Structural basis for regulation of human calcium-sensing receptor by magnesium ions and an unexpected tryptophan derivative co-agonist

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation
Zhang, C., T. Zhang, J. Zou, C. L. Miller, R. Gorkhali, J. Yang, A. Schilmiller, et al. 2016. "Structural basis for regulation of human calcium-sensing receptor by magnesium ions and an unexpected tryptophan derivative co-agonist." Science Advances 2 (5): e1600241. doi:10.1126/sciadv.1600241. http://dx.doi.org/10.1126/sciadv.1600241.

Published Version
doi:10.1126/sciadv.1600241

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:27822149

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Structural basis for regulation of human calcium-sensing receptor by magnesium ions and an unexpected tryptophan derivative co-agonist

Chen Zhang,1* Tuo Zhang,2* Juan Zou,1* Cassandra Lynn Miller,1* Rakshya Gorkhali,1 Jeong-Yeh Yang,3 Anthony Schlimmer,2 Shuo Wang,3 Kenneth Huang,1 Edward M. Brown,4 Kelley W. Moremen,3 Jian Hu,2,5† Jenny J. Yang†

Ca2+-sensing receptors (CaSRs) modulate calcium and magnesium homeostasis and many (patho)physiological processes by responding to extracellular stimuli, including divalent cations and amino acids. We report the first crystal structure of the extracellular domain (ECD) of human CaSR bound with Mg2+ and a tryptophan derivative ligand at 2.1 Å. The structure reveals key determinants for cooperative activation by metal ions and aromatic amino acids. The unexpected tryptophan derivative was bound in the hinge region between two globular ECD subdomains, and the dissection of structure-function relations by mutagenesis, biochemical, and functional studies provides insights into the molecular basis of human diseases arising from CaSR mutations. The data also provide a novel paradigm for understanding the mechanism of CaSR-mediated signaling that is likely shared by the other family C GPCR (G protein–heterotrimeric guanine nucleotide–binding protein)–coupled receptor) members and can facilitate the development of novel CaSR-based therapeutics.

INTRODUCTION

The discovery of the parathyroid Ca2+-sensing receptor (CaSR) established a new paradigm in which extracellular Ca2+ ([Ca2+]o) can act as a first messenger for regulation of diverse cellular processes, including regulating the secretion of parathyroid hormone (PTH) and modulating calcium reabsorption by the kidney, in addition to its well-known role as a second messenger (1, 2). Extracellular divalent cations, particularly [Ca2+]o and magnesium ([Mg2+]o), along with amino acids and neurotransmitters, regulate numerous cellular processes via CaSR and 14 other family C G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors (cGPCRs), including metabotropic glutamate (mGluR) and γ-aminobutyric acid type B (GABA-B) receptors (3–7). Small changes in [Ca2+]o or [Mg2+]o trigger CaSR-mediated intracellular Ca2+ signaling and activate mitogen-activated protein kinase [extracellular signal–regulated kinase (ERK) 1 or 2] (8). CaSRs play a central role in regulating [Ca2+]o and [Mg2+]o homeostasis by stimulating phospholipase C to generate inositol 1,4,5-trisphosphate, which triggers release of calcium from its intracellular calcium stores to increase the intracellular free calcium concentration ([Ca2+]i) and activate [Ca2+]i, signaling (9–11), which, in turn, inhibits PTH release, stimulates calcitonin secretion, and promotes renal Ca2+ excretion (12–15). 1–Amino acids, especially those with aromatic side chains, potentiate high [Ca2+]o–elicited activation of CaSR via positive heterotropic functional cooperativity (5). Like other cGPCRs, CaSR functions as a dimer (16–18), with a long (~600 amino acids) N-terminal extracellular domain (ECD) playing an important role in the receptor’s cooperative responses to its agonists (7). More than 400 mutations in CaSR cause human disorders with abnormal [Ca2+]o and [Mg2+]o homeostasis, including familial hypocalciuric hypercalcemia (FHH), neonatal severe hyperparathyroidism (NSHPT), and autosomal dominant hypocalcemia (ADH); 225 of the mutations map to the ECD, highlighting its critical role (19). To clarify the mechanism for cooperative activation of CaSR by [Ca2+]o, [Mg2+]o and amino acids, we solved the first crystal structure of human CaSR-ECD bound with Mg2+ ions. Unexpectedly, a high-affinity tryptophan derivative was found in the crystal structure of CaSR. Further identification and characterization of the CaSR ligand (CaSLR) suggest that it plays a role in potentiating the function of CaSR.

RESULTS AND DISCUSSION

The Venus flytrap domain of human CaSR ECD (hCaSR-ECD; residues 20 to 541) expressed in human embryonic kidney (HEK) 293S (GnTI) cells was crystallized in the presence of 200 mM Mg2+ and 10 mM Ca2+. The structure was solved by molecular replacement using the structure of mGluR2 [Protein Data Bank (PDB) ID: 4XAQ] as the search template (Fig. 1A and table S1). hCaSR-ECD contains two globular lobes with an overall structure similar to other cGPCR family members, despite a low sequence similarity between these cGPCR family members (20 to 30%) (fig. S1) (20). Both the large lobe (subdomain 1) and the small lobe (subdomain 2) are typical α/β folds where the central parallel β strands are sandwiched by α helices. hCaSR-ECD forms a homodimer in solution (fig. S2) and in the crystal structure, with both protomers in a closed conformation (Fig. 1B) similar to the equivalent closed conformation of mGluR1 bound with glutamate [root mean square deviation of 1.24 Å for C (Fig. 1C)]. In addition, the direct and extensive homodimeric subdomain 2 interactions in hCaSR-ECD are analogous to those observed in the mGluR2 dimer with a bound agonist (PDB ID: 4XAQ), strongly suggesting that hCaSR-ECD crystal structure represents an active conformation (fig. S3) (21).
Our data indicate that Mg\(^{2+}\) binds to hCaSR-ECD and elicits CaSR-mediated [Ca\(^{2+}\)]\(_{i}\) signaling and ERK1/2 phosphorylation in CaSR-expressing cells with a lower potency than Ca\(^{2+}\) (Figs. 2, A and B, and 3C and fig. S4A) (22). Similar to [Ca\(^{2+}\)]\(_{o}\) activation, [Mg\(^{2+}\)]\(_{o}\) activation is further potentiated by the known CaSR co-agonist, l-Phe (Fig. 3F) (5, 7). [Ca\(^{2+}\)]\(_{o}\) potentiates [Mg\(^{2+}\)]\(_{o}\)-stimulated intracellular response mediated by CaSR because an increase of [Ca\(^{2+}\)]\(_{o}\) from 0.5 to 1.5 mM results in a reduction of the median effective concentration (EC\(_{50}\)) of [Mg\(^{2+}\)]\(_{o}\) from 7.2 ± 0.4 to 4.5 ± 0.3 mM for stimulation of [Ca\(^{2+}\)]\(_{i}\) signaling. These results suggest that there is an additive effect of both Ca\(^{2+}\) and Mg\(^{2+}\) and that they share a similar activation mechanism (Fig. 2A, fig. S5, and table S2) (22, 23). The binding of Mg\(^{2+}\) can be visualized by the reduction of intrinsic Trp fluorescence upon addition of Mg\(^{2+}\) to the purified ECD and the reduction of Tb\(^{3+}\)-sensitized energy transfer by Mg\(^{2+}\) competition (fig. S6). In the crystal structure, two Mg\(^{2+}\) binding sites were identified at positions designated as site 1 and site 2 (Fig. 2B). Site 1 is located at the dimerization interface of subdomain 2 and the bound Mg\(^{2+}\) coordinates with S\(_{240}\) and four water molecules with an ideal geometry for a Mg\(^{2+}\) ion. Notably, site 1 is surrounded by highly conserved residues (E\(_{228}\), E\(_{231}\), and E\(_{241}\)* (* means from the other protomer) within 5 Å from an “acidic patch” composed of negatively charged residues on subdomain 2 (fig. S7). Site 2 is found on the periphery of subdomain 1, coordinated by S\(_{84}\) and backbone interactions with I\(_{81}\), L\(_{87}\), and L\(_{88}\), as well as two water molecules. An equivalent cation binding site has been observed in mGluR (20) and likely plays a structural role. To locate additional high off-rate metal binding sites, we generated Gd\(^{3+}\)-derived crystals and identified another metal binding site (site 3) on the acidic patch in proximity to the subdomain 2 di-merization interface (Fig. 2C) and adjacent to Mg\(^{2+}\) binding site 1. Site 3 largely overlaps with a previously predicted Ca\(^{2+}\) binding site 3 (24). Mutation of site 3 coordinating residues (E228I or an E228I/E229I double mutant) reduced Ca\(^{2+}\)/Mg\(^{2+}\) sensing as well as Mg\(^{2+}\)-evoked intracellular Ca\(^{2+}\) mobilization. These results suggest the critical role of these metal binding sites on the acidic patch in both metal sensing (24) and regulation of CaSR function (Fig. 2G, figs. S7 and S9, and tables S3 and S4).

Unexpectedly, an elongated planar electron density was observed in the hinge region between the two subdomains where orthosteric ligand binding is thought to occur (Fig. 3A). No naturally occurring CaSRLs or reagents that were used in sample preparation and crystallization or any currently known CaSRLs fit the density well, suggesting a novel CaSRL. High-resolution liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) of the purified protein preparation (Fig. 3B) identified a species that was eluted at ~4.65 min with a mass/charge ratio (m/z) of 215.0824 in negative-ion mode. The predicted elemental formula based on the observed mass corresponds to C\(_{12}\)H\(_{11}\)N\(_{2}\)O\(_{2}\) (calculated mass, 215.0821; mass accuracy, 1.4 ppm) (Fig. 3B and fig. S8). A search of PubChem identified a tryptophan derivative, 1,1,2,3,4-tetrahydronorharman-3-carboxylic acid (TNCA) with the predicted molecular weight (Mr) and shape of the observed density. When compared to tryptophan, TNCA contains one extra carbon atom linking the amine nitrogen atom and the C2 atom of the indole ring. TNCA can be detected in various food and biological systems and is likely produced by tryptophan reacting with formaldehyde in humans (25), and is perhaps generated during production of the recombinant protein in HEK cells. Elution time, molecular weight, and MS fragmentation of synthetic TNCA matched those of the CaSRL, confirming the identity of the

---

**Fig. 1. Crystal structure of hCaSR-ECD.** (A) Monomeric hCaSR-ECD with labeled secondary structural elements. (B) Homodimer of hCaSR-ECD. (C) Structural overlap of hCaSR-ECD with rat mGluR1 (rmGluR1) in the closed conformation (PDB ID: 1EWK).
compound as TNCA (Fig. 3B and fig. S8). The ligand will be referred to hereafter as TNCA.

TNCA is a strong co-agonist with \([\text{Mg}^2+]_o\) in activating \([\text{Ca}^{2+}]_o\) oscillations and ERK1/2 phosphorylation (Fig. 3, D to F, and fig. S9). Similar to Trp and other amino acids, addition of exogenous TNCA alone cannot activate the receptor. However, TNCA is \(\sim 1000\)-fold more potent than Phe in reducing the EC50 for \([\text{Mg}^2+]_o\) or \([\text{Ca}^{2+}]_o\) activation of \([\text{Ca}^{2+}]_i\) signaling in both wild-type and mutant CaSRs (Fig. 3, Fa n dG), with an apparent EC50 of \(\leq 2 \text{mM}\) (Fig. 3H). The apparent EC50 of TNCA was indirectly determined through the EC50 change of \([\text{Mg}^2+]_o\) when incubated with different concentrations of TNCA (Fig. 3, F to H). Because the bound TNCA can be partially replaced by incubation with 150 mM Phe as assessed by MS (fig. S10), TNCA and Phe likely share the similar binding site in CaSR-ECD. Together, TNCA is a novel, high-affinity co-agonist of CaSR in the activation of both \([\text{Ca}^{2+}]_i\) signaling and ERK activity.

CaSR strongly prefers aromatic amino acid ligands, such as Phe and Trp, over negatively charged Glu, which is the ligand for mGluRs. Structural comparison of the ligand binding pocket in the hinge region between subdomains 1 and 2 of hCaSR-ECD homodimer. Mg\(^{2+}\) and Gd\(^{3+}\) are depicted as hot pink and dark blue spheres, respectively. An anomalous difference map of Gd\(^{3+}\) (\(\sigma = 8.0\)) is shown in purple. W, water molecules. (D to F) Both site 1 (E) and site 3 (D) are on the “acidic patch” at the dimerization interface of subdomain 2 (fig. S7), whereas Mg\(^{2+}\) at site 2 in subdomain 1 (F) is primarily coordinated by the backbone carbonyl oxygen atoms. (G) Single mutations of E228I on the acidic patch significantly reduce CaSR-mediated \([\text{Ca}^{2+}]_i\) responses in the cell population assay.

Mapping of disease-associated mutations on the structure of hCaSR-ECD shows that the mutations are clustered in two regions: the hinge region between subdomains 1 and 2 and the dimerization interface (fig. S12) (26, 27). Indeed, our structural and functional data strongly support the pivotal roles of these two regions in CaSR function. The hinge region between subdomains 1 and 2 harbors the binding site of TNCA, supporting its role as a co-agonist of CaSR. Two other co-agonists of CaSR, Phe and Trp, likely bind in the same position (fig. S10). We did not observe metal binding at the previously proposed site 1 for Ca\(^{2+}\) (6, 24, 28). A close inspection of the structure reveals that the side chain of E\(^{227}\), a critical residue predicted for Ca\(^{2+}\) binding, is not exposed to the medium, suggesting that Ca\(^{2+}\) binding at site 1 is not likely. The structural basis for Mg\(^{2+}/\text{Ca}^{2+}\) modulated CaSR activities (A) CaSR-mediated \([\text{Ca}^{2+}]_i\) responses measured by imaging of single-cell calcium oscillations with Fura-2 using HEK293 cells transfected with CaSR in the presence of various concentrations of \([\text{Ca}^{2+}]_o\) and \([\text{Mg}^2+]_o\) and fit to the Hill equation. (B) ERK1/2 activities upon stimulation by agonists were detected using Western blot and further quantified using ImageJ. The measurements were plotted against different concentrations of \([\text{Ca}^{2+}]_o\) or \([\text{Mg}^{2+}]_o\) and fit to the Hill equation. (C) Identified metal binding sites in the structure of hCaSR-ECD homodimer. Mg\(^{2+}\) and Gd\(^{3+}\) are depicted as hot pink and dark blue spheres, respectively. An anomalous difference map of Gd\(^{3+}\) (\(\sigma = 8.0\)) is shown in purple. W, water molecules. (D to F) Both site 1 (E) and site 3 (D) are on the “acidic patch” at the dimerization interface of subdomain 2 (fig. S7), whereas Mg\(^{2+}\) at site 2 in subdomain 1 (F) is primarily coordinated by the backbone carbonyl oxygen atoms. (G) Single mutations of E228I on the acidic patch significantly reduce CaSR-mediated \([\text{Ca}^{2+}]_i\) responses in the cell population assay.
Fig. 3. Identification and characterization of a tryptophan derivative bound to hCaSR-ECD as a novel high-affinity co-agonist of CaSR. (A) $F_o - F_c$ omit map ($F_o$ and $F_c$ are the observed and the calculated structure factor amplitudes, respectively) of TNCA at $\sigma = 4.5$. The protein is shown in ribbon mode, and the ligand is shown in stick mode. The residues around TNCA are labeled in the zoomed-in figure. (B) LC-ESI-MS of protein sample (top), buffer (middle), and the standard compound (bottom) in negative-ion mode. The high-resolution isotopic MS spectra of the indicated peaks are shown in the inserted figures. (C and D) A representative oscillation pattern from a single HEK293 cell stimulated with various concentrations of extracellular Ca$^{2+}$ or Mg$^{2+}$ in the absence (C) and presence (D) of 0.25 mM TNCA. (E) Frequency distribution of the [Ca$^{2+}$], oscillation frequency (peak/min) in HEK293 cells transfected with wild-type CaSR stimulated with metals in the presence (red bar) and absence (black bar) of TNCA. The frequency was recorded at the point when more than 50% single cells started to oscillate. Around 40 cells were analyzed and further plotted as a bar chart. (F and G) TNCA potentiates [Mg$^{2+}$]- or [Ca$^{2+}$]-evoked [Ca$^{2+}$]$_i$ responses in a population assay in 5001 cells measured by Fura-2 acetoxymethyl (AM) in the absence (black square) or presence of Phe (blue triangular) or TNCA (red closed circle). (H) A maximally active concentration of 0.1 to 0.5 mM TNCA markedly reduces the EC$_{50}$ for activation of [Ca$^{2+}$], signaling by [Mg$^{2+}$], in the presence of 0.5 mM [Ca$^{2+}$]. Inset: The EC$_{50}$ changes of [Mg$^{2+}$], are shown over a narrow concentration range of TNCA.
binding in this proposed site 1, swings away from the other residues in site 1 (S\textsubscript{170}, D\textsubscript{190}, Q\textsubscript{193}, and Y\textsubscript{218}), probably due to the extra carbon atom and the rigid structure of TNCA, ultimately resulting in its failure to capture the Ca\textsuperscript{2+} ion in combination with other site 1 residues (fig. S13). Nevertheless, the essential role of E\textsubscript{297} in Ca\textsuperscript{2+} sensing has been supported by previous mutational studies (7, 24) and in the abrogated Mg\textsuperscript{2+} sensing of the E297I mutant (tables S3 and S4). A bicarbonate anion was also identified in the hinge region in proximity with TNCA, coordinated by the side chains of R\textsubscript{66}, R\textsubscript{69}, W\textsubscript{70}, and S\textsubscript{417} and the backbone amide nitrogen atoms of I\textsubscript{416} and S\textsubscript{417} (figs. S11 and S14), potentially contributing to the known pH sensitivity of the CaSR (29).

Figure 4A summarizes our present model for receptor activation. The presumed conformational change induced by ligand/metal binding at the hinge region between subdomains 1 and 2, with homodimerization of protomer subdomains 1 through loops 1 and 2, facilitates the approach of subdomain 2 from their respective protomers. By neutralizing the repulsive effects of the conserved negatively charged acidic patch, metal binding would stabilize subdomain 2 interactions (fig. S15). Dimerization of subdomain 2 is also critical for the activation of mGluRs and GABA\textsubscript{B} receptors (31, 32) and therefore appears to be a common activation mechanism among cGPCRs that presumably leads to conformational changes of the transmembrane domain, through which the intracellular signal cascades are initiated. Thus, our proposed model, which is based on structural and associated biochemical and cellular studies on wild-type and mutant proteins, reveals the structural basis of agonist/co-agonist binding to CaSR and provides a framework for further studies on the mechanism of receptor activation. The discovery of an unexpected tryptophan derivative ligand (TNCA) with unusually high affinity, which potentiates the activation of CaSR by [Ca\textsuperscript{2+}]\textsubscript{i} and [Mg\textsuperscript{2+}]\textsubscript{i} also opens new avenues for the development of agonists and antagonists as therapeutics for CaSR-related diseases (33).

CONCLUSION

We report here the first crystal structure of the ECD of human CaSR bound with Mg\textsuperscript{2+} and a tryptophan derivative ligand at 2.1 Å. The structure reveals important determinants for cooperative activation of the CaSR by metal ions and aromatic amino acids, including key residues in the ECD participating in Mg\textsuperscript{2+} and amino acid binding. In addition, extensive interactions between the disulfide-linked dimeric ECDs and their respective subdomains that produce a closed and likely active conformation of the Venus flytrap motifs of the two ECDs are present. The unexpected tryptophan derivative that was bound in the hinge region between two ECD subdomains represents a novel high-affinity co-agonist of CaSR that potentiates activation of the full-length CaSR expressed in HEK293 cells by Ca\textsuperscript{2+} and Mg\textsuperscript{2+}. Our dissection, by mutagenesis, biochemical, and functional studies, of structure function relations provides insights into the molecular basis.
of human diseases arising from CaSR mutations. Finally, these data also provide a novel paradigm for understanding the mechanism of CaSR-mediated signaling that is likely shared by the other members of the family C GPCRs and can facilitate the identification and development of novel CaSR-based therapeutics.

**MATERIALS AND METHODS**

**Purification of hCaSR-ECD secreted from HEK293S GnTI cells**

hCaSR-ECD (from residues Tyr20 to Phe612) (fig. S1) was expressed in suspension culture of HEK293S GnTI cells and purified from the culture medium by Ni2+-nitrilotriacetic acid (NTA) chromatography, as previously described (34). To deglycosylate the purified protein, hCaSR-ECD was incubated with recombinant endoglycosidase F1 (Endo F1) at a 1:100 mass ratio of Endo F1 to hCaSR-ECD overnight at 21°C in 10 mM tris buffer (pH 7.4). Further separation of hCaSR-ECD from Endo F1 was achieved by size exclusion chromatography (SEC) in 10 mM Hepes buffer (pH 7.3). hCaSR-ECD forms a homodimer, as determined by the elution volume observed in SEC. The electrophoretic mobility in reducing/nonreducing SDS–polyacrylamide gel electrophoresis (SDS-PAGE) indicates that intermolecular disulfide bonds contribute to dimerization (fig. S2).

**Crystallization, data collection, and structure determination**

The dimeric hCaSR-ECD was concentrated to 10 mg/ml and crystallized in 10% polyethylene glycol (PEG)–8000, 200 mM MgCl2, 10 mM CaCl2, and 100 mM tris-HCl (pH 7.0), using the sitting drop approach at 21°C. No crystals were formed in the absence of Ca2+ or Mg2+. The plate-shaped crystals were cryoprotected using 25% glycerol and were flash-frozen in liquid nitrogen. Dehydration by soaking the crystal in 12% PEG-8000 overnight improved the resolution from 3.5 to 2.3 Å. The diffraction data of the crystals were collected on the beamline of 21-ID-D at LS-CAT (Life Sciences Collaborative of the National Institute of Health) and were processed by HKL2000 (35). To avoid the overexposure of space groups, crystals with the best crystals were selected for data collection. The structure of chain A of mGluR2 (36) with a bound agonist (PDB ID: 4XAQ) was used as the search template (21). The electron density map after molecular replacement was clear enough to identify the unique features of hCaSR-ECD, and iterative model building and refinement were performed using COOT (38) and Refmac5 in the CCP4 (39) suite, respectively. The restraints of TNCA (CaSRL) were generated by JLigand in COOT.

To generate the Gd3+ derivative, the native crystals were soaked with a solution containing 12% PEG–8000, 200 mM MgCl2, 10 mM CaCl2, 100 mM tris-HCl (pH 7.0), and 0.5 mM GdCl3 overnight at 21°C. The anomalous signals of a data set at 2.7 Å collected at the wavelength of 1.6985 Å were used to locate Gd3+ in the structure. The structure was solved by molecular replacement using the previously determined structure as the search template. All the figures of protein structures were generated by PyMOL version 1.3 (Schrodinger LLC).

Using local geometric restraints and electron density intensity as major criteria, Mg2+ bound at site 1 and site 2 (fig. 2B) were unambiguously identified. Evidence also suggested a potential Mg2+ binding site in the hinge region in proximity with the bound TNCA (fig. S16). Modeling a water molecule at this position led to a B factor (26 to 32 Å2) substantially smaller than the coordinating atoms (35 to 40 Å2), suggestive of a slightly heavier atom occupying this position. Considering the negatively charged coordination sphere (A(21), D(316), Y(218), S(27), D(275), and water), it was possible that this density corresponded to a bound Mg2+. Mutations such as Y218K and D275I resulted in diminished intracellular calcium responses using both the cell population and single-cell calcium imaging assays, although these mutations retained their surface expression (fig. S16B). Alternatively, it may be a highly ordered water molecule trapped in the hinge region because the distances to the coordinating oxygen atoms (2.5 to 2.8 Å) were significantly greater than typically observed Mg-O distance in biomolecules (2.1 Å) (40). Nevertheless, the low occupancy of Mg2+ due to its weak affinity may also cause a distorted geometry. At this stage, we have placed a water molecule in the model; however, given the importance of the hinge region in CaSR function and regulation, more functional and structural studies are warranted to further investigate the potential Mg2+ binding site identified here.

**High-resolution LC-ESI-MS and identification of TNCA**

As shown in Fig. 3A, there was an unidentified ligand (CaSRL) bound at the putative orthosteric ligand binding site of CaSR-ECD. We examined the known CaSRLs, including phenylalanine, tryptophan, glutathione, and polyanines, as well as the reagents used in sample preparation and crystallization, but none of them fit the density well. Among these initial trials, tryptophan appeared to be the best fit to the electron density of the unknown ligand, but an additional density was unaccounted for when tryptophan was used to fit the electron density. The size of the density suggested that the CaSRL contained 14 to 18 heavy atoms (C/N/O/S/P), and the absence of anomalous signal indicated that it did not contain sulfur or phosphate. Accordingly, the M, of CaSRL must be within the range of ~180 to 250 daltons.

Considering that the CaSRL was tightly bound with hCaSR-ECD, it was conceivable that hCaSR-ECD had to be denatured to release CaSRL. To extract CaSRL, 50 µl of purified hCaSR-ECD (10 mg/ml) was mixed with 120 µl of acetonitrile and was vortexed. After high-speed centrifugation, 10 µl of the CaSRL extract was injected onto a reverse-phase ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7-µm particle size; Waters). Column temperature was maintained at 40°C. The flow rate was 0.3 ml/min with starting conditions at 99% solvent A (water + 0.1% formic acid) and 1% solvent B (acetonitrile). The 15-min gradient profile for elution was as follows: starting at 1% solvent B, hold for 1 min, then ramp to 98% B at 10 min, hold at 98% B to 12 min, return to 99% A/1% B at 12.01 min, and maintain until 15 min. The samples were analyzed using a Waters Xevo G2-XS QToF LC-MS interfaced to a Waters ACQUITY UPLC system. The MS settings were as follows: electrospray ionization in negative-ion mode, capillary voltage of 2.00 kV, source temperature of 100°C, desolvation temperature of 350°C, desolvation nitrogen gas flow rate of 600 liters/hour, cone voltage of 35 V, and mass range of m/z 50 to 1500 with spectra accumulated at 0.1 s per function. Three separate acquisition functions were performed to generate spectra at different collision energies (5, 25, and 60 eV) providing both nonfragmenting and fragmenting conditions. Analyses of samples by electrospray ionization in positive-ion mode were performed under the same conditions as negative-ion mode except for the collision energies (5, 20, and 40 eV). Fragmentation, formula, and abundances were analyzed with Waters MassLynx Software.

Using the above approach, we identified a species eluting at ~4.65 min, detected by MS in both positive-ion mode (m/z of...
217.0990) and negative-ion mode (m/z of 215.0824), exclusively present in protein samples from several different batches but not in the sample buffer. The predicted elemental compositions based on mass are C12H11N2O2 (calculated mass = 217.0977 daltons) for positive-ion mode and C12H11N2O2 (calculated mass = 215.0824 daltons) for negative-ion mode. A thorough search in the PubChem database led to a list of candidates containing up to 200 compounds with the same mass and formula. By manually fitting the density map with these compounds, only TNCA fit the density perfectly. Synthetic TNCA dissolved in the SEC buffer was treated in the same way as the protein samples in the LC-ESI-MS experiment, and resulted in a peak detected at the same retention time and having the same mass spectrum. In the LC-ESI-MS experiment, we also noticed a minor species that eluted at ~4.57 min (fig. S10), which was detectable only in the positive-ion mode (m/z of 215.0836) and having a predicted elemental formula of C12H11N2O2. The 2-dalton smaller species Ms for this related compound suggested that it was a derivative of TNCA, likely due to a double bond formation between the backbone N and a neighboring C. Because it is also likely to be a tryptophan derivative, we cannot exclude the possibility that it binds hCaSR-ECD with high affinity. This compound may also form during extraction of TNCA from the protein sample. Nevertheless, TNCA perfectly fits the electron density at 2.1 Å, and any extra double bonds in the CaSR1 structure would likely be detrimental to fitting the density.

A phenylalanine replacement experiment was carried out by mixing purified hCaSR-ECD protein (0.26 mg/ml) with phenylalanine (final concentrations are 0, 50, and 150 mM, respectively). After overnight incubation at room temperature, hCaSR-ECD in each sample was repurified with Ni2+-NTA beads. The protein samples were adjusted to the same concentration using SEC buffer and analyzed by LC-ESI-MS.

Monitoring Mg2+ binding to CaSR-ECD by fluorescence spectroscopy

The imidazole in fractions of hCaSR-ECD eluted from the Ni2+-NTA column was removed by passing the protein through desalting columns in Hepes buffer (10 mM Hepes at pH 7.2). The Trp fluorescence spectra of hCaSR-ECD were recorded on a QM1 fluorescence spectrophotometer (Photon Technology International) in a 1-cm-pathlength cell with a xenon short-arc lamp at ambient temperature. The emission between 300 and 400 nm was acquired during excitation at 282 nm. A solution containing 2 μM hCaSR-ECD in 10 mM Hepes (pH 7.2), 120 mM NaCl, and 10 mM KCl was gradually titrated by adding of Ca2+ prepared in the same Hepes buffer. The binding constants of Mg2+ to CaSR-ECD were calculated by fitting the titration curve with the Hill equation. The Ca2+-Tb3+ competition experiments were performed in solutions containing 35 μM Tb3+ and 2 μM hCaSR-ECD as the starting point. MgCl2 was added to the mixture from a 1 M stock solution while maintaining a constant Tb3+ concentration in the solution. The Mg2+ binding affinity of the protein was calculated by fitting normalized fluorescence intensity data using the Hill equation

\[ \Delta S = \frac{[M]^n}{K_d + [M]^n} \]  

where \( \Delta S \) is the total signal change in the equation, \( K_d \) is the apparent binding affinity, \( n \) is the Hill coefficient, and \([M]\) is the free metal concentration.

TNCA and Mg2+ binding site mutation design

All of the full-length CaSR mutants were generated by site-directed mutagenesis on the basis of the sequence of the human CaSR in the pcDNA3.1(+) expression vector (provided by E. Brown). Site-directed mutagenesis was performed using the QuikChange kit (Stratagene) according to the manufacturer’s instructions. Briefly, a pair of complementary primers of 27 to 35 bases was designed for generating each mutant with the mutation placed at the middle of the primers. The template human CaSR in pcDNA3.1(+) was amplified using Pfu DNA polymerase (Stratagene) with these primers for 16 cycles in a polymerase chain reaction instrument (Techne). After digestion of the template DNA with Dpn I (New England Biolabs), the amplified mutant DNA was transformed into XL10-Gold Ultracompetent cells. All the DNA sequences were verified by Genewiz (www.genewiz.com).

Cell culture and transfection

Monolayer cultures of HEK293 cells were purchased from American Type Culture Collection (ATCC CRL-1573) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and high glucose (4.5 g/liter) at 37°C. Wild-type CaSR or its mutants were transfected into HEK293 cells using Lipofectamine 2000 (Life Technologies) by following the manufacturer’s instructions.

Immunostaining

Cells transfected with hCaSR-pcDNA3.1(+) were used in the immunostaining experiments, and this construct contained a FLAG tag between Asp731 and Thr772. After 48 hours post-transfection, cells were fixed with 3.7% formaldehyde for 15 min at room temperature, followed by washing with phosphate-buffered saline (PBS) three times. Mouse anti-FLAG monoclonal antibody was diluted 500 times and incubated with cells overnight at 4°C to stain the cell-surface CaSR. The cells were subsequently washed with PBS and stained with goat anti-mouse Alexa 488–conjugated secondary antibody for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole. Fluorescence was visualized using a Zeiss LSM780 confocal microscope.

Measurement of [Ca2+]i changes triggered by [Mg2+]o in single CaSR-transfected cells

Measurement of intracellular free Ca2+ was assessed as described by Huang et al. (6). Briefly, wild-type CaSR or its mutants were transiently transfected into HEK293 cells grown on coverslips and cultured for 48 hours. The cells were subsequently loaded for 15 min using 2 μM Fura-2 AM in 2 ml of physiological saline buffer (10 mM Hepes, 140 mM NaCl, 5 mM KCl, and 1.0 mM MgCl2) (pH 7.4). The coverslips were mounted on a bath chamber on the stage of a Leica DM6000 fluorescence microscope. The cells were alternately illuminated with 340- or 380-nm light, and the fluorescence at an emission wavelength of 510 nm was recorded in real time as the [Ca2+]i and/or [Mg2+]o was increased in a stepwise manner in the presence or absence of 0.25 mM TNCA in buffer (10 mM Hepes, 155 mM NaCl, 5 mM KCl, 2 mM NaH2PO4, and 0.5 mM MgCl2) (pH 7.4). The ratio of the emitted fluorescence intensities resulting (7) from excitation at both wavelengths was utilized as a surrogate for changes in [Ca2+]i, and was further plotted and analyzed as a function of [Ca2+]i. All experiments were performed at room temperature. The signals from 20 to 100 single cells were recorded for each measurement. Oscillations

Zhang et al. Sci. Adv. 2016;2:e1600241 27 May 2016
were identified as three successive fluctuations in [Ca^{2+}], after the initial peak.

**Determination of the effect of TNCA on Mg^{2+}-evoked [Ca^{2+}]_i signaling by stimulation of CaSR in cell populations**

Changes in [Ca^{2+}]_i elicited by [Mg^{2+}]_o, in a population of cells were measured by fluorometry as previously described (6). A cell line stably expressing CaSR (5001) was seeded on 13.5 × 20-mm coverslips and cultured in DMEM. After reaching 95% confluence, cells were washed three times using loading buffer (20 mM HEPES (pH 7.4), 125 mM NaCl, 5 mM KCl, 1.25 mM CaCl_2, 1 mM MgCl_2, 1 mM NaH_2PO_4, 1% glucose, and 1% bovine serum albumin (BSA)) and subsequently incubated with 4 μM Fura-2 and 4 μM Pluronic F127 for 20 min at 37°C to enable sufficient dye loading in the same buffer. After removing the excess Fura-2, coverslips with cells were diagonally positioned in a quartz cuvette filled with 3 ml of experimental buffer (125 mM NaCl, 5 mM KCl, 0.5 mM CaCl_2, 0.5 mM MgCl_2, 1% glucose, and 1% BSA). Measurements of Fura-2 fluorescence at 510 nm when excited at 340 or 380 nm were performed on a QM1 fluorescence spectrophotometer (Photon Technology International). The emission ratio of 340/380 nm was used to calculate the changes in [Ca^{2+}]_i, when different concentrations of [Mg^{2+}]_o were applied to the cells.

To examine the caoactivation of CaSR by TNCA and [Mg^{2+}]_o, or [Ca^{2+}]_o, different concentrations of TNCA were placed in the experimental buffer with a fixed concentration of [Ca^{2+}]_o, and varying concentrations of [Mg^{2+}]_o, or vice versa, as described in the Results section. The effects of other ligands were analyzed by comparing the changes in [Ca^{2+}]_i, produced by [Mg^{2+}]_o, alone or by copackulation of Mg^{2+} with other ligands. The EC_{50} of [Mg^{2+}]_o, obtained during incubation with various concentrations of TNCA is compared with that observed in the presence of [Mg^{2+}]_o, alone. The EC_{50} changes were plotted as a function of TNCA concentration, and the curve was fit to the Hill equation. The activation of CaSR by the TNCA, functioning as a co-agonist with [Mg^{2+}]_o, was indicated by the increasingly left-shifted EC_{50} for [Mg^{2+}]_o as the concentration of TNCA increases (table S5).

**Determination of ERK1/2 phosphorylation**

The 5001 cell line stably expressing hCaSR was starved in serum-free DMEM medium supplemented with 0.2% (w/v) BSA overnight, followed by washing with Hank’s balanced salt solution (HBSS) three times and a subsequent 10-min HBSS incubation in the morning of the second day. To induce ERK1/2 phosphorylation, varying concentrations of [Mg^{2+}]_o (0 to 50 mM) or [Ca^{2+}]_o (0 to 30 mM) with or without 0.5 mM TNCA were added to cells and incubated for 10 min at 37°C. The cells were then lysed with Pierce radioimmunoprecipitation assay buffer (Thermo Scientific). Total protein concentration was measured using the Bio-Rad assay. Lysates containing 100 μg of total protein were loaded onto 4 to 20% gradient SDS-PAGE gels for separation. After electrophoresis, proteins on the gel were transferred to nitrocellulose membranes and were further analyzed by Western blotting. Anti–phospho-p44/42 ERK (1:1000 dilution) and anti-p44/42 (1:2000) polyclonal antibodies were utilized as probes to detect the phospho-ERK1/2 and total ERK1/2, respectively. A chemiluminescent detection method (AP Conjugate Substrate Kit, Bio-Rad) was applied to detect phospho-ERK1/2 and total ERK1/2. The respective bands on Western blots were evaluated by densitometry. The EC_{50} of [Mg^{2+}]_o- or [Ca^{2+}]_o-dependent responses were obtained by fitting the [Mg^{2+}]_o or [Ca^{2+}]_o concentration-response curves with the Hill equation (Eq. 1).

**SUPPLEMENTARY MATERIALS**

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/5/e1600241/DC1

**SUPPLEMENTARY MATERIALS**

| Table | Title |
|-------|-------|
| S1 | Crystallographic statistics of hCaSR-ECD and hCaSR-ECD/Gd^{3+}. |
| S2 | EC_{50} of [Mg^{2+}]_o for stimulation of [Ca^{2+}]_i signaling in single cell assay with or without TNCA. |
| S3 | EC_{50} of [Mg^{2+}]_o for stimulation of [Ca^{2+}]_i signaling in cell population assay with or without TNCA. |
| S4 | EC_{50} of [Mg^{2+}]_o for stimulation of [Ca^{2+}]_i signaling in cell population assay with coapplication of various concentrations of TNCA. |
| S5 | EC_{50} of [Mg^{2+}]_o-elicited [Ca^{2+}]_i responses in cell population assay with or without TNCA. |

**REFERENCES AND NOTES**

1. E. M. Brown, G. Gamba, D. Riccardi, M. Lombardi, R. Butters, O. Kifor, A. Sun, M. A. Hediger, J. L. Lytton, S. C. Hebert, Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. Nature 366, 575–580 (1993).
2. M. P. Grant, A. Stepanchick, A. Cavanaugh, G. E. Breitwieser, Agonist-driven maturation and plasma membrane insertion of calcium-sensing receptors dynamically control signal amplification. Sci. Signal. 4, ra78 (2011).
3. A. A. Semporkay, D. H. Kretsinger, Cell signaling, beyond cytosolic calcium in eukaryotes. J. Inorg. Biochem. 103, 77–86 (2009).
4. Y. Kubo, T. Miyashita, Y. Murata, Structural basis for a Ca^{2+}-sensing function of the metabotropic glutamate receptors. Science 279, 1722–1725 (1998).
5. A. D. Coniglione, S. J. Quinn, E. M. Brown, L-α-amino acid sensing by the extracellular Ca^{2+}-sensing receptor. Proc. Natl. Acad. Sci. U.S.A. 97, 4814–4819 (2000).
6. Y. Huang, Y. Zhou, W. Yang, R. Butters, H.-W. Lee, S. Li, A. Castiblanco, E. M. Brown, J. J. Yang, Identification and dissection of Ca^{2+}-binding sites in the extracellular domain of Ca^{2+}-sensing receptor. J. Biol. Chem. 282, 19000–19010 (2007).
7. C. Zhang, Y. Huang, Y. Jiang, N. Mulpuri, L. Wei, D. Hamelberg, E. M. Brown, J. J. Yang, Identification of an L-phenylalanine binding site enhancing the cooperative responses of the calcium-sensing receptor to calcium. J. Biol. Chem. 289, 5296–5309 (2014).
8. B. W. Bapty, L.-J. Dais, G. Ritchie, F. Jirik, L. Canaff, G. N. Hendy, G. A. Quammme, Extracellular Mg^{2+} and Ca^{2+}-sensing in mouse distal convoluted tubule cells. Kidney Int. 53, 583–592 (1998).
9. A. L. Magno, B. K. Ward, T. Ratajczak, The calcium-sensing receptor: A molecular perspective. Endocr. Rev. 32, 3–30 (2010).
10. E. M. Brown, R. J. MacLeod, Extracellular calcium sensing and extracellular calcium signaling. Physiol. Rev. 81, 239–297 (2001).
11. W. Chang, D. Shoback, Extracellular Ca^{2+}-sensing receptors—an overview. Cell Calcium 35, 183–196 (2004).
12. N. J. Fudge, C. S. Kovacs, Physiological studies in heterozygous calcium sensing receptor (CaSR) gene-ablated mice confirm that the CaSR regulates calcitonin release in vivo. BMC Physiol. 4, 5 (2004).
13. S. C. Hebert, Extracellular calcium-sensing receptor: Implications for calcium and magnesium handling in the kidney. Kidney Int. 50, 2129–2139 (1996).
14. C. Ho, D. A. Conner, M. R. Pollak, D. J. Ladd, O. Kifor, H. B. Warren, E. M. Brown, J. G. Seidman, C. E. Seidman, A mouse model of human familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. Nat. Genet. 11, 389–394 (1995).
15. A. M. Hofer, E. M. Brown, Extracellular calcium sensing and signalling. Nat. Rev. Mol. Cell Biol. 4, 530–538 (2003).
16. M. Bai, Structure and function of the extracellular calcium-sensing receptor (Review). Int. J. Mol. Med. 4, 115–125 (1999).
17. K. Ray, B. C. Hauschild, P. J. Steinbach, P. K. Goldsmith, O. Hausache, A. M. Spiegel, Identification of the cysteine residues in the amino-terminal extracellular domain of the human Ca$^{2+}$ receptor critical for dimerization. Implications for function of monomeric Ca$^{2+}$ receptor. J. Biol. Chem. 274, 27642–27650 (1999).
18. Y. Suzuki, E. Moriyoshi, D. Tsuchiya, H. Jingami, Negative cooperativity of glutamate binding in the dimeric metabotropic glutamate receptor subtype 1. J. Biol. Chem. 279, 35526–35534 (2004).
19. S. Pidasheva, L. D’Souza-Li, L. Canaff, D. E. C. Cole, G. N. Hendy, CaSRdb: Calcium-sensing receptor locus-specific database for mutations causing familial (benign) hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia. Hum. Mutat. 24, 107–111 (2004).
20. N. Kunishima, Y. Shimada, Y. Tsuji, T. Sato, M. Yamamoto, T. Kumasaka, S. Nakanishi, H. Jingami, K. Morikawa, Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature 407, 971–977 (2000).
21. J. A. Monn, L. Prieto, L. Taboada, C. Pedregal, J. Hao, M. R. Reinhard, S. S. Henry, P. J. Goldsmith, C. D. Beadle, L. Walton, T. Man, H. Rudyk, B. Clark, D. Tupper, S. R. Baker, C. Lamas, C. Montero, A. Marcos, J. Blanco, M. Bures, D. K. Dawson, K. Atwell, F. Lu, W. Jiang, M. Russell, B. A. Heinz, X. Wang, J. H. Carter, C. Xiang, J. T. Caflow, S. Swanson, H. Sanger, L. L. Broad, M. P. Johnson, K. Knopp, R. M. A. Simmons, B. G. Johnson, D. B. Shaw, D. L. McIntosh, Synthesis and pharmacological characterization of C4-disubstituted analogs of 15,25,5R,6S-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate: Identification of a potent, selective metabotropic glutamate receptor agonist and determination of agonist-bound human mGlu2 and mGlu3 amino terminal domain structures. J. Med. Chem. 58, 1776–1794 (2015).
22. S. J. Quinn, A. R. B. Thomsen, O. Egbuna, J. Pang, K. Baxi, D. Goltzman, M. Pollak, 30. H. Minamia, T. Yoshida, K. Oikawa, Q. Zhang, S. Inoue, I. Aotsuy, Direct determination of the ligand-binding cores of a metabotropic glutamate receptor complexed with an antagonist and both glutamate and Ga$^{3+}$. Proc. Natl. Acad. Sci. U.S.A. 99, 2660–2665 (2002).
23. Y. Geng, M. Bush, L. Mosyak, F. Wang, O. R. Fan, Structural mechanism of ligand activation in human GABA$\beta$ receptor. Nature 504, 254–259 (2013).
24. E. F. Nemeth, W. G. Goodman, Calcimimetic and calcilytic drugs: Feats, flaws, and futures. Calcif. Tissue Int. 98, 341–358 (2015).
25. C. Zhang, Y. Zhuo, H. A. Moniz, S. Wang, K. W. Moremen, J. H. Prestegard, E. M. Brown, J. J. Yang, Direct determination of multiple ligand interactions with the extracellular domain of the calcium-sensing receptor. J. Biol. Chem. 289, 33529–33542 (2014).
26. L. Meng, F. Forouhar, D. Thieker, Z. Gao, A. Ramiah, H. Moniz, Y. Xiang, J. Seetharaman, S. Milaninia, M. Su, R. Bridger, L. Veillon, A. Gornhaber, L. Wells, T. G. Montelione, R. J. Woods, L. Tong, K. W. Moremen, Enzymatic basis for N-glycan sialylation: Structure of rat o2,6-sialyltransferase (ST6GAL1) revealed conserved and unique features for sialic acid sialylation. J. Biol. Chem. 288, 34680–34698 (2013).
27. F. M. Hannan, R. V. Thakker, Calcium-sensing receptor (CaSR) mutations and disorders of parathyroid homeostasis. Best Pract. Res. Clin. Endocrinol. Metab. 27, 307–326 (1997).
28. P. D. Adams, P. V. Afonine, G. Bunkóczi, V. B. Chen, I. W. Davis, N. Echols, J. J. Headd, L.-W. Hung, G. J. Kapral, R. W. Grosse-Kunstleve, A. J. McCoy, N. W. Moriarty, R. Oeffner, R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger, P. H. Zwart, PHENIX: A comprehensive Python-based system for macromolecular structure solution. Acta Cryst. D66, 213–221 (2010).
29. P. Emsley, B. Lohkamp, W. G. Scott, K. Cowtan, Features and development of Coot. Acta Cryst. D66, 486–501 (2010).
30. M. D. Winn, C. C. Ballard, K. D. Cowtan, E. J. Dodson, P. Emsley, P. R. Evans, R. M. Keegan, E. B. Krissinel, A. G. Leslie, A. McCoy, J. S. McNicholas, G. N. Murshudov, S. Pannu, E. A. Potterton, H. R. Powell, R. J. Read, A. Vagin, K. S. Wilson, Overview of the CCP4 suite and current developments. Acta Cryst. D67, 235–242 (2011).
31. W. Yang, H.-W. Lee, H. Hellinga, J. J. Yang, Structural analysis, identification, and design of calcium-binding sites in proteins. Proteins 47, 344–356 (2002).

Acknowledgments: We thank R. Das and D. Cox for their help with the confocal experiments. We also thank D. A. Jones at Michigan State University for experimental instructions and thoughtful discussion of mass spectrometry experiments. We thank Y. Zhou for his critical review of this manuscript. We also thank D. A. Jones at Michigan State University for experimental instructions and thoughtful discussion. We thank R. Das and D. Cox for their help with the confocal experiments.