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Structural Mechanism of Cooperative Regulation of Calcium-Sensing Receptor-Mediated Cellular Signaling

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Abstract

Calcaium sensing receptors (CaSRs) play a central role in regulating extracellular calcium (Ca^{2+}) homeostasis and many (patho)physiological processes. This regulation is primarily orchestrated in response to extracellular stimuli via the extracellular domain (ECD). This paper first reviews the modeled structure of the CaSR ECD and the prediction and investigation of the Ca^{2+} and amino acid binding sites. Several recently solved X-ray structures are then compared to support a proposed CaSR activation model involving functional cooperativity. The review also discusses recent implications for drug development. These studies provide new insights into the molecular basis of diseases and the design of therapeutic agents that target CaSR and other family C G protein-coupled receptors (cGPCRs).

Introduction

The discovery of CaSRs by Dr. Ed Brown et al. in 1993 established a new paradigm of Ca^{2+} signaling [1, 2]. CaSRs have been found in key tissues involved in extracellular Ca^{2+} and Mg^{2+} homeostasis (e.g., parathyroid, thyroid, kidney, bone) and in other non-homeostatic tissues (e.g., brain, skin, etc.), of different species [3, 4]. In response to small changes in extracellular concentrations, the ECD of CaSR exhibits a strong positive homotropic cooperative response to Ca^{2+} and Mg^{2+} and heterotropic cooperativity to amino acids,

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Declaration of interests

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metabolites, anions and pH [5]. This functional cooperativity is essential for intracellular Ca^{2+} signaling, inhibition of parathyroid hormone (PTH) release in parathyroid cells, and stimulation of calcitonin secretion in C-cells in both calcitropic and non-calcitropic systems [6–10]. The ability of CaSRs to integrate diverse extracellular stimuli through multiple signaling pathways is shared by other members of cGPCRs, including the metabotropic glutmate receptor (mGluRs) [11–15]. More than 200 mutations and polymorphisms have been found in the ECD of CaSR, either associated with calcitropic diseases, such as primary/secondary hyperparathyroidism, chronic kidney diseases (CKD) and autosomal dominant hypocalcemia (ADH), or associated with non-calcitropic diseases such as tumorigenesis and neuron degeneration [16, 17]. However, only a few positive allosteric modulators, including cinacalcet and etelcalcetide (AMG-416), have been approved after over 20 years of CaSR being recognized as an important therapeutic target. Further development of calcilytic and calcimimetics with biased signaling properties, fewer side effects, and better tissue selectivity of treatment for pre-dialysis patients, requires a deeper understanding of the molecular mechanisms of CaSR regulation [18]. However, detailed binding and structural studies are significantly hampered by difficulties in purification of membrane proteins, heavy heterogeneous glycosylation, and lack of proper assays to characterize binding events with weak affinities [19, 20].

This review will summarize achievements in molecular insights on the structure and functional cooperativity of CaSR ECD using various biochemical and biophysical approaches coupled with functional studies. Efforts to elucidate a working model of CaSR regulation include recent breakthroughs in the structural determination of CaSR ECD by X-ray crystallography. Implications for drug developments via allosteric modulations of both the ECD and 7TM will also be discussed.

Structural Basis of homotropic cooperativity among metal binding sites at the ECD of CaSR

Huang et al. reported in 2007 a modeled structure of the ECD of CaSR based on the 27% sequence identity between the CaSR ECD and the X-ray structures of mGluR1 (PDB IDs 1EWR and 1ISR) [19]. In contrast with previous modeling efforts [8, 21, 22], a flexible loop region that was not visible in the mGluR1 structure was further modeled in these studies. Using computational algorithms based on statistical analysis of coordination properties of known Ca^{2+} binding proteins [23–26], five Ca^{2+} binding sites were predicted in each monomer of the CaSR ECD: Site 1 (S147, S170, D190, Y218 and E297), Site 2 (D215, L242, S244, D248, and Q253), Site 3 (E224, E228, E229, E231 and E232), Site 4 (E350, E353, E354, N386, and S388), and Site 5 (E378, E379, T396, D398 and E399) [19, 27, 28]. The intrinsic Ca^{2+} binding affinities of predicted Ca^{2+} binding sites 1, 3 and 5 were determined by grafting them individually into a non-Ca^{2+} binding protein CD2 scaffold, with a flexible linker [19]. Additionally, subdomains encompassing several wild-type or mutated Ca^{2+} binding sites were created to study functional cooperativity using Tb-FRET binding and Ca^{2+} competition assays, Trp fluorescence, and 1D ^{1}H NMR [28]. Predicted Site 1 was shown to play an important role in positive homotropic cooperativity among multiple Ca^{2+} sites within the CaSR ECD [28].

By developing mammalian expression of a functional dimer with reduced glycosylation, the first structure determination of a Mg^{2+}-bound form of native CaSR ECD dimer (1–540) at pH 7.0 at a resolution of 2.1 Å (PDB ID 5FBK) and a Gd^{3+}-loaded form at 2.7 Å (PDB ID 5FBK) was achieved. Both structures are similar to the modeled structure (Fig. 1) [20]. Zhang et al. revealed a Mg^{2+}/ Ca^{2+} binding site at the hinge region (D216, D275 and S272) similar to Site 1, predicted by Yang et al. (Fig. 1). Additionally, one metal binding site was also identified at the homodimer interface of lobe 2, formed by negatively charged residues E228, E231, S240 and E241. After soaking the crystal in the presence of Gd^{3+}, this site moved slightly (E228, E229 and E232), and was found to be very close to the original predicted site 3. E228I/E229I mutations were found to dramatically reduce CaSR activity, indicating their potentially important role in Ca^{2+}-induced conformational changes and dimerization [20]. The long loop with two predicted binding sites (Sites 4 and 5) was not visible due to lack of electron density and high flexibility. Interestingly, this structure revealed a metal binding site formed by mainchain (backbone) carbonyl oxygen atoms from uncharged residues (new site: I81, S84, L87 and L88) (Fig. 1).

A subsequent study by Geng et al. determined both apo and holo forms of the CaSR ECD with mutations in glycosylation-sites and the Cys-rich domain under different pH conditions and varied Ca^{2+}, phosphate, and sulfate concentrations (PDB IDs 5K5S and 5K5T) (Fig. 2) [29]. Analysis of structures of the Venus Fly Trap (VFT) domains between the apo and holo forms were similar, with an RMSD of 4.4 Å. The holo-ECD structure (PDB ID 5K5S) with mutations (N386Q and S402N, and/or N468Q) was determined at 2.6 Å resolution in 1.6 M NaH_2PO_4, 0.4 M K_2HPO_4, 100 mM Na_2HPO_4/citric acid, 10 mM $CaCl_2$, and 10 mM L-Trp at pH 4.2. Four Ca^{2+} ions were identified in each monomer. The apo-ECD structure (PDB ID 5K5T) was determined at 3.1 Å in 1.5 M Li_2SO_4, 100 mM Tris, 2 mM $CaCl_2$ at pH 8.5, but only a single Ca^{2+} ion was observed in each monomer. A summary and comparison of these crystallization conditions and crystalized metal binding sites, along with the predicted metal binding sites based on the modeled structure is provided in Table. 1. Interestingly, the new site identified by Zhang et al. was also identified in the 5K5T structure, and a metal binding site at a slightly lower position compared to Zhang et al., coordinated by D234, E231 and G557, was identified in the dimer interface. However, the other Ca^{2+} binding sites, 2 (T100, N102 and T145) and 3 (S302 and S303), in the 5K5T structure, which lacked negatively charged ligand residues, were interpreted as chloride ion binding sites in the 5FBK structure. The difference in crystallization conditions, such as the presence of high phosphate or sulfate acids and low pH, may be accounted for the different density interpretation. Collectively, the structures reported by Zhang et al. revealed metal binding sites in support of the initial working model of homotropic cooperativity among metal ions in the ECD domain (Fig. 3). Future studies will be required to address how different metal binding sites cooperatively induce selective CaSR conformational changes.

Mechanistic insights in heterotropic cooperativity orchestrated by allosteric binding at ECD

To understand how an L-amino acid enhances Ca^{2+} activation of CaSR by heterotropic cooperativity, a L-Phe binding pocket was identified within the hinge region adjacent to the

predicted Ca^{2+} binding site of the CaSR ECD, using structure modeling, molecular docking and functional assays. Utilizing saturation transfer difference (STD) NMR and the purified CaSR ECD, Zhang et al. determined the binding affinity of L-Phe to the CaSR ECD and this binding was enhanced in the presence of Ca^{2+} [30]. Multiple disease-related mutations are located either within or in close proximity to both the Ca^{2+} and amino acid binding sites, and these may alter Ca^{2+} binding and CaSR activation via disruption of cooperativity [30–33]. A working model for the co-activation of the CaSR via both Ca^{2+} and amino acid at the hinge region of the ECD was proposed (Fig. 3) [31, 32].

Unexpectedly, a tryptophan derivative, L-1,2,3,4-tetrahydronorharman-3-carboxylic acid (TNCA), was identified at the hinge region in the 5FBK crystal structure bound by S147, S170, D190, Y218 and E297. To interpret the unusual electron density, mass spectroscopy experiment was conducted, leading to the identification of TNCA (Fig. 1). Conversely, Geng et al proposed that this same region in the 5K5S structure was occupied by L-Trp, rather than TNCA (Fig. 2). Geng also concluded that binding of L-Trp was essential for activation of the receptor, while Ca^{2+}-binding played a secondary and non-activating role. This was inconsistent with previous functional studies [19, 20, 28, 30–32], as results from the Yang group and others suggested that Ca^{2+} is an agonist, whereas L-Trp, other L-amino acids, and TNCA are co-agonists, facilitating Ca^{2+} binding but not activating CaSR by themselves (Fig. 3).

MD simulations based on crystal structure of CaSR ECD (PDB ID 5FBK) revealed that TNCA binding residues have strong correlated motions with Ca^{2+} binding sites at the hinge pocket, as well as in the dimer acidic groove. Using K-means clustering algorithm, the relative domain motion between Lobe1 and Lobe 2 using both holo (PDB ID 5FBK) and apo (PDB ID 5K5T) forms of CaSR ECD was analyzed. The bending residues, fixed domain and moving domain, are described in Fig. 4. From the simulations, the moving domain, mainly Lobe 2 of the apo form, rotated 30.6 degree and translated 1.8 Å relative to the holo form. Additionally, the hinge motion measured using the distance between the residue pair S147 and D217 was 5.6 Å for the holo form and 11.6 Å for the apo form. Moreover, the separation between E231 on each chain which characterizes the movement of the dimer interface was 14.2 Å for the holo form and 37.7 Å for the apo form (Fig. 4). Interestingly, mutations such as E228I at the dimeric groove dramatically reduced cooperative Ca^{2+} responses orchestrated by TNCA, which further suggests a dual action between Ca^{2+} and amino acid or TNCA via hetero-tropic cooperativity (Fig. 3) [32, 34]. In addition to the reported metal binding sites, TNCA was also strongly correlated to several mobile loops, such as loop 1 (A40 to V63), loop 3 (N357-R415), and loop 6 (L496-S502) indicating molecular connectivity among different regions of the ECD.

CaSR is also likely modulated by γ-glutamyl peptides, which share the same pocket as L-Phe/TNCA, based on observations that the combined mutations T145A and S170T diminished activation of CaSR by in the presence of bot γ-glutamyl and L-Phe [35]. Structural modeling studies also suggested that glutathione and γ-glutamyl peptides likely bind at the same hinge region of the CaSR ECD [7, 32].

The amino acid binding site adjacent to a Ca^{2+} binding site in the hinge region of CaSR ECD structure is analogous to a similar structure in mGluR1, in which a Ca^{2+} binding site is next to the ligand binding site [36]. Using a Ca^{2+} binding site prediction algorithm and the structure of mGluR1, Jiang et al. reported a Ca^{2+} binding site adjacent to the L-Glu binding site in the hinge region of the ECD domain of mGluR1. Both Ca^{2+} and L-Glu could individually activate the receptor for intracellular response, while together they synergistically led to increased activation [36].

A recent crystal structure of taste receptor type 1 uncovered a binding site for various amino acids in the cleft between LB1 and LB2, employing residues S142 and S165, which are conserved in most cGPCRs [37]. Moreover, these ligand-induced closing motions, which occur after binding to agonists at the hinge region, are reportedly critical to the function of CaSR, taste receptors, metabotropic glutamate receptor type1 (mGluR1), and $GABA_B$ receptors [38, 39]. For the $GABA_B$ receptor, the only GBR1b motion exhibited occurs between the open and closed conformations associated with agonist binding, while GBR2 exhibits no such motion upon agonist binding.

Negative regulation by anion binding and pH effect

CaSR has been reported to have a strong pH sensitivity. In the physiological pH range 6.0–8.0, an increase in pH activates CaSR, while a decrease in pH inactivates CaSR. In contrast, at pH < 5.5, CaSR becomes more sensitive to both Ca^{2+} and agonists [40]. Moreover, small changes in extracellular pH from 7.4 ± 0.2, without corresponding changes in intracellular pH, rapidly inhibits or activates the receptor as assessed by the production of PTH [41]. Similar phenomena were observed in mGluRs that exhibited subtype dependent pH sensitivity towards glutamate-induced activation, where mGluR4 responded to pH-dependent agonist activation while mGluR1, mGluR5 and mGluR8 did not [42]. Similar behavior toward protons was also reported in a recent work by Campion et al [41]. Speculation that this non-linear pH sensitivity was due to the presence of His residues in the CaSR ECD was refuted by the Campion study, when mutations of these His residues failed to alter pH sensitivity, thereby implying the involvement of other residues [41].

Zhang et al. identified a bicarbonate binding site in close proximity to the TNCA site and the metal binding sites in the hinge region of the crystal structure of CaSR ECD (PDB ID 5FBK). The flat triangular shape of the electron density at 2.1 Å strongly suggested that the bound ligand was a bicarbonate, which was trapped in a positively charged pocket consisting of conserved residues R66, R69, W70, R415, I416 and S417. Remarkably, a sequence alignment of CaSR from different species including mammals, birds, and fishes, indicated that R66, R69, W70, R415, I416 and S417, are highly conserved, except for fish, which occupy an environment with different pH and salt conditions. Furthermore, abnormal changes in bicarbonate levels (normally 22–29 mM) play a vital role in cardiovascular damage and chronic kidney disease by changing serum [Ca^{2+}], which negatively impairs the glomerular filtration rate [43]. Interestingly, the L-type Ca^{2+} channel also has a carboxylate cluster responsible for Ca^{2+} selectivity and can sense change in pH [44]. Because of the pH sensitivity, CaSR is expected to have different EC_{50}s for Ca^{2+} activation arising at different environmental pH values in the organs where it is located, such as acidic stomach and

kidney. Additionally, diet may alter the function of CaSR by affecting the local pH. Activating mutations of glutamate resides in patients reduces the pH modulation affect [40]. The complexity and mechanism of pH sensitivity for CaSR remains an interesting topic for regulation of CaSR function.

The bicarbonate binding site identified by Zhang et al in the 5FBH structure, was alternatively identified as a phosphate binding site in 5K5S structure. This site, along with several other sites in the structure were identified as a result of the presence of very high concentrations of phosphate in their crystallization solution (Table. 1) [29]. A narrow concentration range of PO_4^{3-} (~0.8–1.5 mM) coupled with regulation of fibroblast growth factor 23 (FGF23) has been shown to directly impact parathyroid mineral metabolism [45, 46]. Both FGF23 and CaSR are associated with Ca^{2+}, Mg^{2+}, and phosphate homeostasis, and are related to dysfunction in FHH, ADH and chronic kidney disease [47, 48]. Recent work by Ward et al. directly characterized the mechanism of phosphate-induced parathyroid hormone secretion. Mutation of R62A abolished the inhibition of CaSR function by phosphate, although such an effect may result from the impairment of the salt bridge between the upper and lower lobes [49].

Structural dimerization and Implications for drug development

A recently approved CaSR agonist, AMG-416, was proposed to bind to the hinge region of CaSR ECD through the formation of a mixed disulfide bond between the agonist and C482 of the CaSR ECD. However, C482 appears to play a non-essential role in normal CaSR function [50]. Zhang et al. identified three disulfide bonds in each of the two monomers of CaSR ECD. The inter-monomer disulfide bonds between C129 and C131 in loop 2 were not identified because the loop was missing in one monomer. Because the loop 2 regions from the two monomers are anti-parallel, and due to the proximity of these two Cys residues, Zhang et al. believed that two inter-monomer disulfide bonds were formed between C129(A) – C131(B). Mutation studies subsequently demonstrated that mutating either C129 or C131, or both, did not completely inhibit dimerization, indicating that the inter-monomer disulfide bond is not the only contributor for dimerization. Furthermore, based on the structure of CaSR ECD, salt bridges between E456-R54, R172-D215, and R227-E231 were identified as essential for agonist-induced homodimerization of CaSR. Such interactions have been observed in the taste receptor, as well [37]. However, recent studies have reported increasing heterodimerization of CaSR with $GABA_{B1}R$ in patients with primary or secondary hyperparathyroidism, and this dimerization resulted from hydrophobic interaction between the CaSR and $GABA_{B1}R$ monomer rather than through disulfide bonds formation, suggesting a new mechanism for drug development to prevent PTH secretion [51].

Numerous attempts have been made to uncover the molecular mechanism of positive and negative allosteric modulation, specifically targeting to the 7TM. Leach et al. combined site-directed mutagenesis and Ca^{2+} mobilization assays to elucidate the shared pocket at 7TM for NPS R568, cinacalcet and AC265347, regardless of minor differences in binding residues and cooperativity. Interestingly, they also found a putative Ca^{2+} binding site that overlaps with the allosteric binding site in the 7TM [52, 53]. However, Leach et al later determined that negative allosteric modulators share both overlapping and distinct binding sites at 7TM

[54]. Furthermore, Bräuner-Osborn et al. reported that in order to completely block inhibition, two allosteric sites need to be prevented from binding negative allosteric modulators, but as long as one allosteric site is bound per CaSR dimer by allosteric modulators, it is sufficient for achieving a positive allosteric effect [55]. Although this picture is incomplete, the results of these studies demonstrate that efforts to solve the CaSR structure and understand the respective ligand binding sites will benefit future drug development towards CaSR function.

Conclusion and perspectives

Based on multiple approaches including computational studies and predictions, mutagenesis, and various functional and binding assays, Yang et al. has developed a central working model for the regulation of the CaSR where cooperative binding of Ca^{2+} at key hot spots of the CaSR ECD, including the hinge region and dimerization sites, selectively alters key conformational states linked to receptor's activity (Fig. 3). Binding near these spots by agonists/co-agonists, (i.e., amino acids, TNCA, and anions), and pH changes, alters heterotropic cooperativity. This, in turn, biases selection of intracellular signaling pathways required for physiological responses. Recent structural determination of the ligand binding domain and ECD domain of CaSR under different conditions strongly supports the proposed working model and provide several new insights related to anion binding regulation and pH effects. The newly determined structures further extend the understanding of the cooperative Ca^{2+} activation orchestrated by multiple Ca^{2+} binding sites and of the positive cooperativity induced by TNCA/L-amino acid binding sensitivity, and anion dependent CaSR activation. Many disease-associated mutations have been identified at or near the ligand binding sites [20]. Further work is required to differentiate the working models proposed by Yang et al. and Geng et al., to determine whether Ca^{2+} or L-amino acid plays the initial activation role in regulating the receptor's activity. Future studies to understand functional cooperativity will require the application of innovative approaches including high resolution cellular imaging to monitor trafficking and biosynthesis, novel calcium indicators to report subcellular calcium responses, development of cell assays to detect weak binding, mass spectrometry for proteomics, and applications of newly developed cryo EM for structure determination. These studies will provide key data relevant to the molecular mechanism that integrates calcium signaling between the extracellular and intracellular environments, elucidate the molecular basis of CaSR-related clinical disorders in various organs and patients, as well as lead to an exciting new era for the development of novel receptor-based therapeutics for CaSR and other cGPCRs.

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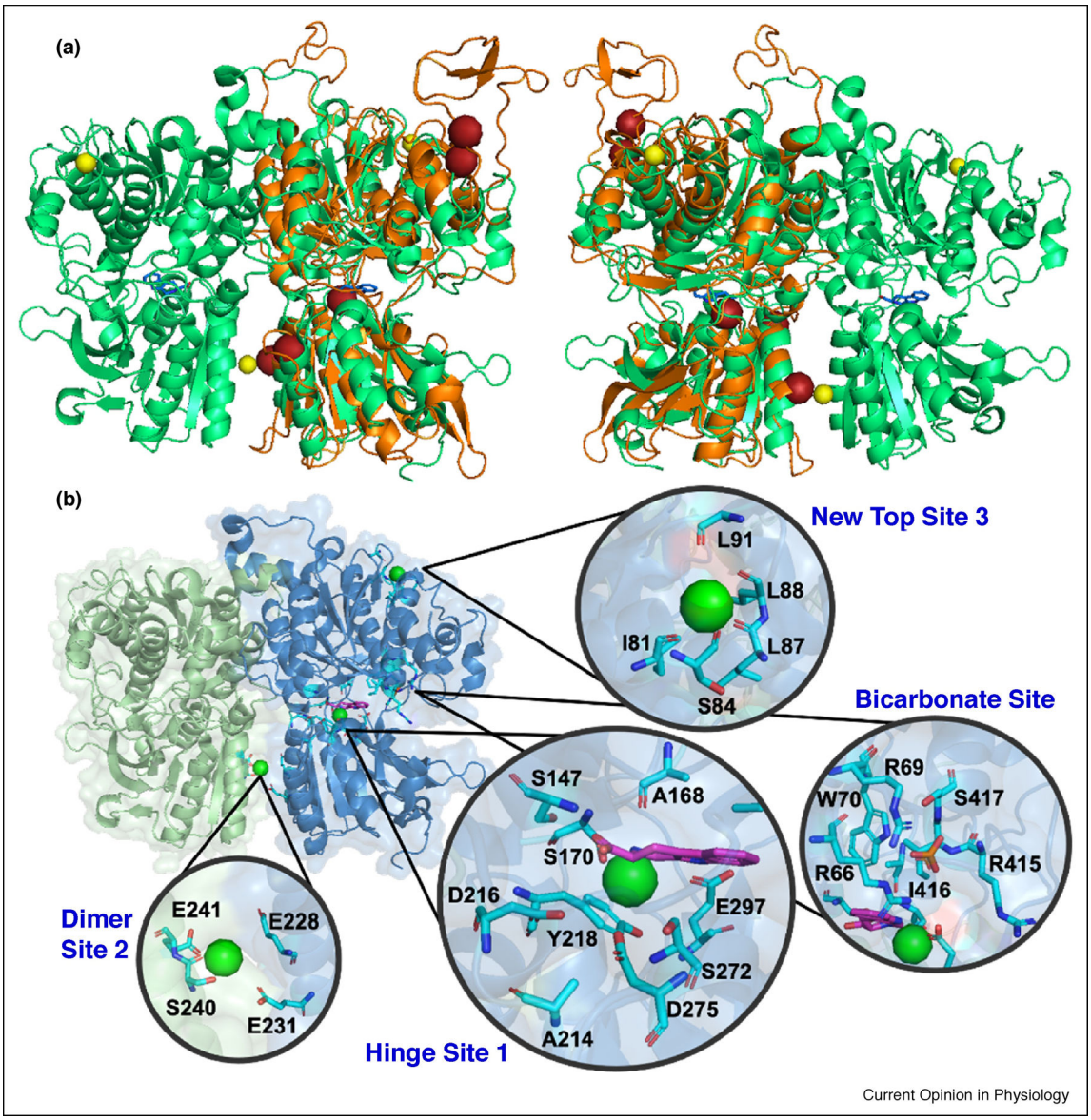

Fig. 1.

(**A**) Overlapping structures of modeled ECD (orange) and determined structure (PDB ID 5FBK). Mg^{2+} and putative Ca^{2+} are shown in yellow and red spheres respectively. (**B**) X-ray Structure of CaSR ECD (PDB ID 5FBK) and key areas to be studied with zoomed Mg^{2+} Site I, Mg^{2+} Site II, Mg^{2+} new Site, hinge ligand binding site and anion binding site. Green spheres represent Ca^{2+}. bicarbonate is shown in orange sticks. TNCA is indicated by magenta sticks.

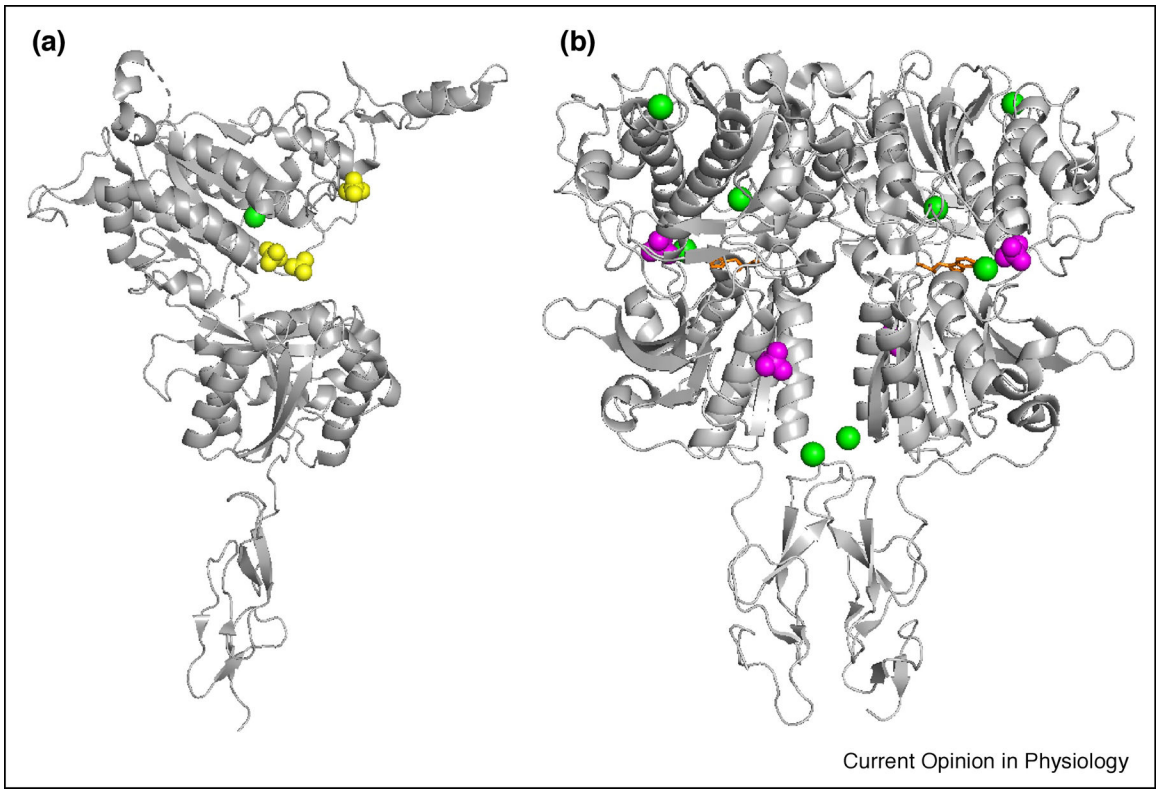

Fig.2.

X-ray Structure of both (**A**) apo and (**B**) holo form of CaSR ECD with Cys-rich domain (PDB IDs 5K5T and 5K5S). Green spheres represent Ca^{2+}.SO_4^{2-} and PO_4^{3-} are shown in yellow and magenta spheres respectively. L-Trp is indicated by orange sticks.

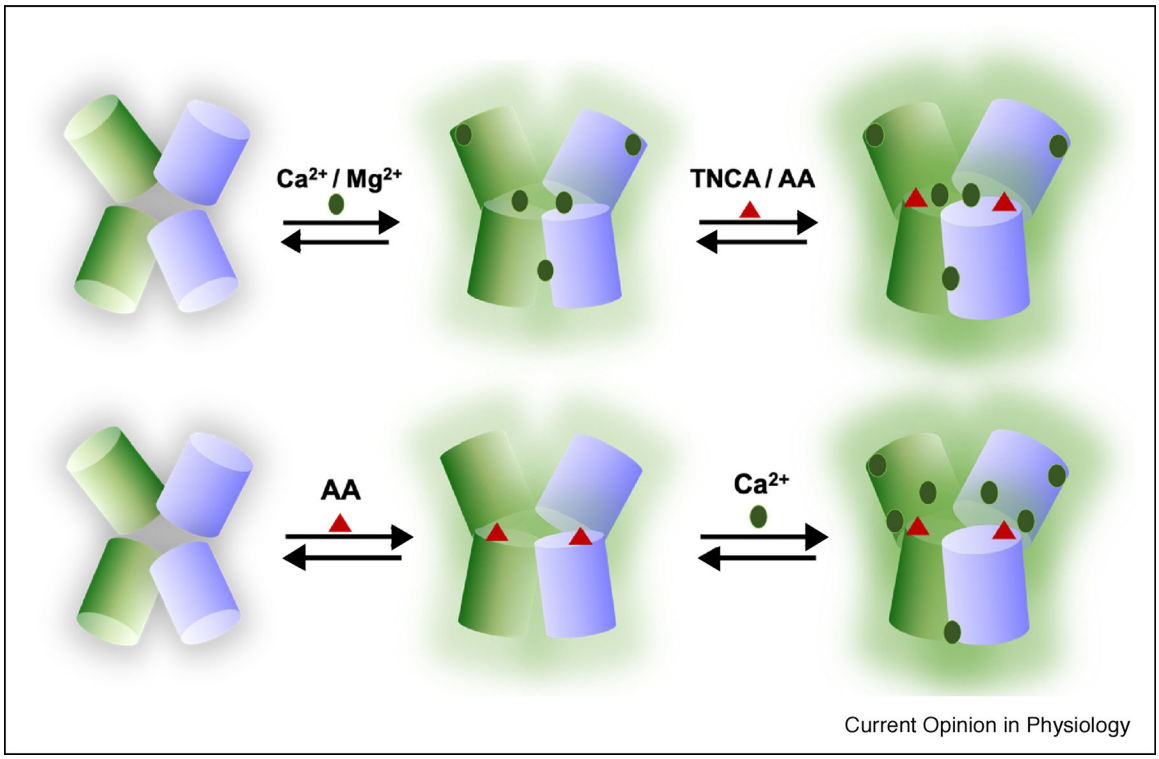

Fig. 3.

(**Top**) Working model proposed by Yang et al. Ca^{2+} activates CaSR first, then activation is enhanced by amino acid (AA)/TNCA. (**Bottom**) Working model proposed by Geng et al. L-amino acid activates the receptor, and Ca^{2+} stabilizes the active state to further activate the receptor.

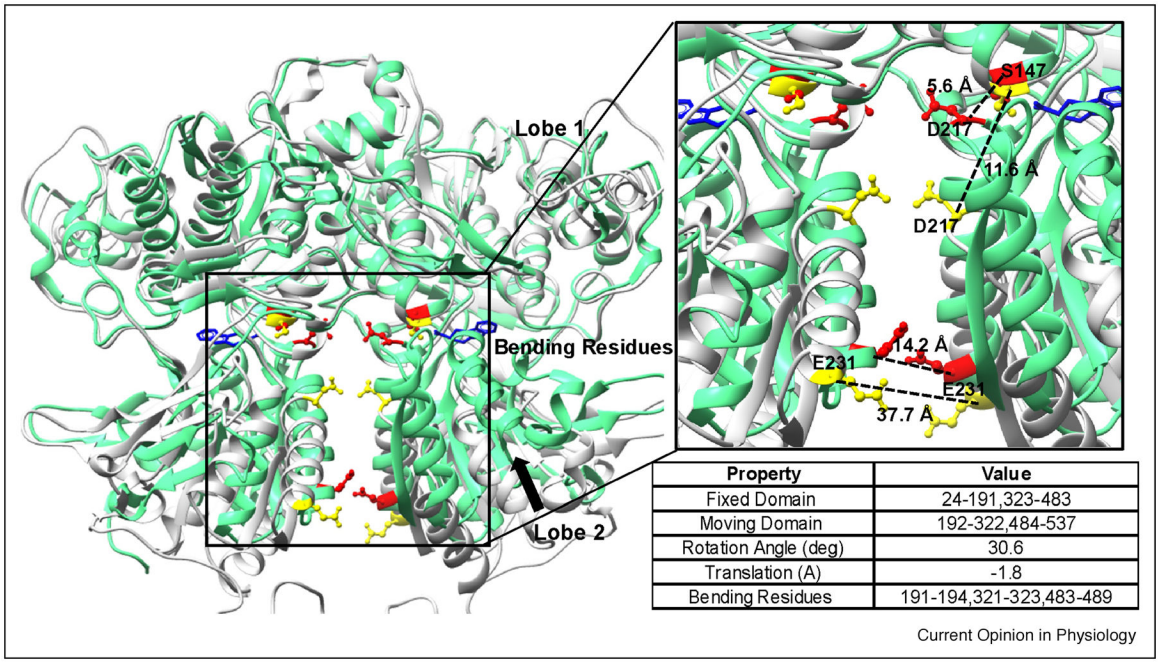

Fig. 4.

Relative domain motion between Lobe 2 and Lobe1 in both the holo (PDB ID 5FBK, green ribbons with highlighted residues in red sticks) and apo (PDB ID 5K5T, gray ribbons with highlighted residues in yellow sticks) forms. Detailed information about the relative domain motion are listed in the table.

Table. 1.

Summary of crystallization conditions and crystalized metal binding sites, along with the predicted metal binding sites based on the modeled structure.

Structures	Binding sites	
5FBK/5FBH/modeled	Determined metal binding sites	E228 E231 E241 S240
		I81 S84 L87 L88
	Modeled metal binding sites	S147 S170 D190 Y218 E297
		D215 L242 S244 D248 E253
		E350 E353 E354 N386 S388
		E378 E379 T396 D398 E399
	Determined L-Trp binding sites	S147 A168 S170 Y218 E297
5K5T/5K5S	Determined metal binding sites	I81 N82 S84 L87 L91
		T100 N102 T145 G146
		S302 S303
		E231 D234 G567
	Determined TNCA binding sites	S147 A168 S170 Y218 E297

Structures	Crystallization conditions
5FBK	200 mM $MgCl_2$, 10 mM $CaCl_2$, and 100 mM Tris-HCl pH 7.0.
5FBH	200 mM $MgCl_2$, 10 mM $CaCl_2$, 100 mM Tris-HCl pH 7.0, and 0.5 mM $GdCl_3$.
5K5T	1.5 M Li_2SO_4, 100 mM Tris pH 8.5 in the absence and presence of 2 mM $CaCl_2$.
5K5S	1.6 M NaH_2PO_4, 0.4 M K_2HPO_4, 100 mM Na_2HPO_4/citric acid pH 4.2, 10 mM $CaCl_2$, and 10 mM L-Trp.