatory elements from the E. coli Tn10-encoded tetracycline (Tet) resistance operon. Tetracycline regulation in the T-Rex™ System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest.

Cell culture:

After transfection, the transfected cells may be cultured using standard culturing conditions well known in the art. It will be apparent to the skilled person that different cells require different culture conditions including approriate temperature and cell culture media.

CSR::T1R receptor protein recovery:

If desired, the protein may be recovered from the cell culture using standard techniques. For example, the cells may be burst open either mechanically or by osmotic shock before being subject to precipitation and chromatography steps, the nature and sequence of which will depend on the particular recombinant material to be recovered. Alternatively, the recombinant protein may be recovered from the culture medium in which the recombinant cells had been cultured.

Modulators that may be identified by the assays:

Modulators (various types including ligands, agonists, partial agonists, antagonists, inverse agonists, inhibitors, enhancers) of CSR::T1R receptor activity can be identified as described herein below.
The type of a modulator may include more than one type at a time, and may depend on the concentration. For example, an agent may act as an agonist in a certain concentration range, but act as a modulator or enhancer of another agonist (for example a sweetener or sugar) in another concentration range. Therefore, agents should be tested at different concentrations to identify them as modulators.

There now follows a definition of the agents to be identified in the methods described herein.

A modulator is an agent that effects an increase or decrease of one or more of the following: the cell surface expression of a receptor, the binding of a ligand to a receptor, the intracellular response initiated by an active form of the receptor (either in the presence or absence or an agonist). The modulator can itself be an agonist that binds to the receptor, activates it and thereby modulates an increase in the cellular response.

Modulators include various types of compounds, including small molecules, peptides, proteins, nucleic acids, antibodies or fragments thereof. These can be derived from various sources including synthetic or natural, extracts of natural material, for example from animal, mammalian, insect, plant, bacterial or fungal cell material or cultured cells, or conditioned medium of such cells.

A ligand is an agent that binds to the receptor; it may be an agonist, partial agonist, enhancer, antagonist, or inverse agonist.

An agonist is a ligand of the CSR::T1R chimeric protein receptor that activates the receptor and increases an intracellular response when it binds to a receptor compared to the intracellular response in the absence of the agonist. Additionally or alternatively, an agonist may decrease internalization of a cell surface receptor such that the cell surface expression of a receptor is increased as compared to the number of cell surface receptors present on the surface of a cell in the absence of an agonist.
Agonists of CSR::T1R include, for example, calcium, perillartine, cyclamate, NDHC, and cinnamonitrile.

A ligand of the CSR::T1R chimeric protein can be divided into two types, a CSR-domain-ligand which binds in the CSR part of the chimeric protein (calcium), or a TSR-domain ligand, which binds in the T1R-part of the chimeric protein (modulators of the sweet taste response).

A partial agonist is an agonist that only partially activates the receptor in comparison to other agonists that maximally activate the receptor.

An antagonist is a ligand which binds to the receptor at the same (competitive antagonist) or at a different site (alllosteric antagonist) as an agonist, but does not

activate an intracellular response initiated by an active form of a receptor, thereby inhibiting the intracellular response induced by an agonist as compared to the intracellular response in the presence of an agonist and in the absence of an antagonist.

An inverse agonist, binding to a receptor, decreases the constitutive intracellular response mediated by a receptor as compared to the intracellular response in the absence of the inverse agonist.

An inhibitor decreases the binding of an agonist to the receptor as compared to the binding of the agonist in the absence of inhibitor, and/or decreases the intracellular response induced by an agonist.

An enhancer increases the binding of an agonist to the receptor as compared to the binding of the agonist in the absence of enhancer, and/or increases the intracellular response induced by an agonist.

The activity, or changes in activity, of a receptor binding a ligand and transmitting the signal through, for example, a G-protein (i.e. due to different interactions with modulators) can be determined by the assays described herein-below.

Assays to identify modulators of the CSR::T1R receptor:

Modulators can be identified using a wide variety of *in vitro* and *in vivo* assays to determine and compare functional effects/parameters, or alternatively by binding assays. The effects of the test agents upon the function of the receptors can be measured by examining a suitable functional parameters. Any physiological change that affects receptor activity can be used to identify modulators.

Such functional assays are well-known in the art, for example assays using intact cells or tissues isolated from animals based on measuring the concentration or activity or their change of a secondary messenger (including, for example, intracellular calcium (Ca2+), cAMP, cGMP, inositol phospate (IP3), diacylglycerol/DAG, arachinoid acid, MAP kinase or tyrosine kinase), ion flux, phosphorylation levels, transcription levels, neurotransmitter levels, and assays based on GTP-binding, GTPase, adenylate cyclase, phospholipid-breakdown, diacylglycerol, inositol triphosphate, arachidonic acid release, PKC, kinase and

transcriptional reporters. Some suitable assays are, for example, described in WO 01 18050.

Receptor activation typically initiates subsequent intracellular events, for example, increases in second messengers, for example, IP3, which releases intracellular stores of calcium ions. Activation of some G-protein coupled receptors stimulates the formation of inositol triphosphate (IP3) through phospholipase C-mediated hydrolysis of phosphatidylinositol. IP3 in turn stimulates the release of intracellular calcium ion stores. Thus, a change in cytoplasmic calcium ion levels, or a change in second messenger levels such as IP3 can be used to determine G-protein coupled receptor activity.

All functional assays may be performed by samples containing cells expressing the receptor on their surfaces or on isolated cell membrane fractions. Useful cells are described herein-above. Instead of samples with separate cells or cell membranes, tissues from transgenic animals may be used.

The screening methods described herein are particularly useful to identify modulators of the sweet taste response, for example, sweetness enhancers.

To identify a modulator which is not an agonist itself (e.g. an antagonist, partial agonist, inverse agonist, inhibitor, or enhancer), samples with and without test agent both containing an agonist are compared. As agonist, for example, calcium can be used. Using calcium has the advantage that both TMDs will be accessible. Other known or identified agonists can also be used, for example, perillartine, cyclamate, neohesperidine dihydrochalone (NDHC), and cinnamonitrile), but will partially occupy ligand/agonist binding sites which may coincide with the modulator binding site of the to be identified modulator, and may cause lower signals. For example, a control (with agonist but without modulator) is assigned a relative receptor activity value of 100. A decrease in activity relative to the control identifies an inhibitor, antagonist or inverse agonist, an increase identifies an enhancer. Usually, an increase or decrease in the measured activity of 10% or more in a sample with test agent compared to a sample without test agent or compared to a sample with test agent but based on cells that do not express CSR::T1R (mock-transfected cells) can be considered significant.

To identify a sweetness enhancer, samples with and without test agent are compared. For example, a control (with agonist, for example calcium chloride, but

without modulator) is assigned a relative receptor activity value of 100. An increase identifies an enhancer. Usually, an increase or decrease in the measured activity of 10% or more in a sample with test agent compared to a sample without test agent or compared to a sample with test agent but based on cells that do not express CSR::T1R (mock-transfected cells) can be considered significant.
For screens that employ the CSR::TSR1 chimeric protein, calcium can be used as agonist. Alternatively, agonists binding in the relevant parts of the T1R2 and/or T1R3 fragments of CSR::TSR1 may be used. These agonists include, for example, perillartine, cyclamate, NDHC, and cinnamonitrile.

Identification of agonists or partial agonists:

To identify an agonist or partial agonist that does not bind in the VFT domains, a sample with test agent is compared to a positive control with an agonist (for example calcium chloride, perillartine, cyclamate, neohesperidin dihydrochalcone (NDHC), cinnamonitrile, or another identified ligand/agonist).
Alternatively/additionally, samples with and without test agent are compared in their activity of the CSR::T1R chimeric protein.

For example, an agonist or partial agonist will have a biological activity corresponding to at least 10% of the maximal biological activity of the positive control sweet agonist when the agonist or partial agonist is present at 100 mM or less, for example it may have a maximal biologial activity comparable to the agonist or higher. Maximal biological activity is defined as the maximal achievable receptor response to an agonist, for example calcium chloride, perillartine, cyclamate, neohesperidin dihydrochalcone (NDHC), cinnamonitrile that can be achieved within a given receptor assay format and this response fails to increase further despite application of increasing concentrations of that same agonist.

The above-mentioned agonists may, at a different concentration, also act as an enhancer of an agonist of the CSR::T1R chimeric protein. This may be tested in a screening method by using calcium or other agonist to test the agonist-test agent for signals indicating a sweetness enhancing effect.

Alternatively, an increase in the measured activity of, for example, 10% or more in a sample with test agent is compared to a sample without test agent or is compared to

a sample with test agent but based on cells that do not express CSR::T1R (mock-transfected cells).

To identify antagonists, receptor activity in the presence of a known agonist with and without a test agent is compared. Antagonists show a reduction of agonist-stimulated receptor activity, for example by at least 10%.

To identify inverse agonists, receptor activity in the presence of a known agonist with and without a test agent is compared in samples comprising animals/cells/membranes that overexpress the receptor as described herein-above. Inverse agonists show a reduction of constitutive activity of the receptor, for example by at least 10%.

Various examples of suitable detection methods that measure CSR::T1R receptor activity in assays described herein-above follow.

Many screens rely on calcium activity, and for these a buffer system low in calcium should be used to avoid unspecific stimulation of cells, receptor, enzyme or reporter genes)

Detection of changes of cytoplasmic ions or membrane voltage:

Cells are loaded with ion sensitive dyes to report receptor activity, as described in detail in "G-protein coupled receptors (Signal Transduction Series)", CRC Press 1999; 1st Edition; Eds Haga and Berstein. Changes in the concentration of ions in the cytoplasm or membrane voltage are measured using an ion sensitive or membrane voltage fluorescent indicator, respectively.

Calcium flux:

Intracellular calcium release induced by the activation of GPCRs is detected using cell-permeant dyes that bind to calcium. The calcium-bound dyes generate a fluorescence signal that is proportional to the rise in intracellular calcium. The methods allows for rapid and quantitative measurement of receptor activity.

Cells used are transfected cells that co-express the CSR::T1R GPCR and a G-protein which allows for coupling to the phospholipase C pathway as described herein-above. Negative controls include cells or their membranes not expressing

CSR::T1R (mock transfected), to exclude possible non-specific effects of the candidate compound.
The calcium flux detection protocol is described in detail in "G-protein coupled receptors (Signal Transduction Series)"; Editors: Tatsuya Haga and Gabriel Berstein, 1st ed., 424pp.CRC Press - Boca Raton FL; September 1999, and an adapted version with is summarised below:

Day 0: 96-well plates are seeded with 8.5K cells per well and maintained at 37°C overnight in nutritive growth media.

Day 1: Cells are transfected using 150 ng of GPCR DNA and 0.3 μl of Lipofectamine 2000 (Invitrogen) per well. Transfected cells are maintained at 37°C overnight in nutritive growth media.

Day 2: Growth media is discarded and cells are incubated for 1 hour (at room temperature in the dark) with 50 μl of calcium assay solution consisting of 1.5 μM Fluo-4 AM (Molecular Probes) and 2.5 μM probenicid dissolved in a reduced calcium C1 buffer solution which contains 130 mM NaCl, 5 mM KCl, 10 mM Hepes, 0.5 mM CaCl2 and 10 mM glucose (pH 7.4) at 37°C. 125 μl of the reduced calcium C1 buffer is added to each well and the plate is further incubated for 30 minutes at room temperature in the dark.
Buffer solutions are discarded and plate is washed 5 times with 100 μl reduced calcium C1 buffer as a washing buffer and cells are reconstituted in 200 μl of reduced calcium C1 buffer.

Then the plate is placed in a fluorescent microplate reader, for example, the Flexstation (Molecular Devices) or the FLIPR (Molecular Devices) and receptor activation is initiated following addition of 20 μl of a 10X concentrated ligand stock solution. Fluorescence is continuously monitored for 15 seconds prior to ligand addition and for 45 – 110 seconds after ligand addition. Receptor activation levels are defined as by the two following equations: % Activation = (Maximum fluorescence – baseline fluorescence/baseline fluorescence) * 100 or Fluorescence Increase = Maximum Fluorescence – baseline fluorescence, where baseline fluorescence represents the average fluorescence levels prior to ligand addition.

Useful cells are, without limitation, mammalian cells as described herein-above, for example HEK293T cells and HEK293 T-RexTM cells. Cells may be transfected with a GPCR and a G-Protein transiently or stably as is well known in the art. An excellent heterologous expression system is described in detail in WO 2004/055048.

A calcium flux assay can be performed, for example, as described in example 1 herein-below.

The identification of a modulator is performed as described above subject to the following modifications. The signals are compared to the baseline level of CSR::T1R activity obtained from recombinant cells expressing CSR::T1R in the presence of an agonist but in the absence of a test agent. An increase or decrease in CSR::T1R activity, for example of at least 2 fold, at least 5 fold, at least 10 fold , at least a 100 fold, or more identifies a modulator.

Alternatively, the identification involves an increase or decrease fluorescence intensity of, for example, 10% or more, when compared to a sample without modulator, or when compared to a sample with modulator but in cells that do not express the CSR::T1R polypeptide (mock-transfected cells).

Adenylate Cyclase activity:

Assays for adenylate cyclase activity are performed, for example, as described in detail by Kenimer & Nirenberg, 1981, Mol. Pharmacol. 20: 585-591. Reaction mixtures are incubated usually at 37° C for less than 10 minutes. Following incubation, reaction mixtures are deproteinized by the addition of 0.9 ml of cold 6% trichloroacetic acid. Tubes are centrifuged and each supernatant solution is added to a Dowex AG50W-X4 column. The cAMP fraction from the column is eluted with 4 ml of 0.1 mM imidazole-HCl (pH 7.5) into a counting vial in order to measure the levels of cAMP generated following receptor activation by the agonist. Control reactions should also be performed using protein homogenate from cells that do not express a CSR::T1R polypeptide.

IP3/ Ca^{2+} signals:

In cells expressing G-proteins, signals corrseponding to inositol triphosphate (IP3)/Ca^{2+} and thereby receptor activity can be detected using fluorescence. Cells expressing a GPCR may exhibit increased cytoplasmic calcium levels as a result of contribution from both intracellular stores and via activation of ion channels, in which case it may be desirable, although not necessary, to conduct such assays in calcium-free buffer, optionally supplemented with a chelating agent such as EDTA, to distinguish fluorescence response resulting from calcium release from internal stores.

Phospholipase C/intracellular Ca^{2+} signals:

CSR::T1R is expressed in a cell with a G-protein that links the receptor to a phospholipase C signal transduction pathway. Changes in intracellular Ca^{2+} concentration are measured, for example using fluorescent Ca^{2+} indicator dyes and/or fluorometric imaging.

GTPase/GTP Binding:

For a GPCR including CSR::T1R, a measure of receptor activity is the binding of GTP by cell membranes containing the GPCR. Measured is the G-protein coupling to membranes by detecting the binding of labelled GTP.

Membranes isolated from cells expressing the receptor are incubated in a buffer containing 35S-GTPγS and unlabelled GDP. Active GTPase releases the label as inorganic phosphate, which is detected by separation of free inorganic phosphate in a 5% suspension of activated charcoal in 20 mM H_3PO_4, followed by scintillation counting. The mixture is incubated and unbound labelled GTP is removed by filtration onto GF/B filters. Bound and labelled GTP is measured by liquid scintillation counting. Controls include assays using membranes isolated from cells not expressing CSR::T1R (mock-transfected), in order to exclude possible non-specific effects of the test agent. The method is described in detail by Traynor and Nahorski, 1995, Mol. Pharmacol. 47: 848-854.

To identify modulators, as described herein-above, a change (increase or decrease) of 10% or more in GTP binding or GTPase activity is usually sufficient. However, to identify agonists, the assays described herein-above are performed subject to the following modifications. An agent is identified as an agonist usually if the activity is at least 50% of that of a known agonist (for example perillartine) when the compound is present at 100 mM or less, for example 10 to 500 µM, for example about 100 µM, or if it will induce a level the same as or higher than that induced by a known agonist.

Microphysiometer or biosensor:

Such assays can be performed as described in detail in Hafner, 2000, Biosens. Bioelectron. 15: 149-158.

Arachinoid acid:

The intracellular level of arachinoid acid is employed as an indicator of receptor activity. Such a method is described in detail by Gijon et al., 2000,J. Biol. Chem., 275:20146-20156.

cAMP/cGMP:

Intracellular or extracellular cAMP is measured using a cAMP radioimmunoassay (RIA) or cAMP binding protein, for example as described by Horton & Baxendale, 1995, Methods Mol. Biol. 41: 91-105. Alternatively, a number of kits for the measurement of cAMP are commercially available, for example the High Efficiency Fluorescence Polarization-based homogeneous assay by LJL Biosystems and NEN Life Science Products. Alternatively, the intracellular or extracellular levels of cGMP may measured using an immunoassay. For example, the method described in Felley-Bosco et al., Am. J. Resp. Cell and Mol. Biol., 11:159-164 (1994), may be used to determine the level of cGMP. Alternatively an assay kit for measuring cAMP and/or cGMP as described in US 4,115,538 can be used.

Negative controls with mock-transfected cells or extracts thereof to exclude possible non-specific effects of test agents may be used.

DAG/IP3:

Second messengers Diacylglycerol (DAG) and/or inositol triphosphate (IP3), which are released by Phospholipid breakdown, that is caused by receptor activity, can be detected and used as an indicator of GPCR (CSR::T1R) activity, for example as described in Phospholipid Signalling Protocols, edited by Ian M. Bird, Totowa, N.J., Humana Press, 1998. Alternatively, kits for the measurement of inositol triphosphates are available commercially from Perkin Elmer and CisBio International.

Negative controls with mock-transfected cells or extracts thereof to exclude possible non-specific effects of test agents may be used.

PKC Activity :

Growth factor receptor tyrosine kinases can signal via a pathway involving activation of Protein Kinase C (PKC), which is a family of phospholipid- and calcium-activated protein kinases.

Increases in gene products induced by PKC show PKC activation and thereby receptor activity. These gene products include, for example, proto-oncogene transcription factor-encoding genes (including c-fos, c-myc and c-jun), proteases, protease inhibitors (including collagenase type I and plasminogen activator inhibitor), and adhesion molecules (including intracellular adhesion molecule I (ICAM I)).

PKC activity may be directly measured as described by Kikkawa et al., 1982, J. Biol. Chem. 257: 13341, where the phosphorylation of a PKC substrate peptide, which is subsequently separated by binding to phosphocellulose paper, is measured. It can be used to measure activity of purified kinase, or in crude cellular extracts. Protein kinase C sample can be diluted in 20 mM HEPES/ 2 mM DTT immediately prior to the assay.

An alternative assay can be performed using the Protein Kinase C Assay Kit commercially available by PanVera.

The above-described PKC assays are performed on extracts from cells expressing the GPCR (CSR::T1R).

Alternatively, activity can be measured through the use of reporter gene constructs driven by the control sequences of genes activated by PKC activation.

Negative controls with mock-transfected cells or extracts thereof to exclude possible non-specific effects of test agents may be used.

MAP Kinase activity:

MAP kinase activity can be measured using commercially available kits, for example, the p38 MAP Kinase assay kit by New England Biolabs, or the FlashPlate™ MAP Kinase assays by Perkin-Elmer Life Sciences. p42/44 MAP kinases or ERK1/2 can be measured to show GPCR (CSR::T1R) activity when cells with Gq and Gi coupled GPCRs are used, and an ERK1/2 assay kit is commercially available by TGR

Biosciences, which measures the phosphorylation of endogenous ERK1/2 kinases following GPCR activation.

Alternatively, direct measurements of tyrosine kinase activity through known synthetic or natural tyrosine kinase substrates and labelled phosphate are well known; the activity of other types of kinases (for example, Serine/Threonine kinases) can be measured similarly.

All kinase assays can be performed with both purified kinases and crude extracts prepared from cells expressing one or more CSR::T1R polypeptide.
The substrates of kinases that are used can be either full-length protein or synthetic peptides representing the substrate. Pinna & Ruzzene (1996, Biochem. Biophys. Acta 1314: 191-225) lists a number of phosphorylation substrate sites useful for detecting kinase activities. A number of kinase substrate peptides are commercially available. One that is particularly useful is the "Src-related peptide," RRLIEDAEYAARG (commercially available from Sigma), which is a substrate for many receptor and nonreceptor tyrosine kinases. Some methods require the binding of peptide substrates to filters, then the peptide substrates should have a net positive charge to facilitate binding. Generally, peptide substrates should have at least 2 basic residues and a free-amino terminus. Reactions generally use a peptide concentration of 0.7-1.5 mM.

Negative controls with mock-transfected cells or extracts thereof to exclude possible non-specific effects of test agents may be used.

Transcriptional reporters/ CSR::T1R-responsive promoter/reporter gene:
To identify modulators with reporter gene assays, an at least 2-fold increase or 10% decrease in the signal is significant. An agonist stimulates for example at least 2-fold, 5-fold, 10-fold or more when comparing activity in presence and absence of the test agent.
The intracellular signal initiated by binding of an agonist to CSR::T1R sets in motion a cascade of intracellular events, the ultimate consequence of which is a rapid and detectable change in the transcription or translation of one or more genes.

The activity of the receptor can therefore be determined by measuring the expression of a reporter gene driven by a promoter responsive to CSR::T1R activation.

A "promoter" as used herein is one or more transcriptional control elements or sequences necessary for receptor-mediated regulation of gene expression, including one or more of basal promoter, enhancers and transcription-factor binding sites necessary for receptor-regulated expression. Promoters responsive to the intracellular signals resulting from agonist binding to CSR::T1R are selected and operatively linked to a corresponding promoter-controlled reporter gene whose transcription, translation or ultimate activity is readily detectable and measurable.

Reporter genes may be selected, for example, from luciferase, CAT, GFP, β-lactamase, β-galactosidase, and the so-called "immediate early" genes, c-fos proto-oncogene, transcription factor CREB, vasoactive intestinal peptide (VIP) gene, the somatostatin gene, the proenkephalin gene, the phosphoenolpyruvate carboxy-kinase (PEPCK) gene, genes responsive to NF-κB, and AP-1-responsive genes (including the genes for Fos and Jun, Fos-related antigens (Fra) 1 and 2, IκBα, ornithine decarboxylase, and annexins I and II).

Promoters will be selected according to the selected reporter gene, as will be apparent to the skilled person.

Luciferase, CAT, GFP, β-lactamase, β-galactosidase and assays for the detection of their products are well known in the art. Examples of further reporter genes are described herein-below.

The "immediate early" genes are suitable and are rapidly induced (for example within minutes of contact between the receptor and the effector protein or ligand). Desirable properties in reporter genes include one or more of the following: rapid responsiveness to ligand binding, low or undetectable expression in quiescent cells; induction that is transient and independent of new protein synthesis; subsequent shut-off of transcription requires new protein synthesis; and mRNAs transcribed from these genes which have a short half-life of several minutes to a few hours. Similarly, the promoter may have one, several or all of these properties.

The c-fos proto-oncogene is an example of a gene that is responsive to a number of different stimuli and has an rapid induction. The c-fos regulatory elements include a TATA box that is required for transcription initiation; two upstream elements for basal transcription, and an enhancer, which includes an element with dyad symmetry and which is required for induction by TPA, serum, EGF, and PMA. The 20 bp c-fos

transcriptional enhancer element located between -317 and -298 bp upstream from the c-fos mRNA cap site, is essential for serum induction in serum starved NIH 3T3 cells. One of the two upstream elements is located at -63 to -57 and it resembles the consensus sequence for cAMP regulation.

The transcription factor CREB (cyclic AMP responsive element binding protein) is responsive to levels of intracellular cAMP. Therefore, the activation of a receptor that signals via modulation of cAMP levels can be determined by detecting either the binding of the transcription factor, or the expression of a reporter gene linked to a CREB-binding element (termed the CRE, or cAMP response element). The DNA sequence of the CRE is TGACGTCA. Reporter constructs responsive to CREB binding activity are described in US 5,919,649.

Other suitable reporter genes and their promoters include the vasoactive intestinal peptide (VIP) gene and its promoter which is cAMP responsive; the somatostatin gene and its promoter which is cAMP responsive; the proenkephalin and its promoter which is responsive to cAMP, nicotinic agonists, and phorbol esters; and the phosphoenolpyruvate carboxy-kinase (PEPCK) gene and its promoter which is cAMP responsive.

Additional examples of reporter genes and their promoters that are responsive to changes in GPCR activity include the AP-1 transcription factor and NF-κB.
The AP-1 promoter is characterised by a consensus AP-1 binding site which is the palindrome TGA(C/G)TCA. The AP-1 site is also responsible for mediating induction by tumor promoters including the phorbol ester 12-O-tetradecanoylphorbol-β-acetate (TPA), and are therefore sometimes also referred to as a TRE, for TPA-response element. AP-1 activates numerous genes that are involved in the early response of cells to growth stimuli. Examples of AP-1-responsive genes include the genes for Fos and Jun (which proteins themselves make up AP-1 activity), Fos-related antigens (Fra) 1 and 2, IκBα, ornithine decarboxylase, and annexins I and II.

The NF-κB promoter/binding element has the consensus sequence GGGGACTTTCC. A large number of genes have been identified as NF-κB responsive, and their control elements can be linked to a reporter gene to monitor GPCR activity. Genes responsive to NF-κB include for example those encoding IL-1β, TNF-α, CCR5, P-selection, Fas ligand, GM-CSF and IκBα. Vectors encoding NF-κB-responsive reporters are known in the art or can be readily formed using ordinary

skill in the art, for example, synthetic NF-κB elements and a minimal promoter, or using the NF-κB-responsive sequences of a gene known to be subject to NF-κB regulation. Further, NF-κB responsive reporter constructs are commercially available from, for example, CLONTECH.

A given promoter construct can easily be tested by exposing GPCR (CSR::T1R)-expressing cells, transfected with the construct, to an agonist (for example perillartine). An increase of at least 2-fold in the expression of reporter gene in response to the agonist indicates that the reporter is suitable to measure GPCR (CSR::T1R) activity.

Controls for transcription assays include both cells not expressing GPCR (CSR::T1R), but carrying the reporter construct, and cells with a promoterless reporter construct.

Agents that modulate GPCR (CSR::T1R) activity as shown by reporter gene activation can be verified by using other promoters and/or other receptors to verify GPCR (CSR::T1R) specificity of the signal and determine the spectrum of their activity, thereby excluding any non-specific signals, for example non-specific signals via the reporter gene pathway.

Inositol Phosphates (IP) Measurement:

Phosphatidyl inositol (PI) hydrolysis may be determined as described in US 5,436,128, which involves labelling of cells with 3H-myoinositol for at least 48 hours or more. The labelled cells are contacted with a test agent for one hour, then these cells are lysed and extracted in chloroform-methanol-water. This is followed by separating the inositol phosphates by ion exchange chromatography and quantifying them by scintillation counting. For agonists, fold stimulation is determined by calculating the ratio of counts per minute (cpm) in the presence of tested agent, to cpm in the presence of buffer control. Likewise, for inhibitors, antagonists and inverse agonists, fold inhibition is determined by calculating the ratio of cpm in the presence of test agent, to cpm in the presence of buffer control (which may or may not contain an agonist).

Binding assays:

Alternatively to the functional assays described herein-above that measure a change in parameters caused by a funtional response to ligand binding, ligand binding may be determined by binding assays that measure the binding of a ligand to a CSR::T1R receptor.

Binding assays are well known in the art and can be tested in solution, in a bilayer membrane, optionally attached to a solid phase, in a lipid monolayer, or in vesicles. Binding of a modulator to a CSR::T1R polypeptide can be determined, for example, by measuring changes in spectroscopic characteristics (for example fluorescence, absorbance, or refractive index), hydrodynamic methods (employing for example shape), chromatography, measuring solubility properties of a CSR::T1R polypeptide. In one embodiment, binding assays are biochemical and use membrane extracts from cells/tissue expressing recombinant CSR::T1R polypeptides. A binding assay may, for example, be performed as described for T1Rs by Adler et al. in US20050032158, paragraphs [0169] to [0198].

CSR::T1R receptor polypeptide and nucleic acid, and substantially homologous polypeptides and nucleic acids:

The CSR::T1R chimeric protein useful in methods described herein may be selected from the group consisting of the polypeptide selected from SEQ ID NO:2 (CSR::T1R2-a), SEQ ID NO:4 (CSR::T1R3-a), SEQ ID NO: 20 (CSR::T1R2-b), SEQ ID NO: 22 (CSR::T1R3-b), the chimeric heterodimer of SEQ ID NO: 2 and SEQ ID NO:4 (CSR::T1R2-a/ CSR::T1R3-a), the chimeric heterodimer of SEQ ID NO: 20 and SEQ ID NO: 22 (CSR::T1R2-b/ CSR::T1R3-b), the chimeric heterodimer of SEQ ID NO: 2 and SEQ ID NO: 22 (CSR::T1R2-a/ CSR::T1R3-b), the chimeric heterodimer of SEQ ID NO: 20 and SEQ ID NO: 4 (CSR::T1R2-b/ CSR::T1R3-a), a heterodimer of SEQ ID NO:2 or 20 with wildtype T1R3 (CSR::T1R2-a/ T1R3 or CSR::T1R2-b/ T1R3), a heterodimer of SEQ ID NO: 4 or 22 with wildtype T1R2 (T1R2/ CSR::T1R3-a or T1R2/ CSR::T1R3-b).
Alternatively, the CSR::T1R chimeric protein (or nucleic acid encoding the CSR::T1R) may be a receptor (or nucleotide sequence to form such a CSR::T1R receptor) which is subtantially homologous and remains functional (i.e. binds to ligands and/or is activated by ligands, or encodes such a receptor).

A substantially homologous CSR::T1R chimeric protein includes such proteins where the T1R2 or T1R3 part of CSR::T1R2 and/or CSR::T1R3 is replaced with the relevant part of an allelic variant or different species, including T1R2 and/or T1R3 from mouse, rat, hamster, ape, and dog.

Further, substantially homologous CSR::T1R nucleotide or polypeptide sequences may be formed by conservative mutations and/or point mutations and include any conservatively modified variant as detailed below.

With respect to nucleic acid sequences, conservatively modified variants means nucleic acids which encode identical or essentially identical amino acid sequences (conservatively substituted amino acids, i.e. lysine switched to arginine and further examples as explained herein-below).

Because of the degeneracy of the genetic code, a large number of nucleic acids different in sequence but functionally identical encode any given polypeptide/protein. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Each nucleic acid sequence which encodes a polypeptide also describes every possible silent variation of the nucleic acid. Therefore, each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical nucleic acid sequence that will produce an identical polypeptide. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each given nucleic acid sequence.

With respect to amino acid sequences, amino acid substitutions may be introduced using known protocols of recombinant gene technology including PCR, gene cloning, site-directed mutagenesis of cDNA, transfection of host cells, and in-vitro transcription which may be used to introduce such changes to the CSR::T1R sequence. The variants can then be screened for taste-cell-specific GPCR functional activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, one exemplary guideline to select conservative substitutions includes (original residue followed by exemplary substitution): ala/gly or ser; arg/lys; asn/gln or his; asp/glu; cys/ser; gln/asn; gly/asp; gly/ala or pro; his/asn or gln; ile/leu or val; leu/ile or val; lys/arg or gin or glu; met/leu or tyr or ile; phe/met or leu or tyr; ser/thr; thr/ser; trp/tyr; tyr/trp or phe; val/ile or leu.

An alternative exemplary guideline uses the following six groups, each containing amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (1); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Another alternative guideline is to allow for all charged amino acids as conservative substitutions for each other whether they are positive or negative.
In addition, individual substitutions, deletions or additions that alter, add or delete a single amino acid or a small percentage (for example up to 26%, or up to 20%, or up to 10%) of amino acids in an encoded sequence are also considered to be conservatively modified variations.

Substantially homologous nucleotide or polypeptide sequences have the degree of sequence identity or hybridize under certain stringent hybridization conditions as indicated below.

%Sequence identity:
A substantially homologous nucleotide sequence has a % sequence identity of at least at least 90%, at least 95%, or at least 98%.
A substantially homologous polypeptide sequence has a % sequence identity of at least at least 90%, at least 95% or at least 98%.

Calculation of % Sequence Identity is determined as follows.
BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastn which is available at http://www.ncbi.nlm.nih.gov.
To determine % identity of a nucleotide query sequence against another nucleotide sequence, Blastn is used, using default parameters of BLAST version 2.2.1.3, including an EXPECT (statistical significance threshold for reporting matches against database sequences) of 10, and DUST filtering.
To determine % identity of a polypeptide query sequence against another polypeptide sequence, Blastp is used, using default parameters of BLAST version 2.2.1.3, including an EXPECT of 10, and DUST filtering.

Stringent hybridization conditions:
Nucleotide sequences are considered substantially homologous provided that they are capable of selectively hybridizing to the nucleotide sequences presented herein, or to their complement, under stringent hybrdisation conditions detailed below. Stringent conditions are temperature of 42° C in a solution consisting of 50% formamide, 5×SSC, and 1% SDS and washing at 65° C in a solution consisting of 0.2×SSC and 0.1% SDS (1×SSC=0.15 M NaCl, 0.015 M Na3 Citrate pH 7.0).

Background hybridization may occur because of other nucleotide sequences present, for example, in the cDNA or genomic DNA library being screened.

A signal that is less than 10 fold as intense as the specific interaction observed with the target DNA is considered background. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with 32P.

Kit to identify a modulator :

A kit, for example, a screening kit or high throughput screening kit, that comprises recombinant cells that express the CSR::T1R, or a substantially homologous sequence thereto; and that comprises an agonist of the CSR::T1R, for example, without limitation, calcium chloride, perillartine, NDHC, cyclamate, and cinnamonitrile.

Using a kit comprising calcium has the advantage of binding to and activating the chimeric protein only, but not the wild-type receptor or the T1R2 and T1R3 part of the chimeric protein.

Optionally, the cells further comprise a G-protein for example for calcium signalling. Suitable G-proteins are known and described herein-above; the skilled person is aware how to introduce them to the cells if necessary. A very useful chimeric G-protein is Galpha16-gustducin 44.

The agonist is provided in suitable concentrations, for example 1nM to 10 mM, or 0.1 microM to1 milliM, for example 0.1 microM to 100 microM.

Useful concentrations are, for example, for calcium chloride 0.2 to 20 mM, for perillartine 5 to 500 μM, for cinnamonitrile 10 to 1000 μM, for cyclamate 0.01 to 5 mM, for Neohesperidin Dihydrochalcone (NDHC) 0.033 to 3.3 mM.

Optional kit components may include a suitable medium for culturing the recombinant cells provided, and a solid support to grow the cells on, for example, a cell culture dish or microtiter plate, these optional components will be readily available to the skilled person.

The kit may be used as follows:
(i) Recombinant cells that express the CSR::T1R chimeric protein are grown on the solid support.
(ii) test agents at concentrations from about 1 nM to 100 mM or more are added to the culture medium of defined plates or wells in the presence of the agonist in a suitable concentration
(iii) a change in a functional response of the cells is determined by comparing the response in presence and absence of the test agent, and the test agent is thereby identified as a modulator.

For example, (iii) may be performed according to any one of the assays described-herein above, in combination with any one of the detection methods that report receptor activity described herein-above. This may require specifically chosen or adapted recombinant cells, which are also described herein-above.
A suitable assay is, for example, the calcium flux assay to determine activation of CSR::T1R and its change in response to a test agent.

The kit may be used to identify an enhancer as follows:
(i) Recombinant cells that express the CSR::T1R chimeric protein are grown on the solid support.
(ii) rest agents at concentrations from about 1 nM to 100 mM or more are added to the culture medium of defined plates or wells in the presence of the calcium agonist (for example, without limitation, in form of calcium chloride) in a suitable concentration.
(iii) a change in a functional response of the cells to calcium is determined by comparing the response in presence and absence of the test agent, and the test agent is thereby identified as an enhancer.

A suitable calcium chloride concentration is, for example, from about 0.2 to 20 mM, or 0.5 to 10 mM, or about 1 mM.

Confirmation of identified modulators:
A modulator identified by a method described herein-above may easily be confirmed by simple sensory experiments using a panel of flavorists or test persons to taste the identified modulators. The compounds are tasted e.g. in water to confirm sweet taste or together with sweet tastants in comparison to a negative control without modulator to confirm a modultor that enhances the sweet taste.

Large scale screening assays:

Transcriptional reporter assays and most cell-based assays described herein-above are well suited for screening libraries for agents that modulate CSR::T1R activity.

The assays may be designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to the assays, which are typically run in parallel (for example in microtiter formats on microtiter plates in robotic assays).

Assays may be run in high throughput screening methods that involve providing a combinatorial chemical or peptide library containing a large number of potential modulators. Such libraries are then screened in one or more assays described herein-above to identify those library agents (particular chemical species or subclasses) that display the activity described herein-above. The modulators thus identified can be directly used or may serve as leads to identify further modulators by making and testing derivatives.

Synthetic compound libraries are commercially available from a number of companies including Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, N.J.), Brandon Associates (Merrimack, N.H.), and Microsource (New Milford, Conn.).

Libraries of test agents:

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given

compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

A rare chemical library is available from Aldrich (Milwaukee, Wis.).

Libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are commercially available for example from Pan Laboratories (Bothell, Wash.) or MycoSearch (NC), or are readily produceable by methods well known in the art. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means.

Other libraries include protein/expression libraries, cDNA libraries from natural sources, including, for example, foods, plants, animals, bacteria, libraries expressing randomly or systematically mutated variants of one or more polypeptides, genomic libraries in viral vectors that are used to express the mRNA content of one cell or tissue.

In a high throughput assay, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 modulators. If 1536 well plates are used, then a single plate can easily assay from about 100 to about 1500 different compounds. It is possible to assay several different plates per day; assay screens for up to about 6,000-20,000 different compounds is possible.

Types of test agents that may be tested for their CSR::T1R modulating effect in the assay methods:

The test agents may be any agent including small chemical compounds, chemical polymers, biological polymers, peptides, proteins, sugars, carbohydrates, nucleic acids and lipids. An agent can be a synthetic compound, a mixture of compounds, a natural product or natural sample, for example plant extract, culture supernatant, or tissue sample.

As examples of compounds that may modifiy sweet taste there may be mentioned methyl chavicol, Theasaponin E1, Acesulfame K, Alitame, Aspartame, CH 401, Dulcin, Neotame, sodium Cyclamate, Sucralose, Superaspartame, Cynarin, Glycyphyllin, Rebaudioside C, Abrusoside A, Abrusoside B, Abrusoside C, Abrusoside D, Abrusoside E, Apioglycyrrhizin, Araboglycyrrhizin, Baiyunoside, Brazzein, Bryodulcoside, Carnosifloside V, Carnosifloside VI, D. cumminsii, Cyclocarioside A, Cyclocarioside I, Dulcoside A, Glycyrrhizic Acid, Hernandulcin, Hernandulcin, 4beta-hydroxy-Hesperitin-7-Glucoside Dihydrochalcone, Huangqioside E, Huangqioside E, 3-Hydroxyphloridzin, 2,3-Dihydro-6-Methoxy 3-O-Acetate, Mabinlin Maltosyl-Alpha-(1,6)-Neohesperidin Dihydrochalcone, Mogroside IIE, Mogroside III, Mogroside IIIE, Mogroside IV, Mogroside V, 11-Oxo Mogroside V, Monatin, Monoammonium Glycyrrhizinate (Mag), Mukuroziosíde Iib, Naringin Dihydrochalcone, Neohesperidin Dihydrochalcone (NHDHC), Neomogroside, Osladin, Periandrin I, Periandrin II, Periandrin III, Periandrin IV, Periandrin V, Phlomisoside I, Phlorizin, Phyllodulcin, Polypodoside A, Potassium magnesium calcium glycyrrhizin, Pterocaryosides A, Pterocaryosides B, Rebaudioside A, Rebaudioside B, Rubusoside, Scandenoside R6, Siamenoside I, Sodium glycyrrhizinate, Steviolbioside, Stevioside, Stevioside, alpha-Glycosyl Suavioside A, Suavioside B, Suavioside G, Suavioside H, Suavioside I, Suavioside J, Thaumatin, Triammonium Glycyrrhizinate (TAG), Trilobatin Curculin, Strogin 1, Strogin 2, Strogin 4, Miraculin, Hodulcin, Jujubasaponin II, Jujubasaponin III, Abrusoside E, Periandrinic acd I, monoglucuronide, Periandrinic acid II, monoglycuronide, Chlorogenic Acid, beta- (1,3-Hydroxy-4-methoxybenzyl)-Hespertin Dihydrochalcone, 3'-Carboxy-Hespertin Dihydrochalcone, 3'- Stevioside analogue.

Identified modulators of sweet tastants may include, for example, modulators of artificial sweeteners that are able to elicit a sweet taste sensation.

Consumables include food products, beverages, oral care products, and compositions for admixture to such products, in particular flavour compositions. Flavour compositions may be added to processed foods or beverages during their processing, or they may actually be consumables in their own right, e.g. condiments such as sauces and the like. Sweet tastants are particularly interesting in confectionary and other sweet consumables including desserts, but also in savoury and sweet-sour consumables. Examples of consumables include confectionary products, cakes, cereal products, baker's products, bread products, gums, chewing gums, sauces (condiments), soups, processed foods, cooked fruits and vegetable products, meat and meat products, egg products, milk and dairy products, cheese

products, butter and butter substitute products, milk substitute products, soy products, edible oils and fat products, medicaments, beverages, alcoholic drinks, beers, soft drinks, food extracts, plant extracts, meat extracts, condiments, sweeteners, nutraceuticals, pharmaceutical and non-pharmaceutical gums, tablets, lozenges, drops, emulsions, elixirs, syrups and other preparations for making beverages, instant beverages and effervescent tablets.

Sequences of nucleic acids and proteins:

The sequences employed in the constructs and methods described-herein can be found in the sequence listing herein-below.

SEQ ID NO:1 and 19 correspond to the nucleotide/nucleic acid sequence encoding the CSR::T1R2 chimeric proteins (-a/-b), SEQ ID NO: 2 and 20correspond to the polypeptide/amino acid sequence of the CSR::T1R2 chimeric proteins (-a and –b) . SEQ ID NO:3 and 21 correspond to the nucleotide/nucleic acid sequence encoding the CSR::T1R3 chimeric proteins (-a and –b), SEQ ID NO: 4 and 22 correspond to the polypeptide/amino acid sequence of the CSR::T1R3 chimeric proteins (-a and –b).
Together as a complex comprising two subunits, the CSR::T1R2 chimeric protein and the CSR::T1R3 chimeric protein form a functional chimeric sweet receptor. The resulting complex may comprise the two –a variants, the two –b variants, or combinations (CSR::T1R2-a with CSR::T1R3-b or CSR::T1R2-b with CSR::T1R3-b), or homologous variants of these that retain their function as herein described.

In the transfected construct, the nucleic acid coding for the novel chimeric protein (SEQ ID NO:1 or 3 for variants-a, and SEQ ID NO: 19 and 21 for variants-b) is followed by the HSV tag at the C-terminus (SEQ ID NO:5).
The resulting proteins will accordingly comprise the following amino acids: amino acids of SEQ ID NO:1 followed by SEQ ID NO:5, SEQ ID NO:19 followed by SEQ ID NO:5, SEQ ID NO: 3 followed by SEQ ID NO:5, or SEQ ID NO:21 followed by SEQ ID NO:5.

The known full-length nucleic acid and protein sequences of the known T1R2 and T1R3 subunits of the T1R2/T1R3 receptor complex are given in SEQ ID NO: 7 + 8 for T1R2, and SEQ ID NO: 9 +10 for T1R3.

The known full length hCaSR receptor nucleic acid and protein sequences are given in SEQ ID NO: 11 + 12.

SEQ ID NO: 1 + 2: CSR::T1R2-a nucleic acid + protein
SEQ ID NO: 3 + 4: CSR::T1R3-a nucleic acid + protein
SEQ ID NO:5 + 6: HSV tag at C-terminus nucleic acid + protein
SEQ ID NO:7 + 8: T1R2 (full length coding sequence) nucleic acid + protein
SEQ ID NO: 9 + 10: T1R3 (full length coding sequence) nucleic acid + protein
SEQ ID NO:11 + 12: hCaSR nucleic acid + protein
SEQ ID NO: 13-18: primer sequences, compare example 2a&b and example 3a&b
SEQ ID NO: 19 + 20: CSR::T1R2-b nucleic acid + protein
SEQ ID NO: 21 + 22: CSR::T1R3-b nucleic acid + protein
SEQ ID NO: 23-25: primer sequences, compare examples 2b and 3b

There now follows a series of examples that serve to illustrate the above-described methods. The following examples are merely illustrative and should not be construed as limiting the methods or kit in any manner.

Examples

All examples use the DNA sequences derived from human T1R2, T1R3 and hCaSR.

Overview examples

1: Fluo-4 Calcium Assay

2a: Preparation of CSR::T1R2-a vector construct

2b: Preparation of CSR::T1R2-b vector construct

3a: Preparation of the CSR::T1R3-a vector construct

3b: Preparation of the CSR::T1R3-b vector construct

4: Preparation of the T1R2, T1R3 vector constructs (wildtype receptors for comparison)

5: Transfections of CSR::T1R2/CSR::T1R3, and T1R2/T1R3 heterologous expression

5.2: Preparation of Stable Cell Lines expressing the CSR:T1R2/CSR:T1R3 heterodimer

6a: Activation of CSR::T1R2-a/CSR::T1R3-a

6b: Activation of CSR::T1R2-b/CSR::T1R3-b

Example 1

Fluo-4 Calcium Assay

Fluo-4 is a fluorescent indicator for intracellular calcium and allows to determine changes in the calcium concentration, in particular an increase in response to receptor activation occurring after ligand addition.

HEK293 cells stably expressing Gα16-gustducin 44 were used as host cells and transfected with various constructs as described in example 4.

Black, clear-bottom 96-well plates were used for all assays. They were seeded the day before with 8500 transfected cells per well and maintained at 37° C overnight in an a growth medium appropriate for the cells used. For HEK293 cells, Dulbecco's Modified Eagle medium containing high glucose, L-glutamine, pyroxidine hydrochloride, and supplemented with10% fetal bovine serum was used for growth and maintenance of the HEK293 cells.

At the time of the assay, the growth medium was discarded and cells were incubated for 1 hour (at 37° C in the dark) with 50 µl of a calcium assay solution consisting of 1.5 µM Fluo-4 AM (Molecular ProbesTM, Invitrogen, US) and 2.5 µM probenicid (Sigma-Aldrich) dissolved in a reduced calcium C1 buffer solution. Reduced calcium C1 buffer solution contains 130 mM NaCl, 5 mM KCl, 10 mM Hepes, 0.5 mM CaCl2 (reduced from 2 mM) and 10 mM glucose (pH 7.4).

After the initial 1 hour loading period, the plates were washed 5 times with 100 µl per well of reduced calcium C1 buffer using an automated plate washer (BioTek) and after washing, the plate was further incubated for 30 minutes at room temperature in the dark to allow for complete de-esterification of the Fluo-4-AM. The buffer solutions were discarded, the plate was washed 5 times with 100 µl reduced calcium C1 wash buffer and finally the cells were reconstituted in 180 µl of reduced calcium C1 wash buffer.

For assay reading, the plate was placed in a FLIPR (fluorescence imaging plate reader (FLIPR-Tetra, Molecular Devices)), and receptor activation was initiated following addition of 20 µl of a 10X concentrated ligand stock solution, which were prepared in reduced calcium C1 buffer.

Fluorescence was continuously monitored for 15 seconds prior to ligand addition and for 105 seconds after ligand addition (45 – 105 sec may be sufficient).

Receptor activation is given in relative fluorescence units (RFU) and is defined by the following equation:
Fluorescence Increase = Maximum Fluorescence – baseline fluorescence, wherein the baseline fluorescence represents the mean fluorescence calculated for the first 10 to 15 seconds prior to ligand addition.

As a negative control, mock transfected cells were exposed to the same concentration of ligand and the concentration of calcium traces not corresponding to a signal was determined.

Cells with an activated receptor were identified by the signal (RFU) being significantly above the negative control.

Example 2a

Preparation of CSR::T1R2-a vector construct

The CSR::T1R2-a chimeric cDNA vector construct was generated by joining two DNA fragments generated by PCR via a common restriction enzyme site in both PCR products , namely of a PCR product representing the extracellular amino terminal domain (ATD) of hCaSR (1 – Phe^{539}) to a PCR product representing an "-a" fragment of T1R2 containing the cysteine-rich domain (CRD), transmembrane (TMD) and C-terminus beginning at Ser^{493} (T1R2-a, SEQ ID No: 1 (nucleic acid) and 2 (protein)).

To facilitate the making of the CSR::T1R2-a chimeric DNA, a Sac II site was introduced to the primers that were used to form the two fragments described hereinabove. Using these introduced sites and the appropriate restriction enzyme in buffers and under conditions well known in the art, the fragments were joined by enzymatic ligation.

These Sac II sites in the formed PCR-products/fragments are located at the C-terminal end of the hCaSR ATD fragment and the N-terminal end of the T1R2-a fragment, respectively, allowing for ligation of the two PCR-products/fragments of the chimeric DNA. Incorporation of this Sac II site converts Phe^{539} in the hCaSR into an arginine residue. PCR using Platinum Taq High Fidelity Polymerase was used to amplify the fragments that comprise the CSR:T1R2-a chimeric cDNA fragment using the specific primers of SEQ ID 13-16 which are given below. F designates the forward primer, R the reverse primer.

The underligned letters designate restriction sites located within the primers for subsequent subcloning of the PCR products.

hCaSR-ATD primer F (Seq ID NO:13):
CACCAAGCTTATGGCATTTTATAGCTGC

hCaSR-ATD primer R (Seq ID NO:14):
ATATCCGCGGCACCTCCCTGGAGAACCC

T1R2-fragment primer F (Seq ID NO:15):
ATATCCGCGGTCCATGTGTTCCAAGAGG

T1R2-fragment primer R (Seq ID NO:16):
ATATGCGGCCGCAGTCCCTCCTCATGGT

The template for the PCR amplification was a full length cDNA for either the human CaSR (commercially available from Origene Inc., USA), or the human T1R2, which was isolated from a cDNA library generated from human fungiform papillae taste tissue. PCR reaction parameters were: 94° C for 5 min followed by 35 cycles of 94° C for 45 seconds, 54° C for 15 seconds and 72 ° C for 2 minutes, followed by a final extension cycle of 72° C for 10 minutes.

The resulting nucleic acid fragments were separated by gel electrophoresis, purified and subcloned into the pCR-Topo-II vector (Invitrogen) and the resulting clones were verified by DNA sequencing to ensure absence of mutations arising from the PCR amplification.
After sequencing, the inserts were subcloned into an expression cassette vector construct based on the pcDNA4-TO vector (purchased from Invitrogen, USA) via 3-piece ligation, allowing for assembly of the CSR::T1R2-a chimeric cDNA fragment in the vector construct.

The C-terminus of the formed vector construct encodes the herpes simplex virus (HSV) glycoprotein D epitope, which can be used for immuncytochemistry studies using a specific antibody that binds to this epitope. The resulting CSR:T1R2-a vector construct with CSR:T1R2-a cDNA allows for expression of the CSR:T1R2-a:HSV protein of joined amino acid sequences of Seq ID NO:2 (CSR:T1R2-a) followed by Seq ID NO:6 (HSV epitope) (in amino terminus to C terminus direction).

Example 2b
Preparation of CSR::T1R2-b vector construct

The CSR::T1R2-b chimeric cDNA vector construct was generated by joining two DNA fragments generated by PCR via a common restriction enzyme site in both PCR products , namely of a PCR product representing the extracellular amino terminal domain (ATD) and cysteine-rich domain (CRD) of hCaSR (1 – Ile^{603}) to a PCR product representing a "-b" fragment of T1R2 containing the transmembrane (TMD) and C-terminus beginning at Val^{557} (T1R2-b, SEQ ID No: 19 (nucleic acid) and 20 (protein)).

To facilitate the making of the CSR:T1R2-b chimeric DNA, a BsiW I site was introduced to the primers that were used to form the two fragments described

hereinabove. Using these introduced sites and the appropriate restriction enzyme in buffers and under conditions well known in the art, the fragments were joined by enzymatic ligation.

These BsiW I sites in the formed PCR-products/fragments are located at the C-terminal end of the hCaSR ATD fragment and the N-terminal end of the T1R2-b fragment, respectively, allowing for ligation of the two PCR-products/fragments of the chimeric DNA. Incorporation of this BsiW I site converts Glu^{602}/Ile^{603} in the hCaSR into an Arg/Thr residues. PCR using Platinum Taq High Fidelity Polymerase was used to amplify the fragments that comprise the CSR:T1R2-b chimeric cDNA fragment using the specific primers of SEQ ID 13, 16, 23 and 24 which are given below. F designates the forward primer, R the reverse primer.

The underligned letters designate restriction sites located within the primers for subsequent subcloning of the PCR products.

hCaSR-ATD primer F (Seq ID NO:13):
CACCAAGCTTATGGCATTTTATAGCTGC

hCaSR-ATD primer R (Seq ID NO:23):
ATA TCG TAC GCT TGG CAA TGC AGG AGG T

T1R2-fragment primer F (Seq ID NO:24):
ATA TCG TAC GGT CTT CCT GGA ATG GCA T

T1R2-fragment primer R (Seq ID NO:16):
ATATGCGGCCGCAGTCCCTCCTCATGGT

The template for the PCR amplification was a full length cDNA for either the human CaSR (commercially available from Origene Inc., USA), or the human T1R2, which was isolated from a cDNA library generated from human fungiform papillae taste tissue. PCR reaction parameters were: 94° C for 5 min followed by 35 cycles of 94° C for 45 seconds, 54° C for 15 seconds and 72 ° C for 2 minutes, followed by a final extension cycle of 72° C for 10 minutes.

The resulting nucleic acid fragments were separated by gel electrophoresis, purified and subcloned into the pCR-Topo-II vector (Invitrogen) and the resulting clones were verified by DNA sequencing to ensure absence of mutations arising from the PCR amplification.

After sequencing, the inserts were subcloned into an expression cassette vector construct based on the pcDNA4-TO vector (purchased from Invitrogen, USA) via 3-piece ligation, allowing for assembly of the CSR::T1R2-b chimeric cDNA fragment in the vector construct.

The C-terminus of the formed vector construct encodes the herpes simplex virus (HSV) glycoprotein D epitope, which can be used for immuncytochemistry studies using a specific antibody that binds to this epitope. The resulting CSR:T1R2-b vector construct with CSR:T1R2-b cDNA allows for expression of the CSR:T1R2-b:HSV protein of joined amino acid sequences of Seq ID NO:20 (CSR:T1R2-b) followed by Seq ID NO:6 (HSV epitope) (in amino terminus to C terminus direction).

Example 3a
Preparation of the CSR::T1R3-a vector construct

The CSR::T1R3-a chimeric cDNA vector construct was generated by joining two DNA fragments generated by PCR via a common restriction enzyme site in both PCR products, namely the joining of a PCR product representing the extracellular amino terminal domain (ATD) of hCaSR (1 – Phe^{539}) to a fragment of T1R3 containing the cysteine-rich domain (CRD), transmembrane (TMD) and C-terminus beginning at Ser^{497}.

To facilitate the making of the CSR::T1R3-a chimeric cDNA vector construct, a Sac II site was introduced into the primers which were used to make the above-described two fragments.

These Sac II sites in the formed PCR-products/fragments are located at the C-terminal end of the hCaSR-ATD fragment and the N-terminal end of the T1R3-a fragment, respectively, allowing for ligation of the two fragments. Incorporation of this Sac II site results in a vector construct that comprises a sequence wherein the Phe^{539} of the previous hCaSR is converted into an arginine residue. Using the introduced ligation sites and the appropriate restriction enzyme in buffers and under conditions well known in the art, the fragments were joined by enzymatic ligation.

PCR using Platinum Taq High Fidelity Polymerase was used to amplify the fragments that comprise the CSR::T1R3-a chimeric cDNA fragment using the specific primers of Seq ID NO: 17 and Seq ID NO:18 listed below. Afterwards, the

amplified PCR-products of T1R3-a and the amplified PCR products of hCaSR (the latter formed as described in example 2 above) were ligated via the restriction sites indicated in the primer listed below. F designates the forward primer, R the reverse primer. The underligned letters designate restriction sites located within the primers for subsequent ligation and subcloning of the amplified PCR products.

hCaSR-ATD F and hCaSR-ATD R:
Seq ID NO: 13 and Seq ID NO: 14 as indicated in example 2a above.

TAS1R3-a-fragment primer F (Seq ID NO:17):
ATATCCGCGGTCCCGGTGCTCGCGGCAG

TAS1R3-fragment primer R (Seq ID NO:18):
ATATGCGGCCGCACTCATGTTTCCCCTGATT

The template for the PCR amplification was a full length cDNA for either the hCaSR (purchased from Origene Inc., USA), or the hT1R3, which was isolated from a cDNA library generated from human fungiform papillae taste tissue.
PCR reaction parameters were: 94° C for 5 min followed by 35 cycles of 94° C for 45 seconds, 54° C for 15 seconds and 72 ° C for 2 minutes, followed by a final extension cycle of 72° C for 10 minutes.

The resulting nucleic acid fragments (ligation is performed later after the fragments are verified) were separated by gel electrophoresis, purified and subcloned into the pCR-Topo-II vector (Invitrogen, USA). The resulting clones were verified by DNA sequencing to ensure absence of mutations arising from the PCR amplification.

After sequencing, the inserts were subcloned into an expression cassette vector construct based on the pcDNA4-TO vector (purchased from Invitrogen, USA) via 3-piece ligation, forming the CSR::T1R3-a vector construct. The C-terminus of the formed vector construct encodes the herpes simplex virus (HSV) glycoprotein D epitope, which can be used for immuncytochemistry studies using a specific antibody that binds to this epitope. The resulting vector construct allows for expression of the CSR::T1R3-a::HSV protein of joined amino acid sequences of Seq ID NO:4 (CSR::T1R3-a) followed by Seq ID NO:6 (HSV epitope) (in amino terminus to C terminus direction).

Example 3b

Preparation of the CSR::T1R3-b vector construct

The CSR::T1R3-b chimeric cDNA vector construct was generated by joining two DNA fragments generated by PCR via a common restriction enzyme site in both PCR products, namely the joining of a PCR product representing the extracellular amino terminal domain (ATD) and cysteine-rich domain (CRD) of hCaSR (1 – Ile^{603}) to a PCR product representing a fragment of T1R3 containing the transmembrane (TMD) and C-terminus beginning at Arg^{560}.

To facilitate the making of the CSR::T1R3-b chimeric cDNA vector construct, a BsiW I site was introduced into the primers which were used to make the above-described two fragments.

These BsiW I sites in the formed PCR-products/fragments are located at the C-terminal end of the hCaSR-ATD fragment and the N-terminal end of the T1R3-b fragment, respectively, allowing for ligation of the two fragments. Incorporation of this BsiW I site results in a vector construct that comprises a sequence wherein the Phe^{539} of the previous hCaSR is converted into an arginine residue. Using the introduced ligation sites and the appropriate restriction enzyme in buffers and under conditions well known in the art, the fragments were joined by enzymatic ligation.

PCR using Platinum Taq High Fidelity Polymerase was used to amplify the fragments that comprise the CSR::T1R3-b chimeric cDNA fragment using the specific primers of Seq ID NO: 25 and Seq ID NO:18 listed below. Afterwards, the amplified PCR-products of T1R3-b and the amplified PCR products of hCaSR (the latter formed as described in example 2b above) were ligated via the restriction sites indicated in the primer listed below. F designates the forward primer, R the reverse primer. The underligned letters designate restriction sites located within the primers for subsequent ligation and subcloning of the amplified PCR products.

hCaSR-ATD F and hCaSR-ATD R:
Seq ID NO: 13 and Seq ID NO: 23 as indicated in example 2b above.

TAS1R3-fragment primer F-b (Seq ID NO:25):
ATA TCG TAC GCG GTT CCT GGC ATG GGG C

TAS1R3-fragment primer R (Seq ID NO:18):
ATATGCGGCCGCACTCATGTTTCCCCTGATT

The template for the PCR amplification was a full length cDNA for either the hCaSR (purchased from Origene Inc., USA), or the hT1R3-b, which was isolated from a cDNA library generated from human fungiform papillae taste tissue.
PCR reaction parameters were: 94° C for 5 min followed by 35 cycles of 94° C for 45 seconds, 54° C for 15 seconds and 72 ° C for 2 minutes, followed by a final extension cycle of 72° C for 10 minutes.

The resulting nucleic acid fragments (ligation is performed later after the fragments are verified) were separated by gel electrophoresis, purified and subcloned into the pCR-Topo-II vector (Invitrogen, USA). The resulting clones were verified by DNA sequencing to ensure absence of mutations arising from the PCR amplification.

After sequencing, the inserts were subcloned into an expression cassette vector construct based on the pcDNA4-TO vector (purchased from Invitrogen, USA) via 3-piece ligation, forming the CSR::T1R3-b vector construct. The C-terminus of the formed vector construct encodes the herpes simplex virus (HSV) glycoprotein D epitope, which can be used for immuncytochemistry studies using a specific antibody that binds to this epitope. The resulting vector construct allows for expression of the CSR::T1R3-b::HSV protein of joined amino acid sequences of Seq ID NO:22 (CSR::T1R3-b) followed by Seq ID NO:6 (HSV epitope) (in amino terminus to C terminus direction).

Example 4
Preparation of the T1R2, T1R3 vector constructs (wildtype receptors for comparison)

To form the T1R2 and T1R3 vector construct, cDNA fragments containing the entire protein coding sequences for human T1R2 and T1R3 were isolated from a human fungiform cDNA library, fully sequenced and then subcloned into pCDNA3.1 (Invitrogen).

Example 5
Transfections of CSR::T1R2/CSR::T1R3, and T1R2/T1R3 heterologous expression

Transfected vector constructs used were those described in examples 2a and 3a, or 2b and 3b, and 4, formed as described above. For hCaSR, a commercially available

pCMV-based vector construct which is based on the full length cDNA was used (TRUECLONE collection, Origene Inc., USA).

HEK293T cells that stably express Gα16-gustducin 44 (formed as described in WO 2004/055048) were transfected with the CSR::T1R2 and CSR::T1R3 vector constructs, or with T1R2 and T1R3, or hCaSR as follows:

On day 0, the HEK293T/ Gα16-gustducin 44 cells were plated in 96-well black, clear-bottom plates at a density of 8500 cells per well and grown overnight in selective growth media. On day 1, the media was changed to an antibiotic-free and serum-free growth medium and the cells were transfected using 75 ng each of CSR:T1R2 (-a or -b) and CSR:T1R3 (total 150 ng) (-a or -b), T1R2 and T1R3 (total 150 ng), or 75 ng hCaSR vector construct DNA and 0.3 μl of Lipofectamine 2000 (Invitrogen).

The hCaSR vector is used as positive control for a GPCR that is sensitive to calcium, as it is sensitive to calcium and the calcium binding site lies in the VFT of this receptor, which is where the VFT for the chimera is derived from.

For transfection of either the CSR:T1R2/CSR:T1R3 (CSR:T1R2-a/CSR:T1R3-a or CSR:T1R2-b/CSR:T1R3-b) or T1R2/T1R3 heterodimers, 75 ng of each vector construct was combined for a total of 150 ng per pair and used together with 0.3 μl of Lipofectamine 2000. 75 ng of hCaSR vector DNA was used for this calcium-sensing monomeric GPCR.

The above-described lipofectamine/DNA mixtures were incubated on the cells for 3-4 hours and then replaced with an antibiotic-free, serum-containing growth medium. The cells were grown overnight and the Fluo-4 calcium assay was performed as described in example1.

The cells transiently transfected with one of the above-described vector constructs were identified using a fluorescence imaging plate reader (FLIPR-Tetra, Molecular Devices) as described in example 1.

Example 5.2
Preparation of Stable Cell Lines expressing the CSR::T1R2-a/CSR::T1R3-a heterodimer

A stable cell line was generated in which CSR::T1R3-a was constitutively over-expressed in the presence of a tetracycline-regulated CSR::T1R2-a to avoid possible cytotoxic effects of constitutive over-expression of both proteins. DNA encoding one subunit of the heterodimer (CSR::T1R2-a) was placed in a tetracycline-regulated vector to allow regulation of its expression level so that viability and functionality of the stable clonal lines can be optimised.

Human cell lines that stably express the chimeric human CSR::T1R2-a/CSR::T1R3-a heterodimer were generated sequentially by first transfecting a linearized pcDNA4-TO vector (Invitrogen) containing the human CSR::T1R2-a into a Gα16gust44 expressing cell line, which was prepared as described in WO 2004/055048. The Gα16gust44 expressing cell line shows enhanced coupling to taste receptors, is tetracycline inducible, stably expresses the Gα16gust44 promiscuous G-protein, and is based on the HEK-293-T-Rex cell line (commercially available from Invitrogen, USA). A clonal cell line expressing CSR:T1R2-a was identified and transfected with a linearized pcDNA3.1-Hygro vector (Invitrogen) containing human CSR::T1R3-a cDNA to obtain a double stable clonal cell line that expresses both CSR::T1R2-a and CSR::T1R3-a.

24 hours after transfection with 4 micrograms of the linearized CSR:T1R2-a/pcDNA4TO construct and 0.3 µl of Lipofectamine 2000 (Invitrogen), cells were re-plated at 10x dilutions up to 1:150,000 in selective medium containing DMEM (Invitrogen) supplemented with 10% FBS, 0.005 mg/ml blasticidin, 0.36 mg/ml G418, and 0.2mg/ml Zeocin at 37 °C. After 2-3 weeks, Zeocin-resistant colonies were individually expanded and stable clones were selected based on a functional response to 50 micromolar perillartine following a 4 hour induction with 10 µg/ml tetracycline to allow for expression of the CSR::T1R2-a cDNA. We identified an individual clone (#17) that exhibited minimal basal expression of the CSR::T1R2-a cDNA and used this as a recipient for the CSR:T1R3-a construct to generate a stable cell line for the heterodimeric receptor complex. Clone 17 containing the inducible CSR::T1R2-a was transfected with 4 µg of the linearized CSR::T1R3-a/pcDNA3.1-Hygro vector construct DNA and 0.3 µl of Lipofectamine 2000 (Invitrogen). The lipofectamine/DNA mixture was incubated on the cells for 3-4 hours and then replaced with an antibiotic-free, serum-containing growth medium. After 24 hours the cells were re-plated in selective medium containing DMEM supplemented with 10% FBS, 0.005 mg/ml blasticidin, 0.36 mg/ml G418, 0.2 mg/ml zeocin, and 0.2 mg/ml hygromycin at 37°C.

Resistant colonies were expanded, and identified as containing the CSR::T1R2-a/CSR::T1R3-a heterodimer on the basis of a response to both perillartine (contributed by binding/activation of CSR::T1R2-a) and to sodium cyclamate (contributed by binding/activation of CSR:T1R3-a), which was determined via automated fluorimetric imaging on the FLIPR-Tetra instrumentation (Molecular Devices) using the methods described in example 1. All potential clones were evaluated for a functional response to sweet tastants following induction with 10 μg/ml tetracycline (to induce over-expression of CSR::T1R2-a). Potential clones were also tested in the absence of tetracycline induction to identify any clones that basally express T1R2-a at a low level, but have sufficient expression of the CSR::T1R2-a receptor to allow for assembly with CSR::T1R3-a resulting in a functional heterodimer complex (the tetracycline-regulated systems such as the T-Rex HEK-293 (Invitrogen) are known to have a low-level basal expression of transgenes due to the inherent leakiness of the system). Stable clones expressing an inducible functional CSR::T1R2-a/CSR::T1R3-a heterodimer were identified on the basis of a response to both 50 microM perillartine and to 5 mM (milliM) sodium cyclamate. One clonal cell line exhibiting the greatest tetracycline-inducible response to multiple sweet tastants was propagated and used for subsequent comparisons. The results for tests with various ligands/sweet tastants are indicated in the table below.

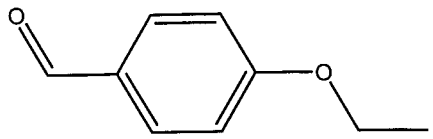
		CSR:T1R2-a/CSR:T1R3-a		T1R2/T1R3		G16gust44 (NEG. CONTROL)	
Ligand	Ligand Concentration	AVG (dF/F)	S.D. (dF/F)	AVG (dF/F)	S.D. (dF/F)	AVG (dF/F)	S.D. (dF/F)
p-ETBZ	100 microM	1.11	0.05	1.029	0.1	0.16	0.01
NDHC	1 mM	1.64	0.23	1.71	0.05	0.23	0.01
NarDHC	1 mM	1.43	0.23	1.40	0.15	0.22	0.07
Cyclamate	5 mM	1.28	0.063	1.60	0.08	0.17	0.06

Table 1

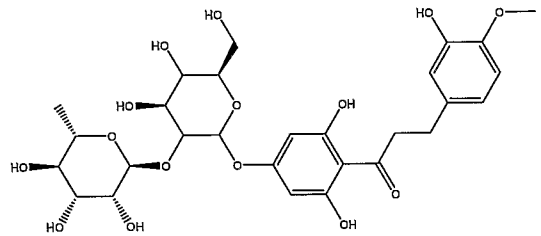
The data indicates the normalized increase in fluorescence over baseline after stimulation (ΔF/F) using the following equation: $\Delta F/F=(F-F_0)/F_0$, where F is the peak fluorescence signal and F_0 is the baseline fluorescence signal, which is determined from the average fluorescence signal measured prior to ligand addition. The ΔF/F value obtained corresponds to the calcium increase of the cell in response to a direct or indirect interaction with the transfected receptor ("signal") (the mean (AVG) and standard deviation (S.D.) of three replicate experiments is given).

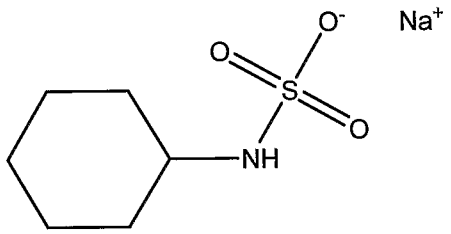
The chemical structure of the ligands is shown below.

p-ETBZ = p-ethoxybenzaldehyde



NDHC





Sodium Cyclamate (Cyclamate is a sweet tastant. The additional presence of sodium ions improves water solubility, but does not further contribute to sweet taste)

Example 6a

Activation of CSR::T1R2-a/CSR::T1R3-a

The intracellular calcium response following stimulation with various ligands was determined in HEK293T cells stably expressing Gα16-gustducin 44 and transfected with CSR::T1R2-a/CSR::T1R3-a chimeric heterodimer. The results were compared to results obtained in cells transfected with the both the T1R2 vector construct and the T1R3 vector construct which is described in example 4 (to form the wildtype T1R2/T1R3 sweet heterodimer) or the hCaSR vector construct described in example 5 (to form monomeric hCaSR).

The transfections were performed as described in example 4. Results were calculated as described in example 1 (data indicates the net increase in fluorescence over baseline after stimulation (Relative Fluorescent Units or RFU); the mean (AVG) and the ± Standard deviation (STD) of six replicate experiments is given). The

following ligands were used for to stimulate the transfected cells in the concentrations as indicated in brackets:
Calcium Chloride (2 mM), Sucralose (0.5 mM), aspartame (0.85 mM), perillartine (50 μM), cinnamonitrile (100 μM), cyclamate (1 mM), Neohesperidin Dihydrochalcone (NDHC) (0.33 mM).
The signals obtained are the fluorescence in RFU corresponding to the calcium increase of the cell in response to a direct or indirect interaction with the transfected receptor ("signal").

Mock transfected HEK293T/ Gα16-gustducin 44 cells transfected without construct that do not express a sweet receptor were used as a negative control to determine signals corresponding merely to the background.

The transfected cells are exposed to the sweeteners as indicated and to a positive control (calcium) for the proteins containing Calcium-sensing domains, and to a negative control (C1 buffer).

The results are shown in the table below.
The AVG column gives the mean fluorescence, the STD column gives the standard deviation. The table below shows the average change in RFUs +/- STD for the 6 replicates for each of the various vector constructs tested.

	CSR::T1R2-a/ CSR::T1R3-a		hCaSR		T1R2/T1R3		Neg. control (mock transfection)	
	AVG	STD	AVG	STD	AVG	STD	AVG	STD
Positive control (Calcium)	3912	295	7610	1776	1361	426	1570	509
Aspartame	72	78	41	97	531	154	-125	116
Sucralose	-75	130	22	92	601	173	-186	36
Perillartine	**2400**	**466**	**73**	**354**	**1840**	**333**	**-379**	**327**
Cinnamonitrile	**1501**	**194**	**197**	**33**	**632**	**484**	**-998**	**36**
Cyclamate	**370**	**213**	**196**	**79**	**341**	**132**	**-324**	**297**
NDHC	**631**	**233**	**-257**	**53**	**331**	**129**	**-524**	**44**
Negative control (C1 buffer)	-115	70	57	101	-63	131	-217	204

Table 2a Results

The negative control/mock transfection shows the signal level corresponding to background signals.

As the positive control (calcium) shows, all transfected cells which have a calcium-sensing domain react to calcium (CSR::T1R2-a/CSR::T1R3-a heterodimer and hCaCSR)
The response of the chimeric heterodimer to calcium can not be compared to those obtained with the sweet heterodimer. Since calcium is not an agonist of the T1R2/T1R3 sweet heterodimer it did not give signals that were greater than mock transfected cells expressing only the Gα16-gustducin 44 g-protein.

For aspartame and sucralose, a signal is detected in the cells transfected with the T1R2/1R3 heterodimer only. Sucralose and aspartame are believed to bind in the VFT of T1R2, which is absent in the CSR:T1R chimera, which explains the lack of signal in the CSR::T1R2-a/CSR::T1R3-a heterodimer.

The hCaSR responded only to calcium chloride and could not be activated by any of the sweet tastants tested.

For calcium chloride, perillartine, cinnamonitrile, cyclamate and NDHC, a significant increase of the signal was observed in cells expressing the CSR::T1R2-a/CSR::T1R3-a chimeric heterodimer.

For perillartine, cinnamonitrile, cyclamate, and NDHC these signals were comparable in intensity to the signal detected for the T1R2/T1R3 heterodimer.

These signals detected in the cells transfected with the chimeric CSR::T1R2-a/CSR::T1R3-a heterodimer were significantly higher than both the signal in the T1R2/T1R3 heterodimer and the background signals obtained in the negative control (mock transfected HEK293T/ Gα16-gustducin 44 cells), and were approximately 50% of the magnitude of signals obtained in cells transfected with the hCaSR receptor.

The results demonstrate that CSR::T1R2-a/CSR::T1R3-a is activated by calcium, perillartine, cyclamate, cinnamonitrile, and neohesperidin dihydrochalcone (NDHC) but not by sucralose or aspartame.

Example 6b

Activation of CSR::T1R2-b/CSR::T1R3-b

The intracellular calcium response following stimulation with various ligands was determined in HEK293T cells stably expressing Gα16-gustducin 44 and transfected with CSR::T1R2-b/CSR::T1R3-b chimeric heterodimer. The results were compared to results obtained in cells transfected with the both the T1R2 vector construct and the T1R3 vector construct which is described in example 4 (to form the T1R2/T1R3 sweet heterodimer) or the hCaSR vector construct described in example 5 (to form monomeric hCaSR).

The transfections were performed as described in example 4. Results were calculated as described in example 1 (data indicates the normalized increase in fluorescence over baseline after stimulation (ΔF/F); the mean (AVG) and the ± Standard deviation (STD) of six replicate experiments is given). Calcium Chloride (2 mM), Sucralose (0.5 mM), perillartine (50 μM), were used as the test ligands.

The calcium mobilization signals obtained are increase in peak fluorescence (F) normalized to the baseline fluorescence (F_0). The data are normalized using the following equation: $\Delta F/F=(F-F_0)/F_0$, where F is the peak fluorescence signal and F_0 is the baseline fluorescence signal, which is determined from the average fluorescence signal measured prior to ligand addition. The ΔF/F value obtained corresponds to the calcium increase of the cell in response to a direct or indirect interaction with the transfected receptor ("signal").

Mock transfected HEK293T/ Gα16-gustducin 44 cells transfected without construct that do not express a sweet receptor were used as a negative control to determine signals corresponding merely to the background.

The transfected cells are exposed to the sweeteners as indicated and to a positive control (calcium) for the proteins containing Calcium-sensing domains, and to a negative control (C1 buffer).

The results are shown in the table below.

The AVG column gives the mean ΔF/F, the STD column gives the standard deviation. The table below shows the average change in ΔF/F +/- STD for the 6 replicates for each of the various vector constructs tested.

	CSR::T1R2-b/ CSR::T1R3-b		CSR::R2-b alone		T1R2/T1R3		Neg. control (mock transfection)	
	AVG	STD	AVG	STD	AVG	STD	AVG	STD
Positive control (Calcium)	0.46	0.11	0.424	0.02	0.19	0.001	0.20	0.01
Sucralose	0.017	0.006	0.016	0.001	0.12	0.006	0.03	0.00
Perillartine	0.169	0.04	0.11	0.02	0.5	0.02	0.06	0.02
Negative control (C1 buffer)	0.017	0.001	0.036	0.008	0.03	0.01	0.03	0.002

Table 2b Results

The negative control/mock transfection shows the signal level corresponding to background signals.

As the positive control (calcium) shows, all transfected cells which have a calcium-sensing domain react to calcium (CSR::T1R2-b/CSR::T1R3-b heterodimer and hCaSR)
The response of the chimeric heterodimer to calcium can not be compared to those obtained with the sweet heterodimer. Since calcium is not an agonist of the T1R2/T1R3 sweet heterodimer it did not give signals that were greater than mock transfected cells expressing only the Gα16-gustducin 44 g-protein.

For sucralose, a signal is detected in the cells transfected with the T1R2/1R3 heterodimer (wildtype) only. Sucralose is believed to bind in the VFT of T1R2, which is absent in the CSR::T1R chimerae, which explains the lack of signal in the CSR::T1R2-b/CSR::T1R3-b heterodimer.

The hCaSR responded only to calcium chloride and could not be activated by any of the sweet tastants tested.

For calcium chloride, perillartine, a significant increase of the signal was observed in cells expressing the CSR::T1R2-b/CSR::T1R3-b chimeric heterodimer as well as the

CSR::T1R2-b homomer expressed in the absence of any T1R3 construct or its variants.

These signals detected in the cells transfected with the chimeric CSR::T1R2-b/CSR::T1R3-b heterodimer were significantly higher than the background signals obtained in the negative control (mock transfected HEK293T/ Gα16-gustducin 44 cells)

The results demonstrate that CSR::T1R2-b/CSR::T1R3-b is activated by calcium and perillartine, but not by sucralose.

While the receptors, nucleic acids, polypeptides, methods and kit have been described above in connection with certain illustrative embodiments, it is to be understood that other similar embodiments may be used or modifications and additions may be made to the described embodiments for performing the same function(s). Further, all embodiments disclosed are not necessarily in the alternative, as various embodiments may be combined to provide the desired characteristics. Variations can be made by one having ordinary skill in the art without departing from the spirit and scope of the disclosure. Therefore, the receptors, nucleic acids, polypeptides, methods and kit should not be limited to any single embodiment, but rather construed in breadth and scope in accordance with the recitation of the attached claims.

Claims

1. A CSR::T1R chimeric protein able to bind to at least one sweetener or sweetness enhancer, comprising one or more CSR::T1R selected from the group consisting of

a CSR::T1R2 polypeptide substantially homologous to SEQ ID NO:2 or SEQ ID NO:20 with a sequence identity of at least 90%,

a CSR:: T1R3 polypeptide substantially homologous to SEQ ID NO:4 or SEQ ID NO: 22 with a sequence identity of at least 90%

.

2. A CSR::T1R chimeric protein according to claim 1 comprising

two polypeptide subunits in form of a heterodimeric protein selected from the group consisting of

a CSR::T1R2/CSR::T1R3 heterodimeric chimeric protein, a CSR::T1R2/ T1R3 heterodimeric chimeric protein, and a T1R2/CSR::T1R3 heterodimeric chimeric protein,

wherein the T1R2 subunit of the heterodimer comprises a polypeptide substantially homologous to SEQ ID NO:8 with a sequence identity of at least 90%;

and wherein the T1R3 subunit of the heterodimer comprises a polypeptide substantially homologous to SEQ ID NO:10 with a sequence identity of at least 90%.

3. A CSR::T1R chimeric protein comprising two polypeptide subunits according to claim 2 which comprises a CSR::T1R2/CSR::T1R3 heterodimeric chimeric protein including but not limited to a CSR::T1R2-a/CSR::T1R3-a heterodimeric protein, a CSR::T1R2-b/CSR::T1R3-b heterodimeric protein, a CSR::T1R2-a/CSR::T1R3-b heterodimeric protein, a CSR::T1R2-b/CSR::T1R3-a heterodimeric protein, or a heterodimeric protein substantially homologous thereto, wherein CSR::T1R2-a corresponds to SEQ ID NO: 2, CSR::T1R2-b corresponds to SEQ ID NO:20, CSR::T1R3-a corresponds to SEQ ID NO:4 and CSR::T1R3-b corresponds to SEQ ID NO:22.

4. A nucleic acid encoding a CSR::T1R chimeric protein able to bind at least one sweetener or sweetness enhancer comprising one or more of

a nucleic acid substantially homologous to a nucleotide sequence selected from the group consisting of SEQ ID NO:1 (CSR::T1R2-a), SEQ ID NO: 19 (CSR::T1R2-b); SEQ ID NO:3 (CSR::T1R3-a) and SEQ ID NO: 21 (CSR::T1R3-b) as determined by sequence identity,

a nucleic acid substantially homologous to a nucleotide sequence selected from the group consisting of SEQ ID NO:1 (CSR::T1R2-a), SEQ ID NO: 19 (CSR::T1R2-b); SEQ ID NO:3 (CSR::T1R3-a) and SEQ ID NO: 21 (CSR::T1R3-b) as determined by hybridisation,

a nucleic acid substantially homologous to a nucleotide sequence encoding the CSR::T1R chimeric protein as defined in claim 1,

wherein the substantially homologous nucleic acid as determined by sequence identity has a sequence identity of at least 90%;

wherein the substantially homologous nucleic acid as determined by hybridisation hybridises under stringent hybridization conditions at a temperature of 42° C in a solution consisting of 50% formamide, 5×SSC, and 1% SDS, and washing at 65° C in a solution consisting of 0.2×SSC and 0.1% SDS;
wherein the nucleic acid optionally comprises SEQ ID NO:6 (HSV tag) at or near its end to form the C-terminus in the corresponding protein.

5. An expression vector comprising the nucleic acid as defined in claim 4.

6. A host cell transfected with an expression vector as defined in claim 5.

7. The host cell of claim 6 stably expressing a CSR::T1R chimeric protein as defined in claim 1 and a G-Protein, optionally a G-Protein substantially homologous to Gaq-Gustducin.

8. The host cell of claim 6 transiently expressing a CSR::T1R chimeric protein as defined in claim 1 and a G-Protein, optionally a G-Protein substantially homologous to Gaq-Gustducin.

9. A method of producing a CSR::T1R chimeric protein as defined in claim 1, 2 or 3, comprising culturing host cells comprising an expression vector encoding for the CSR::T1R chimeric protein under conditions sufficient for expression, thereby forming the CSR::T1R chimeric protein and optionally recovering it from the cells.

10. A method to identify an agent that modulates sweet taste signaling in taste cells, the method comprising:
(i) contacting the cells that express a CSR::T1R chimeric protein that responds to stimuli selected from sweet taste stimuli and calcium stimuli with an agent thereby providing a functional response, optionally in presence of another agent; and
(ii) determining whether at least one agent affects the functional response of said CSR::T1R chimeric protein in said cells by at least one functional response in said cells;
wherein said CSR::T1R chimeric protein is as defined in claims 1, 2 or 3.

11. A method according to claim 10 wherein the cells also express a G-Protein.

12. A method according to claim 11 wherein the G-Protein is a chimeric G-protein substantially homologous to Gaq-Gustducin.

13. A method according to claim 12 wherein the G-Protein is the chimeric G-protein G alpha 16-gustducin 44.

14. A method according to claim 10 wherein step (ii) is performed by measuring a change in or caused by intracellular messengers.

15. A method according to claim 11 wherein the functional response is determined by measuring a change in an intracellular messenger selected from IP3 and calcium^{2+}.

16. The method of claim 10 wherein said cells are selected from the group consisting of bacterial cells, eucaryotic cells, yeast cells, insect cells, mammalian cells, amphibian cells, and worm cells.

17. The method according to claim 16 wherein the cell is a mammalian cell.

18. The method according to claim 17 wherein the cell is a mammalian cell selected from the group consisting of CHO, COS, HeLa and HEK-293 cells.

19. The method according to claim 10 wherein step (i) further comprises contacting the CSR::T1R chimeric protein with a test agent in presence of calcium.

20. The method according to claim 19 wherein the calcium is provided in the form of calcium chloride.

21. A kit comprising:
(i) recombinant cells that express a CSR::T1R chimeric protein as defined in claim 1, 2 or 3, and
(ii) an agonist of the CSR::T1R chimeric protein,
for combined use to identify test agents as modulators of the CSR::T1R chimeric protein.

22. A method of using the kit of claim 21, comprising:
(i) growing recombinant cells that express a CSR::T1R chimeric protein,
(ii) adding test agents in the presence of the agonist in a suitable concentration, and
(iii) determining a change in a functional response of the cells by comparing the response in presence and absence of the test agent, and the test agent is thereby identified as a modulator of the CSR::T1R chimeric protein as defined in claim 1, 2, or 3.

23. A method to identify an agent that modulates the CSR::T1R chimeric protein as defined in claim 1, 2, or 3, the method comprising:
(i) measuring a parameter that changes in response to a ligand binding to the CSR::T1R chimeric protein, and
(ii) determining a change of the parameter in response to a test agent, optionally in presence of a ligand, in comparison to a negative control and thereby identifying a modulator or ligand.

24. Method according to claim 23 wherein the ligand is selected from the group consisting of calcium, calcium ions and calcium chloride.

25. Method according to any one of claims 23 and 24 wherein step (i) is performed by a method selected from the group consisting of fluorescence spectroscopy, NMR spectroscopy, measuring of one or more of absorbance, refractive index, hydrodynamic methods, chromatography, measuring solubility, biochemical methods, wherein the methods measure the properties of the CSR::T1R chimeric protein in a suitable environment selected form the group consisting of solution, bilayer membrane, attached to a solid phase, in a lipid monolayer, bound on a membrane, and in vesicles.

INTERNATIONAL SEARCH REPORT

International application No
PCT/CH2007/000297

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50 C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, EMBASE, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/004992 A (UNIV CALIFORNIA [US]; US GOVERNMENT [US]; ZUKER CHARLES S [US]; RYBA N) 16 January 2003 (2003-01-16)	2,9-25
A	page 3 page 5, line 24 - page 6, line 23 page 17, line 16 - line 31 claims 3,7,14,22; sequences 1-3,9,7-9,15,20,23,25,27,30	1,3-8
X	WO 2005/015158 A (SENOMYX INC [US]; LI XIAODONG [US]; STASZEWSKI LENA [US]; XU HONG [US]) 17 February 2005 (2005-02-17)	2,9-25
A	page 1 - page 4; claims 23,24,45; sequences 6,7,22,23,16,17,20,21,22,26-29,32 page 58, line 24 - line 28 page 67, line 11 - line 26	1,3-8

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 2007	16/11/2007

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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