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Sweet proteins lysozyme and thaumatin are protein-type agonists for the calcium-sensing receptor

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ABSTRACT

In addition to the maintenance of Ca^{2+} homeostasis, the calcium-sensing receptor (CaSR) is involved in many diverse physiological functions in the mammalian body. The receptor works as a *kokumi* taste receptor in taste buds and as a nutrient sensor in the gut, where it regulates the secretion of glycemic response and appetite-related hormones. To identify novel human CaSR (hCaSR) activators from food ingredients, we conducted a screening using a cell-based hCaSR assay. Hen egg-white lysozyme, which is a sweet protein, was found to be a novel orthosteric agonist of hCaSR with an EC_{50} value of 592 μM. Lysozyme hydrolysate was not able to activate hCaSR, thus suggesting that the protein structure of lysozyme is necessary for hCaSR activation. Thaumatin, which is another sweet protein, also activated hCaSR with an EC_{50} value of 71 μM. This is the first report that shows hCaSR activation by proteins with molecular weights exceeding 10,000 Da. These results provide a new avenue for the development of hCaSR activators, which could be applicable in food or drugs that modulate taste perception, appetite, or glucose tolerance, in addition to Ca^{2+} homeostasis.

1. Introduction

The calcium-sensing receptor (CaSR) is a G protein–coupled receptor (GPCR) involved in the maintenance of Ca^{2+} homeostasis in mammals [1–4]. CaSR was initially cloned from the bovine parathyroid gland as the primary protein regulating the secretion of parathyroid hormone [2]. Mutations of human CaSR (hCaSR) have been reported to be associated with metabolic disorders such as hyperparathyroidism and hypercalcemia [3]. Therefore, hCaSR is a therapeutic target for modulating signaling in the body (e.g., cinacalcet, which is a synthetic hCaSR modulator, is used for the treatment of hyperparathyroidism) [3–6].

Recent studies have revealed that CaSR is expressed in various tissues, including the kidney and gastrointestinal tract, in addition to the parathyroid gland [3]. In addition to Ca^{2+}, CaSR is also activated by divalent and trivalent cations, spermine, L-amino acids, and γ-Glu peptides [3–5,7–16]. Considering that CaSR has a broad biodistribution and is activated by diverse activators, the receptor is expected to be involved in a variety of physiological functions in many tissues, in addition to the maintenance of Ca^{2+} homeostasis in the parathyroid gland. Muramatsu et al. [17] reported that the activation of CaSR in the small intestine reduced glycemic response via serotonin secretion under an oral glucose tolerance test in rats, thus suggesting that CaSR can be a novel target for the attenuation of postprandial hyperglycemia. Geibel et al. [18] showed that the activation of CaSR in the stomach stimulated the secretion of gastrin and stomach acid, thus indicating that CaSR activators modulate appetite. In 2009, San et al. [19] found that CaSR is expressed in the taste buds of mice. hCaSR is suggested to function as a novel taste receptor (*kokumi* taste receptor); hCaSR activators induce "*kokumi taste*", which is not a taste on its own, but enhances sweet, salty, and umami tastes and enhanced all three *kokumi* taste characteristics (thickness, continuity, and mouthfulness) in human sensory analyses [14,15,20]. Therefore, the identification of diverse CaSR activators could promote the development of functional food, supplements, and drugs that regulate glucose tolerance, appetite, and taste perception, in addition to Ca^{2+} homeostasis.

In this study, we conducted a screening to identify novel hCaSR activators to advance our understanding of hCaSR activation by

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food ingredients. We identified two sweet proteins, namely, lysozyme and thaumatin, as novel hCaSR activators. As far as we know, this is the first report that shows hCaSR activation by proteins with molecular weights exceeding 10,000 Da. Furthermore, we present a new concept: sweet proteins activate not only human sweet-taste receptors (hT1R2/hT1R3) but also CaSR via electrostatic interactions with these receptors.

2. Materials and methods

2.1. Materials

Lysozyme was purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Thaumatin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). NPS 2143 was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). HEK293T cells were provided by the RIKEN BioResource Research Center (Ibaraki, Japan). pHEK293 Ultra Expression Vector I was purchased from Takara Bio Inc. (Shiga, Japan). Artificial genes encoding hCaSR, hGα15, hGαq, and hGα11 proteins, with codon usages optimized for expression in human cells, were chemically synthesized by Eurofins Genomics (Tokyo, Japan). Supplementary Table S1 shows the DNA sequences and accession IDs.

2.2. Cell culture and transfection

The genes encoding hCaSR, hGα15, hGαq, and hGα11 were independently inserted into the pHEK293 Ultra Expression Vector I to enable overexpression with the transactivation-responsive region–transactivator system. The hCaSR and hGα expression plasmids were transiently cotransfected into HEK293T cells by using Lipofectamine™ 2000 (Invitrogen, MD, USA) according to the protocol procedure. Twenty-one to 26 h after transfection, the cells were evaluated for hCaSR responses. HEK293T cells transfected with the hGα expression plasmid and empty pHEK 293 Ultra Expression Vector I were used as the negative control (mock cells). All cells were cultured at 37 °C in the presence of 5% CO_2 in DMEM + GlutaMax™-I DMEM (Thermo Fisher Scientific, Inc., MA, USA) supplemented with 10% fetal bovine serum (Ireland origin; Biowest, Nuaillé, France).

2.3. Ca^{2+} imaging

For Ca^{2+} imaging, HEK293T cells cotransfected with the hCaSR and hGα expression plasmids were seeded onto 96-well plates (black/clear flat bottom tissue culture-treated plate with Lid, FALCON Corp., NY, USA). After washing with an assay buffer (146 mM NaCl, 5.5 mM glucose, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 2 mM HEPES, pH 7.2), the cells were incubated at 37 °C for 30 min with 3 μM Fluo-8 AM (AAT Biquest, CA, USA). Following washout with the assay buffer, the cells were held for 10 min at 37 °C and were used for Ca^{2+} imaging. The test sample was added to each well by using a FlexStation II microplate reader (Molecular Devices, Inc., CA, USA), and the changes in intracellular Ca^{2+} levels were evaluated by the specific fluorescence signal (Ex = 490 nm, Em = 525 nm). The Ca^{2+} response of cells was calculated by the following equation: response = $(F - F_0)/F_0$, where F_0 (baseline) is defined as the mean fluorescence value at 0–30 s before sample addition, and F (signal intensity) is defined as the highest fluorescence value at 35–65 s after sample administration. The hCaSR response value was calculated by subtracting the response of the HEK293T cells expressing only hGα (mock cells) from that of the hCaSR and hGα coexpressing cells. The EC_{50} values of each activator were estimated by the dose–response curves created using PRISM software version 4.03 (GraphPad Software Inc., San Diego, CA). Each experiment was repeated at least two or three times, and each data point represents the mean ± S.E.

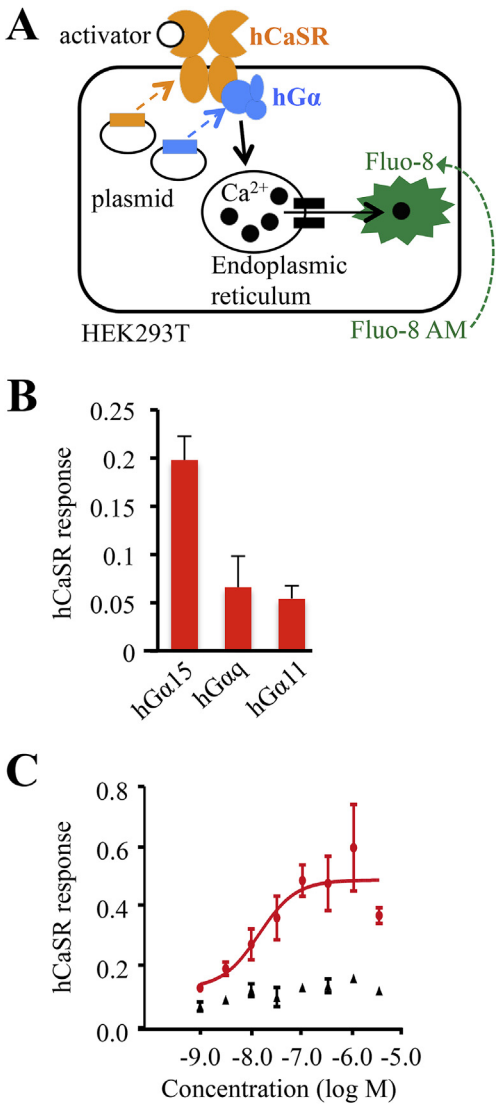

Fig. 1. Establishment of a Ca^{2+} imaging system for the evaluation of hCaSR activity. **A)** hCaSR assay system. hCaSR and hGα proteins were transiently coexpressed in HEK293T cells. Elevation of intracellular Ca^{2+} concentration was detected by a Ca^{2+}-sensitive fluorescent dye, namely, Fluo-8 AM. **B)** Comparison of hCaSR responses to 300 nM cinacalcet in cells expressing different types of Gα protein (hGα15, hGαq, and hGα11). hCaSR response values were calculated by subtracting the responses of the mock cells from those of the hCaSR and hGα coexpressing cells. Data are shown as means (n = 6) with error bars (S.E.). **C)** A dose–response curve for cinacalcet action on hCaSR. The symbols are as follows: red circles, Ca^{2+} responses in the hCaSR and hGα15 coexpressing cells; black triangles, responses in the cells expressing only hGα15 (mock cells). Data are shown as means (n = 3) with error bars (S.E.). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.4. Hydrolysis of lysozyme by protease

Lysozyme was incubated at 40 °C for 4 h with or without proteinase K (Invitrogen, MD, USA). Following lyophilization, the samples were prepared to 1% (w/v) with distilled water and were

subjected to the hCaSR assay.

3. Results and discussion

3.1. Development of a cell-based hCaSR assay system

The interaction of activators with hCaSR expressed on the surface of HEK293T cells leads to the activation of a Gq-type Gα protein, which stimulates the production of inositol 1,4,5-triphosphate (IP_3). IP_3 then induces the release of Ca^{2+} from intracellular calcium stores by binding to the IP_3 receptor on the endoplasmic reticulum. Therefore, the potency of CaSR activators is often evaluated by Ca^{2+} imaging by using Ca^{2+}-dependent fluorescent probes, such as Fluo-8 AM, in hCaSR-expressing HEK293T cells (Fig. 1A) [13–15]. However, it is unclear which Gq-type of Gα protein is suitable for the Ca^{2+} imaging of hCaSR. Therefore, we first examined which Gα protein was suitable for the hCaSR assay by using cinacalcet, which is a well-known CaSR activator (a positive allosteric modulator). Specifically, we compared 300 nM cinacalcet-induced responses on cells coexpressing hCaSR and one of the following Gα proteins: hGα15, hGαq, or hGα11 (Fig. 1B). Considering that the hCaSR response in cells expressing hGα15 was four-times higher than those in cells expressing hGαq or hGα11, we used hGα15 for subsequent hCaSR assays in the present study. Cinacalcet induced the concentration–dependent Ca^{2+} responses in the hCaSR and hGα15 coexpressing cells, but it did not elicit responses in the mock cells (Fig. 1C). These results indicated that the hCaSR assay system worked as expected. The EC_{50} value of cinacalcet on hCaSR was calculated to be 14.2 nM, which is comparable with that of a previous report [5].

3.2. hCaSR activation by lysozyme

We screened hCaSR activators from a library composed of 136 different food-related materials, including peptides and proteins contained in food (data not shown). A significant hCaSR response was induced by the administration of lysozyme, which is a protein derived from hen egg-white. Fig. 2A shows the representative Ca^{2+} responses induced by lysozyme in hCaSR-expressing cells and mock cells.

To clarify whether the lysozyme-induced Ca^{2+} response was attributable to hCaSR activation, NPS 2143, which is a specific hCaSR inhibitor (a negative allosteric modulator) [7], was added along with lysozyme to the hCaSR-expressing cells. The lysozyme-induced responses were attenuated by the coadministration of NPS 2143 in a concentration–dependent manner (Fig. 2B), thus showing that lysozyme provoked Ca^{2+} responses via hCaSR activation.

Lysozyme activated hCaSR in a concentration–dependent manner (Fig. 2C), but it did not elicit responses in the mock cells. The EC_{50} value of lysozyme was calculated to be 592 μM. When we compared the EC_{50} value of lysozyme with those of other CaSR activators, such as spermine (198 μM) and protamine (231 μM), the value was two to three times higher for lysozyme.

3.3. Analysis of mode of hCaSR activation

CaSR activators are classified into two types: orthosteric agonists and positive allosteric modulators. Orthosteric agonists (e.g., Ca^{2+} and Mg^{2+}) are capable of activating CaSR on their own [7–9,16], whereas positive allosteric modulators (e.g., cinacalcet and L-amino acids) bind to allosteric sites on CaSR and require the

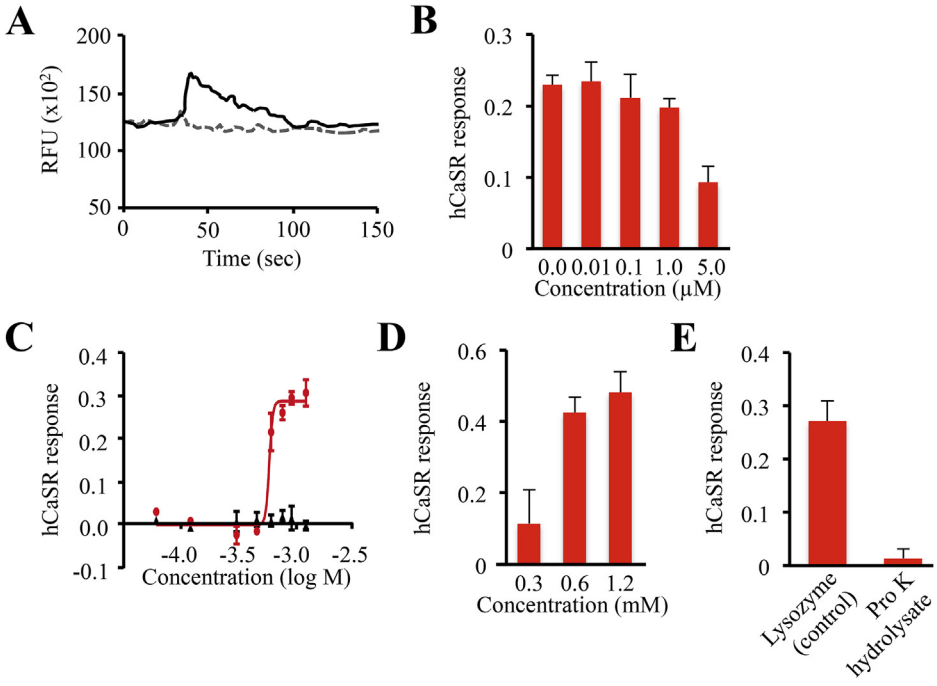

Fig. 2. hCaSR activation by lysozyme. A) Representative Ca^{2+} responses to 0.9 mM lysozyme on hCaSR- and hGα15-expressing cells (black solid line) and mock cells (gray dotted line). Lysozyme was administered to the cells 30 s after taking baseline measurements. X-axis: measurement time (seconds); y-axis: relative fluorescent unit (RFU). **B)** Inhibition of lysozyme-induced hCaSR responses by the coadministration of NPS 2143, which is a negative allosteric modulator of hCaSR. Lysozyme (0.6 mM) was added along with 0, 0.01, 0.1, 1.0, and 5.0 μM NPS 2143 to hCaSR and hGα15 coexpressing cells. **C)** Concentration–response curve of lysozyme on the hCaSR-expressing cells. Ca^{2+} responses on hCaSR and hGα15 coexpressing cells (red circle) and mock cells (black triangle) are shown. **D)** hCaSR activation by lysozyme in the absence of other orthosteric hCaSR agonists (Ca^{2+} and Mg^{2+}). Each concentration of lysozyme (0.3, 0.6, 1.2 mM) was added to the hCaSR and hGα15 coexpressing cells in Ca^{2+}- and Mg^{2+}-free assay buffer. **E)** hCaSR responses elicited by intact lysozyme and lysozyme hydrolysate treated with proteinase K. hCaSR response values were calculated by subtracting the responses of the mock cells from those of the hCaSR and hGα coexpressing cells in B, D and E. Data are shown as average (n = 3) with error bars (S.E.) in B to E. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

binding of an orthosteric agonist to the receptor to produce their effects [5,7,10–15]. In our screening, we used Ca^{2+}- and Mg^{2+}-containing buffer to identify both orthosteric agonists and positive allosteric modulators for hCaSR. We then evaluated the hCaSR response by lysozyme in buffer without Ca^{2+} and Mg^{2+} to clarify whether lysozyme is an orthosteric agonist or a positive allosteric modulator. We found that lysozyme activated hCaSR in the absence of orthosteric agonists Ca^{2+} and Mg^{2+}, thus indicating that lysozyme itself is an orthosteric agonist of hCaSR (Fig. 2D).

3.4. Contribution of lysozyme protein structure for its hCaSR activity

We evaluated the hCaSR response to a lysozyme hydrolysate to clarify whether the protein structure of lysozyme is essential for its ability to activate hCaSR. The decomposition of lysozyme by proteinase K treatment, which is a serine protease exhibiting broad substrate specificity, was confirmed by SDS-PAGE analysis (data not shown). Intact lysozyme produced an hCaSR response, whereas its hydrolysate was not able to activate hCaSR (Fig. 2E). This finding suggests that the protein structure of lysozyme is necessary for its activation of hCaSR. Most CaSR activators, such as L-amino acids (100–200 Da), glutathione (307.33 Da), and spermine (202.34 Da), have low molecular weights [7,9–15]. To the best of our knowledge, this is the first report that shows the activation of hCaSR by a protein with a molecular weight exceeding 10,000 Da.

3.5. Interaction of sweet proteins, lysozyme, and thaumatin with hCaSR and hT1R2/hT1R3

Hen egg-white lysozyme is known to be a sweet protein, and its positively charged moieties composed of basic amino acids are considered important for its sweet-taste activity [21,22]. In particular, five positively charged amino acids, namely, Lys 13, Arg 14, Arg 21, Lys 96, and Arg 73, contribute to the interaction of lysozyme with the human sweet-taste receptor (hT1R2/hT1R3) (Fig. 3A) [21,22]. Interestingly, hT1R2/hT1R3 belongs to the same class of GPCR as hCaSR (i.e., class C). For CaSR, positively charged compounds, such as Ca^{2+}, polyamines, aminoglycoside antibiotics, and protamine, have been identified as its agonists [7–9,16]. These previous reports and the present results led us to hypothesize that lysozyme can bind to both hT1R2/hT1R3 and hCaSR via electrostatic interactions between the positively charged amino acids in lysozyme and the negatively charged regions of the receptors. The basic amino acids located on the surface of the other sweet (or taste-modifying) proteins, namely, thaumatin, brazein, monelin, neoculin, and miraculin, are also thought to be important for electrostatic interactions between these proteins and hT1R2/hT1R3 [21–25]. Thus, we examined the hCaSR activity of thaumatin (a *Thaumatococcus danielli*–derived sweet protein), which is commercially used as a food additive [22]. In thaumatin, Lys 49, Lys 67, Lys 106, Lys 137, and Arg 82 are reported to be important for interaction with hT1R2/hT1R3 (Fig. 3B) [25].

Fig. 3C shows the representative Ca^{2+} responses induced by 90 μM thaumatin on hCaSR-expressing cells and mock cells; thaumatin elicited a response in the hCaSR-expressing cells but not in the mock cells at this concentration. Thaumatin produced concentration–dependent responses in the hCaSR-expressing cells between 11–90 μM, whereas it caused nonspecific responses at concentrations higher than 135 μM in the mock cells (Fig. 3D). The concentration–response curves yielded an EC_{50} value for 71 μM thaumatin. Consequently, we identified the sweet proteins lysozyme and thaumatin as novel hCaSR activators.

Further analyses regarding the interaction of sweet proteins with hCaSR are necessary to achieve a detailed understanding of how sweet proteins activate hCaSR. We are currently in the process of identifying the regions of interaction between hCaSR and sweet proteins.

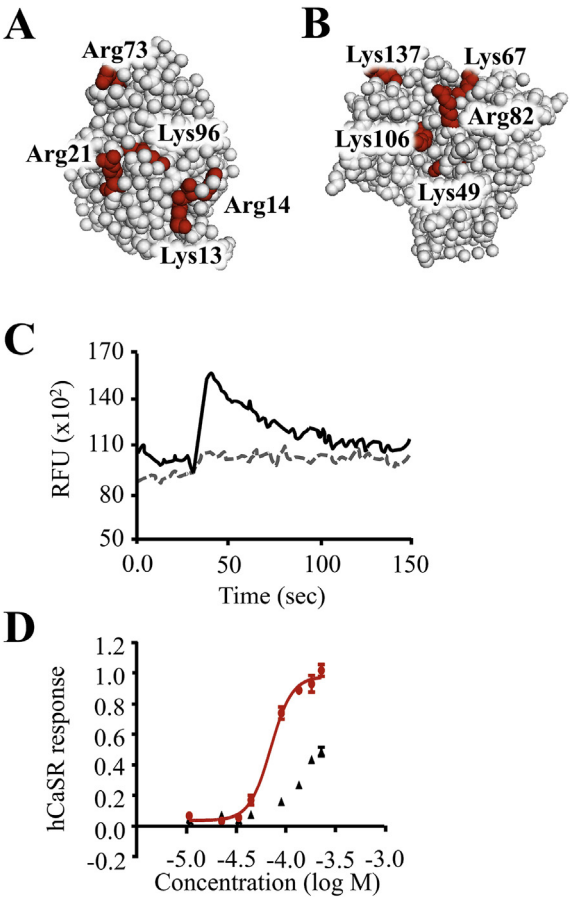

Fig. 3. hCaSR activation by thaumatin. Crystal structures of sweet proteins: **A)** lysozyme (PDB: 193L) and **B)** thaumatin (PDB: 1THW). They are shown as space-filled models. Red amino acids indicate the important residues for interactions with the sweet-taste receptor, hT1R2/hT1R3 [21,22,25]. **C)** Representative Ca^{2+} response from 90 μM thaumatin treatment on hCaSR and hGα15 coexpressing cells (black solid line) and mock cells (gray dotted line). After the measurement of the baseline for 30 s, thaumatin was administered to the cells. X-axis: measurement time (seconds); y-axis: RFU. **D)** A dose–response curve of thaumatin for hCaSR. Thaumatin (10–300 μM) was added to hCaSR and hGα15-expressing cells (red circle) and mock cells (black triangle). Data are shown as means (n = 3) with error bars (S.E.). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

To the best of our knowledge, this is the first report on hCaSR activation by proteins with molecular weights exceeding 10,000 Da (lysozyme and thaumatin). We hope that our work will facilitate the identification of novel protein-type hCaSR activators. CaSR activators would be useful as functional additives for food, supplements, and drugs. Furthermore, the discovery of protein-type hCaSR activators will provide the insights necessary to elucidate the activation mechanisms of hCaSR and other class C GPCRs, including hT1R2/hT1R3.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.10.111.

Transparency document

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