Kokumi Substances, Enhancers of Basic Tastes, Induce Responses in Calcium-Sensing Receptor Expressing Taste Cells

Yutaka Maruyama*, Reiko Yasuda, Motonaka Kuroda, Yuzuru Eto

Institute for Innovation, Ajinomoto Co., Inc., Kawasaki, Japan

Abstract

Recently, we reported that calcium-sensing receptor (CaSR) is a receptor for kokumi substances, which enhance the intensities of salty, sweet and umami tastes. Furthermore, we found that several γ-glutamyl peptides, which are CaSR agonists, are kokumi substances. In this study, we elucidated the receptor cells for kokumi substances, and their physiological properties. For this purpose, we used Calcium Green-1 loaded mouse taste cells in lingual tissue slices and confocal microscopy. Kokumi substances, applied focally around taste pores, induced an increase in the intracellular Ca2+ concentration ([Ca2+]i) in a subset of taste cells. These responses were inhibited by pretreatment with the CaSR inhibitor, NPS2143. However, the kokumi substance-induced responses did not require extracellular Ca2+. CaSR-expressing taste cells are a different subset of cells from the T1R3-expressing umami or sweet taste receptor cells. These observations indicate that CaSR-expressing taste cells are the primary detectors of kokumi substances, and that they are an independent population from the influenced basic taste receptor cells, at least in the case of sweet and umami.

Introduction

The extracellular calcium-sensing receptor, CaSR, is a classic seven-transmembrane-spanning, G protein-coupled receptor (GPCR) belonging to Family C of the superfamily of GPCRs [1]. CaSR has been identified in several cells and tissues, including the parathyroid gland and kidney. It plays a central role in extracellular calcium homeostasis in mammals [2]. An increase in the blood calcium level is sensed by CaSR, which in turn suppresses parathyroid hormone secretion, stimulates calcitonin secretion, and induces urinary calcium excretion to reduce blood calcium to normal levels. It has become apparent that CaSR is expressed not only in the parathyroid glands and kidney, but also in many other tissues such as liver, heart, lung, gastrointestinal tract, pancreas and the central nervous system, suggesting that it is involved in a range of biological functions [3]. It has been reported that CaSR is activated by several types of substances including cations such as Ca2+, Mg2+ and Gd3+, basic peptides such as proteamnine and polylysine, and polyamines such as spermine [3].

CaSR is expressed in a subpopulation of taste cells in mice and rats [4,5], suggesting potential roles for this receptor in taste cellular biology. Ninomiya and colleagues reported that mice have a group of gustatory afferent nerve fibers that respond to calcium and magnesium [6]. Tordoff and co-workers described the taste perception of calcium and the physiological mechanisms underlying calcium intake, appetite and homeostasis, and indicated that calcium deprivation increases the palatability of calcium [7]. These findings indicate the existence of a calcium transduction mechanism in taste cells. However, except for calcium, the physiological role of these CaSR agonists is not clear. Recently, Bystrova et al. reported that CaSR is expressed in a subset of taste cells, and that the agonists, NPS R-568, neomycin and several L-amino acids, induced a response in isolated taste cells [4].

Recently, we reported that various CaSR agonists including γ-glutamyl-cysteinyl-glycine (reduced form of glutathione, GSH) and other γ-glutamyl peptides have enhancing activities on umami, sweet and salty tastes, and that there is a high correlation between CaSR agonist activity and taste intensity [8]. Ueda et al. reported that water extracts from garlic, which contain GSH, enhance umami taste intensity, and they propounded the taste enhancing character as the “kokumi flavor” [9–12]. Furthermore, we identified several γ-glutamyl peptides, which are CaSR agonists that have a kokumi flavor activity, and found that γ-glutamyl-valine-glycine (γEVG) is the most potent kokumi substance [8]. These results suggest that CaSR-expressing taste cells in lingual epithelium respond to kokumi substances.

In the present study, we employed a semi-intact lingual slice preparation in which it is possible to focally apply kokumi stimuli onto the apical tips of the taste buds and measure individual cellular responses with enough time and spatial resolution for Ca2+ imaging. We show that kokumi substances induce a [Ca2+]i,
response in taste cells in the posterior tongue. The results indicate that kokumi substances are detected by CaSR-expressing taste cells.

**Results**

**CaSR is expressed in the taste buds in lingual epithelia**

We tested the expression of CaSR mRNA in taste buds and in non-taste lingual epithelium from a C57BL/6 mouse by RT-PCR. We confirmed that CaSR mRNA was expressed in circumvallate and foliate, but not in non-taste epithelium (Fig. 1A). To determine the presence of CaSR in taste cells, we employed immunohistochemistry on mice lingual tissues. CaSR immunoreactivity was observed in a subset of spindle-shaped taste cells in circumvallate, foliate, fungiform and palate papillae (Fig. 1B–E). In the transverse section of circumvallate taste buds, 8–10 CaSR-immunoreactive taste cells were present in a taste bud (Fig. 2D, H). The specificity of the antibody was confirmed by antigen preabsorption, which resulted in little or no immunoreaction in taste cells (Fig. 1F).

**CaSR is expressed in a subset of type II (receptor) and type III (presynaptic) cells**

Mammalian taste buds contain three distinct classes of cells [13–15]. A heterogeneous population of mammalian taste cells includes morphologically and functionally different taste cells classified into three subtypes, type I (glial-like cells), type II (receptor cells) and type III (presynaptic) taste cells [16]. These classes express different complements of genes related to their functions: receptor (Type II) cells express G-protein coupled taste receptors and transduction machinery. In contrast, presynaptic (Type III) cells express neuronal proteins, including those associated with synapses, and also respond to sour stimuli [17–21].

To characterize the CaSR-immunoreactive taste cells, we investigated the coexpression of CaSR and taste cell markers. Using immunofluorescence, previous reports have shown in circumvallate taste buds the expression of PLC\(_2\) in type II taste cells (receptor cells) [17], and of neural cell adhesion molecule (NCAM) in type III taste cells (presynaptic cells) [22].

Conversely, out of 823 PLC\(_2\)-positive cells, 314 cells expressed NCAM (669 cells out of 1033 cells, 64.7%; Fig. 2H). These populations were in agreement with previous findings regarding the cell-types of CaSR-expressing taste cells [5].

**Taste cells respond to focally applied kokumi substances**

To test whether kokumi substances induce an intracellular Ca\(^{2+}\) response in taste cells and if so, to identify which taste cells are responsible, we employed semi-intact lingual slice preparations, focally applied kokumi substances to the apical chemosensory tips of the taste cells, and imaged the intracellular [Ca\(^{2+}\)] changes in the taste cells with confocal scanning microscopy [23,24]. Focal application of the kokumi substances, cinacalcet (a classic CaSR agonist; 10 \(\mu\)M), glutathione (GSH; 100 \(\mu\)M), or \(\gamma\)-glutamyl-valinyl-glycine (\(\gamma\)EVG; 100 \(\mu\)M), induced Ca\(^{2+}\) responses (\(\Delta\)Ca\(^{2+}\)) in a small fraction of the taste cells (Fig 3A) [8]. These responses were not induced by solution puffing, because no response was observed after ejection of Tyrode’s solution (Fig 3A). Some, but not all, kokumi substance-responsive cells also responded to bath applied KCl (50 mM), which induces a Ca\(^{2+}\) response in the presynaptic (Type III) taste cells via depolarization of the plasma membrane. \(\gamma\)EVG evoked a transient [Ca\(^{2+}\)] increase in 6.5% of the taste cells in the circumvallate papilla (34 of 524 cells obtained from 26 mice). The mean amplitude of Ca\(^{2+}\) responses (\(\Delta\)F/\(F_{0}\) evoked by 100 \(\mu\)M \(\gamma\)EVG was 11.6 ± 1.7% (mean ± SE; n = 21 cells; Fig. 3B). The EC\(_{10}\) value of \(\gamma\)EVG was estimated at approximately 13 \(\mu\)M (Fig. 3B).

**Figure 1. Taste cells express CaSR.** (A) RT-PCR for CaSR expression in taste bud-enriched circumvallate (cv), foliate (foli) and non-taste bud (nt) lingual epithelium, nc - negative control (lacking template); M - molecular standard. (B–E) Immunostaining for CaSR in taste buds. CaSR immunofluorescence is seen in most circumvallate (B), foliate (C), palate (D) and fungiform (E) taste buds. Immunofluorescent images (green) were superimposed on DIC images. (F) Validating the anti-CaSR antibody. The CaSR antiserum was preabsorbed with an excess of antigen peptides. The circumvallate sections reacted with preabsorbed and non-absorbed antibodies and were processed simultaneously. Images were taken under the same illumination conditions and detector settings. Scale bars 20 \(\mu\)m.

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concentrations of γEVG (>30 μM) did not induce Ca2+ responses in additional taste cells. Furthermore, kokumi substance-induced responses were selectively blocked by 3 μM NPS2143, a CaSR inhibitor, which barely affected the umami (MPG 100 mM + IMP 1 mM) and the sweet (SC45647, 100 μM) responses [Fig. 3C] [8,25]. This suggests that the Ca2+ responses we recorded reflect selective stimulation of a specific subpopulation of kokumi substance-responsive taste cells.

**Responses to kokumi substance stimulus involve Ca2+ release**

Next, we investigated the Ca2+-mobilizing pathway that is activated by the kokumi substance stimulus in mouse circumvallate taste cells. We examined responses in acute absence of extracellular Ca2+ by bathing slices in a Ca2+-free Tyrode’s solution (containing 0.2 mM EGTA) 2 min before the focal kokumi substance stimulation. As shown in Fig. 4A, responses evoked by γEVG did not significantly change compared with the presence of extracellular Ca2+ (control, ΔF/F = 7.1 ± 1.8%; Ca2+-free, ΔF/F = 6.7 ± 0.7%; n = 5). In contrast, the depolarization-evoked Ca2+ response, elicited by perfusing the slice with 50 mM KCl that allows Ca2+ influx through voltage-gated Ca2+ channels in Type III presynaptic taste cells [26], was nearly completely abolished in the absence of extracellular Ca2+ under parallel treatments (control, ΔF/F = 16.1 ± 5.6%; Ca2+-free, ΔF/F = 2.8 ± 0.2%; n = 4; Fig. 4B). Part of the γEVG-responsive taste cells showed a Ca2+ response to KCl stimulation; however, the γEVG-induced response in these cells was not affected by Ca2+-free conditions (Fig. 4A and B). These results are consistent with kokumi transduction involving release of stored Ca2+, not Ca2+ influx (Fig. 4C).

Umami, sweet and bitter stimuli trigger the release of stored Ca2+ by activating phospholipase C (PLC) [24,27–30]. To directly test whether kokumi substance-elicited Ca2+ responses arise from PLC activation, we used a non-selective PLC inhibitor, U73122 [31–33]. After incubation with 10 μM U73122 for 10 min, responses elicited by γEVG were almost abolished (control, ΔF/F = 8.3 ± 1.6%; U73122, ΔF/F = 1.7 ± 0.8%; n = 4; Fig. 4D and E). In contrast, depolarization (KCl)-induced responses were not significantly altered by treatment with U73122 (data not shown). These data strongly support the notion that the kokumi mechanisms involve intracellular Ca2+ release.

**CaSR ligand-responsive cells do not respond to L-glutamate stimuli**

It has been reported that CaSR is activated by various γ-glutamyl peptides, including glutathione and γEVG [8,34,35]. When transiently expressed in HEK293 cells, CaSR also induces Ca2+ changes in response to L-glutamate monomer, which is associated with the umami taste [4]. In contrast, receptor (Type II) cells respond to sweet, bitter and umami taste stimuli by elevating cytoplasmic Ca2+ [17,21]. We asked whether the CaSR ligand, γEVG, and glutamate produce responses in the same mouse type II taste cells. These studies have suggested that γEVG mimics the taste of L-glutamate, at least in part, by activating the same taste receptors as umami compounds. To test this interpretation directly, we focally applied γEVG and monopotassium L-glutamate (MPG) + inosine monophosphate (IMP) sequentially on circumvallate taste buds. γEVG (100 μM) evoked transient Ca2+ responses in some taste cells (10 responding cells out of 132 recorded cells; ΔF/F = 7.4 ± 1.3%), but not in those that responded to MPG (100 mM) + IMP (1 mM) (Fig. 5A, C). Conversely, MPG + IMP-responding cells (9 cells out of 132 recorded cells; ΔF/F = 6.7 ± 1.5%) did not respond to γEVG (Fig. 5B, C). These data suggest that separate receptors, found on separate cells, generate Ca2+ responses to γEVG and MPG + IMP (Fig. 5C). In cells that respond to each agonist, we cannot rule out the possibility of subthreshold responses to the other agonist. Nevertheless, these results emphasize that responses to CaSR ligand in native taste tissues are highly heterogeneous and vary markedly from those described for the proposed umami taste receptors.
Figure 3. Taste cell responses (ΔCa\(^{2+}\)) evoked by kokumi stimuli, recorded in a slice preparation of the mouse circumvallate papilla. (A) Taste cells were stimulated sequentially with three kokumi substances, cinacalcet (CCT, 10 μM), glutathione (GSH, 100 μM) and γ-glutamyl-valinylglycine (γEVG, 100 μM), as well as γEVG + NPS2143 (3 μM), a CaSR antagonist. Arrowheads below traces indicate the stimulation. (B) Concentration-response relationship for γEVG (mean ± SE; n = 4 cells). (C) Taste responses elicited by γEVG (100 μM) were inhibited by the CaSR antagonist, NPS2143 (3 μM), but the umami (MPG 100 mM + IMP 1 mM) and the sweet (SC45647, 10 μM) responses were unaffected. Mean amplitudes of γEVG-, MPG+IMP-, and SC45647-induced responses in the presence or absence of 3 μM NPS2143 are shown (mean ± SE; *p<0.05, n = 4 cells). Raw traces are shown in A. doi:10.1371/journal.pone.0034489.g003

Figure 4. Ca\(^{2+}\) response elicited by γEVG involves intracellular Ca\(^{2+}\) stores and phospholipase C. (A) γEVG (100 μM) was focally applied in medium containing Ca\(^{2+}\) (left trace) or in the absence of Ca\(^{2+}\) (Ca-free medium with 0.2 mM EGTA; right trace). (B) Responses elicited by depolarization (bath-applied KCl, 50 mM) and influx of Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels were abolished in the absence of extracellular Ca\(^{2+}\). (C) Mean amplitudes of responses in the presence or absence of Ca\(^{2+}\) in the medium (mean ± SE; *p<0.05, n = 4 cells). (D) Responses to γEVG inhibited by U73122 (10 μM). (E) Mean amplitudes of the responses in the presence or absence of U73122 (mean ± SE; *p<0.05, n = 4 cells). doi:10.1371/journal.pone.0034489.g004
Physiological responses correlate with molecular expression

We recorded Ca\(^{2+}\) responses to 100 μM γEVG and 100 mM MPG in lingual slice preparations as described above. The functional responses of taste cells in lingual slices fell into two distinct classes. Our next step was to test whether the two kinds of responding taste cells, determined by functional imaging, mapped onto the two categories determined by expression of CaSR and an umami receptor subunit, T1R3 [36]. We designed additional methods, which are independent from the methods described above, to distinguish between CaSR-expressing cells and T1R3 cells. To identify these receptors, we used dual immunohistochemistry for CaSR and T1R3 in mouse circumvallate papillae. Examples of immunostained circumvallate papilla are shown in Fig. 6. The presence of CaSR immunofluorescent signals was observed in a subset of taste cells. In 63 circumvallate taste buds, 502 taste cells expressed CaSR, whereas 347 taste cells expressed T1R3. Only three cells (0.6% of CaSR-positive taste cells) expressed both CaSR and T1R3 (Fig. 6). These data demonstrate that most taste cells express CaSR, T1R3, or neither.

Discussion

In this study, we used a preparation of lingual slices from circumvallate papilla that allows one to apply tastants selectively to the apical chemosensory tips of taste cells, while avoiding stimulating non-taste cells and basolateral regions of taste buds [24]. This report presents data that indicate that CaSR ligands induce a cellular response in mouse circumvallate taste cells. We identified a population (6.5%) of taste cells in the circumvallate papillae that responds to kokumi substances. This value is comparable with the immunohistochemistry results (6.8% CaSR-positive cells/taste bud). Furthermore, this compares with previous estimates, in the same preparation, of 28% cells that respond to bitter [37] and 5% to umami (monopotassium L-glutamate) stimuli [24]. The threshold concentration of γEVG needed to activate a Ca\(^{2+}\) response in taste cells was 3 μM, which is consistent with reports on human sensory evaluation analysis [8]. We also observed that both species respond to CaSR agonists with very similar characteristics (Figure S1, Method S1). Hence, the results from mouse experiments can be extrapolated to results from human CaSR experiments. Additionally, NPS2143, a CaSR inhibitor, impaired the CaSR agonist-induced taste cell response in lingual slice preparations, as well as in heterologous experiments. Moreover, taste cells responded to multiple CaSR agonists, including hydrophobic (cinacalcet) and hydrophilic GSH and γEVG kokumi substances. Taste buds contain tight junctions around the taste pore (e.g., claudin 4 and 8), and maintain a highly specific permeability barrier for paracellular diffusion [38]. It is expected that CaSR agonists (kokumi substances), especially the hydrophilic GSH and γEVG, are accessible only to microvilli in the apical tip of the taste cells, and activate CaSR. Taken together, the data from the present study suggest that CaSR is involved in taste transduction in mice.

Human sensory analysis demonstrated that CaSR agonists enhance the intensity of umami and sweet tastes, and are called kokumi flavor [8]. We expected that CaSR agonists would induce a response in the umami or sweet taste cells. Tordoff and colleagues reported that calcium elicits appetitive behavior in mice [7]. Their recent study suggested that CaSR dimerizes with the sweet and umami receptor subunit T1R3 [39]. However, surprisingly, CaSR agonists induced a response in a different group of taste cells from the umami or sweet taste cells. Results from double immunohis-
tochemistry of CaSR and T1R3 also support this observation. Signaling in taste cells, especially for umami, sweet and bitter, are well understood. Taste receptors for umami (T1R1 + T1R3) and sweet (T1R2 + T1R3) agonists have been identified [36,40–42], and activation of these receptors elicits cellular responses (e.g., transient Ca²⁺ changes and ATP secretion) in receptor-expressing taste cells. These findings demonstrate the involvement of CaSR in kokumi signaling, and suggest that CaSR is not directly involved in umami or sweet taste signaling. In cells that respond to each agonist, we cannot rule out the possibility of cell-to-cell signaling within a taste bud.

Our results indicate that kokumi substance-responsive taste cells are both presynaptic (Type III) and non-presynaptic taste cells. Furthermore, the results suggest that certain stimuli evoke the Ca²⁺ response via release from intracellular stores in both presynaptic and non-presynaptic taste cells. The exact classification of kokumi substance-responsive taste cells remains to be determined. San Gabriel et al. reported that CaSR-positive taste cells express PLCβ2, a receptor (Type II) cell marker, or NCAM, a presynaptic cell marker in rat vallate taste buds [5]. Taken together with these observations, CaSR is expressed at least in Type II and Type III taste cells. Activation of kokumi substance-responsive cells may modulate the activity of sensory afferent fibers, and/or neighboring taste cells in the same taste bud [24,43,44]. ATP released from receptor cells may excite primary sensory afferent fibers [45]. ATP may also function as a paracrine transmitter and act on cells within the taste bud [43,46,47].

Our experiments were not designed to distinguish between these possibilities, both of which remain open questions.

Recently, Bystrova et al. reported that only type III taste cells responded to agonists of CaSR. However, in our study, all CaSR agonists tested induced responses in both type II and type III taste cells. We do not know the reason for this discrepancy; enzyme treatment during taste cell isolation might affect cellular responses. In PLCβ2-positive type II taste cells, CaSR is expressed in T1R3-negative taste cells. Whether CaSR is expressed in bitter receptor T2R-positive cells remains unresolved.

Our results suggest that kokumi substance-induced increases in [Ca²⁺], arise principally via mobilization from intracellular stores, because responses were essentially unaffected by depletion of extracellular Ca²⁺. Conversely, responses were strongly abolished by pre-treatment with U73122, demonstrating the involvement of phospholipase C in kokumi transduction. These results provide direct evidence of the existence of a functional kokumi receptor coupled to Ca²⁺ mobilization in taste cells. Generally, [Ca²⁺], response in taste cells seems to depend on two sources of Ca²⁺. For depolarizing stimuli (KCl), Ca²⁺ influx is induced via activation of voltage-gated Ca²⁺ channels in presynaptic taste cells [17,44,48]. Interestingly, neither thapsigargin nor U73122 totally eliminated the Ca²⁺ responses evoked by kokumi stimuli. Whether this represents minor additional pathways remains unresolved.

In summary, our results demonstrate that kokumi substance-responsive cells are presynaptic and non-presynaptic taste cells. We observed that part of the kokumi substance-responsive cells did not respond to umami (MPG) or sweet (SC45647) stimuli. The kokumi flavor is defined as an enhancer of umami and sweet tastes. Kokumi substance-responding taste cells might involve enhancement of these basic tastes.

Materials and Methods

Tissue preparation and functional imaging

All experimental procedures were approved by the Animal Experiment Institution Review Board of Ajinomoto Co., Inc., Institute for Innovation (2008220, 2009085, 2010013 and 2011239), and conformed with the standards for the use of laboratory animals published by the Institute of Laboratory Animal Resources, U.S. National Academy of Sciences. C57BL/6 adult mice (≥7 weeks old, male) were sacrificed by exposure to diethyl ether, followed by cervical dislocation. Tongues were removed and immersed in cold Tyrode’s solution. Liquid slices containing the vallate papilla were obtained, and a Ca²⁺ indicator dye was injected into taste cells following similar procedures previously described by Maruyama et al. [24]. Briefly, the fluorescent Ca²⁺ indicator dye, Calcium Green-1 dextran (CGD; MW 3,000; 0.25 mM in H₂O; Invitrogen, Carlsbad, CA, USA), was injected iontophoretically through a large-diameter-tip glass micropipette (40 μm) into the crypt surrounding the vallate papilla (−3.5 μA square pulses, 10 min). The CGD-loaded tissue was sliced at 100 μm with a vibratome (Leica VT1000S, Nussloch, Germany). Slices containing vallate taste buds were mounted on a glass coverslip coated with Cell-Tak (Becton Dickinson, Franklin Lakes, NJ, USA), put in a recording chamber, and superfused with Tyrode’s solution (30°C) at a rate of 1.5 ml/min. Single glass micropipettes (2 μm tip diameter) were used to directly deliver taste stimuli for apical stimulation of a selected taste bud. Stimuli were ejected for 1 s with air pressure (3.5 psi; Pressure System Ie, Toohy Company, Fairfield, NJ, USA). Different pipettes were mounted for each taste stimulus. All stimulus solutions contained 2 μM fluorescein to monitor stimulus application, duration and concentration.

CGD-loaded taste cells were viewed with a laser-scanning confocal microscope, using an argon laser (Fluoview FV-300, Olympus, Tokyo, Japan). Images were captured at 1.1 s intervals. Fluorometric signals are expressed as relative fluorescence change: ΔF/ΔF₀ = (F-F₀)/F₀, where F₀ denotes the resting fluorescence level corrected for any bleaching that occurred during the recording. Using ΔF/ΔF₀ corrects for variations in baseline fluorescence, cell thickness, total dye concentration and illumination [49]. Peak ΔF/ΔF₀ constituted the response amplitude for statistical quantification.

Data analysis

Statistical analyses using paired Student’s t-tests were applied to determine whether the changes in the response amplitudes (peak ΔF/ΔF₀) to a given treatment were significant. Data presented in bar graphs show mean ± SEM.

Reagents and solutions

γ-glutamyl-valinyl-glycine (γEVG) was synthesized by Kokusan Chemicals (Tokyo, Japan). Cinaaclet, NPS2143 and SC45647 were chemically synthesized in our facility by previously described methods [50–52]. All other chemicals including monopotassium L-glutamate (MPG) were purchased from Sigma Chemical (St Louis, MO, USA). All tastants were freshly dissolved in Tyrode’s solution for each experiment. The standard medium consisted of Tyrode’s solution, which is composed of, in mM: 135 NaCl, 5 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, 10 sodium pyruvate and 5 NaHCO₃, pH 7.2; 318–323 mOsm. For Ca²⁺-free Tyrode’s solution, CaCl₂ was removed, and 0.2 mM EGTA was added.

Immunohistochemistry

Mouse circumvallate tissues were fixed in 4% paraformaldehyde and cryoprotected in 10–30% sucrose for 3.5 h at 4°C. Frozen sections (12 μm) were prepared and blocked in Protein Block (Dako, Glostrup, Denmark) with 1% Triton X-100 (Sigma) for 45 min at room temperature. Sections were then incubated for 1 h with a primary antibody [rat anti-CaSR, diluted 1:400; rabbit
anti-PLCβ2, 1:500 (sc-206, Santa Cruz, Santa Cruz, CA, USA); rabbit anti-NCAM, 1:400 (AB5032, Millipore, Billerica, MA, USA) and rabbit anti-T1R3, 1:800 (kind gift from Dr. Iwatsuki, Ajinomoto Co., Inc.) [53]], followed by a secondary antibody (1:1000, Alexa Fluor 480 labeled donkey anti-rat IgG antibody for anti-CaSR (A-21206, Invitrogen) and Alexa Fluor 568 labeled goat anti-rabbit IgG antibody for anti-PLCβ2, anti-NCAM and anti-T1R3 (A-11036, Invitrogen). CaSR was detected using a specific antibody generated in our lab (host = rat). The anti-mouse-CaSR antibody recognizes the protein sequences: KSNSEDFFQPQEQKQGQPVMGDHQPEIESPD and MRQNSLEAQKSNSTNLNR, corresponding to residues 917–933, 1034–1050 and 994–1010 of the mouse CaSR, respectively. Negative controls without the primary antibody were processed in parallel in every experiment. Images were obtained with a laser-scanning confocal microscope (Olympus). We estimated a thickness of ~3 μm for the optical section taken by the confocal microscope.

RT-PCR analysis

RT-PCR amplification was performed using primers that amplify the mouse CaSR, PLCβ2, Snap25 and β-actin. Briefly, dissected tongue containing circumvallate and foliate papillae was injected into the submucosal layer with a mixture of 1 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN, USA), 2.5 mg/ml Dispase II (Roche Applied Science) and 1 mg/ml trypsin inhibitor (Sigma), and then incubated for 20 min at room temperature. The papilla-containing epithelium was peeled from the underlying connective tissue. Total RNA was isolated from the epithelial papillae and from the epithelium without taste buds (RNA micro kit, Agilent Technologies, Santa Clara, CA, USA). Purified RNA was denatured, and first-strand cDNA was synthesized using oligo(dT)12-18 primer and reverse transcriptase (Super Script III, Invitrogen). cDNA was used as a template in a 20 μl PCR mixture with Taq polymerase (Invitrogen). PCR conditions were as follows: 94°C for 2 min, followed by 29–35 cycles of 94°C for 30 s, 58°C for 20 s, and 72°C for 45 s. The PCR products were analyzed by gel electrophoresis with GelRed staining (Biotium, Hayward, CA, USA). The primers used were as follows: CaSR, 5'-ctcgagaccctccatgccgac-3' (forward) and 5'-aagtcttcccgccacctgta-3' (reverse); PLCβ2, 5'-ctcctggttgaggatgtgcc-3' (forward) and 5'-gctctgtgcttgggtgctta-3' (reverse); Snap25, 5'-

gcataaattgaggagtag-3' (forward) and 5'-agatttaaccctctccgca-3' (reverse); β-actin, 5'-caccctggctgctcacc-3' (forward) and 5'-cagctttacctctcag-3' (reverse).

Supporting Information

Figure S1 Human and mouse CaSR have similar properties for kokumi substances. Concentration-response curves for cinacalcet (A), glutathione (GSH; B) and γ-glutamyl-valinyl-glycine (γEVG; C) in human (filled) or mouse (open) CaSR-expressing HEK cells. For the tested CaSR agonists, we observed very similar EC50 values in both species of CaSR. The EC50 values for cinacalcet, GSH and γEVG were 0.207, 0.058 and 0.033 μM for human CaSR, and 0.380, 0.058 and 0.032 μM for mouse CaSR, respectively. (TIF)

Method S1 Determination of CaSR activity using HEK293 cells. Full-length human and mouse CaSR cDNA were isolated by RT-PCR and validated by sequencing. Both constructs were cloned into pcDNA3.1 (Invitrogen) for functional experiments. HEK cells were transfected in parallel using Fugene 6 (Roche, Indianapolis, IN, USA) with constructs of human- or mouse-CaSR, or with the empty expression vector, pcDNA3.1. After 24 h, cells were harvested and seeded in a 96-well plate. Cells were loaded with the calcium indicator dye, Calcium 3 (Molecular Devices, Sunnyvale, CA, USA), and responses were measured with FRIPR or FLEX Station (Molecular Devices). (DOC)

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Author Contributions

Conceived and designed the experiments: YM MK YE. Performed the experiments: YM RY. Analyzed the data: YM RY. Contributed reagents/materials/analysis tools: YM MK YE. Wrote the paper: YM YE.

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Author Contributions

Conceived and designed the experiments: YM MK YE. Performed the experiments: YM RY. Analyzed the data: YM RY. Contributed reagents/materials/analysis tools: YM MK YE. Wrote the paper: YM YE.
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