Integration Of Extracellular And Intracellular Signals Via The Calcium Sensing Receptor (CASR)

Chen Zhang
INTEGRATION OF EXTRACELLULAR AND INTRACELLULAR SIGNALS VIA THE CALCIUM SENSING RECEPTOR (CASR)

by

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Under the Direction of Dr. Jenny J. Yang

ABSTRACT

It has long been recognized that Ca\(^{2+}\) acts as a second messenger to regulate diverse crucial cellular processes in response to external stimuli. The discovery of the parathyroid Ca\(^{2+}\)-sensing receptor (CaSR) has established a new paradigm of Ca\(^{2+}\) signaling as a first messenger. The CaSR regulates the calcium homeostasis in the human body via sensing fluctuations in the extracellular Ca\(^{2+}\) concentration. Naturally occurring mutations in the CaSR could result in abnormal receptor responses toward [Ca\(^{2+}\)]\(_{o}\) which causes inherited Ca\(^{2+}\) regulation disorders. L-amino acids have been found to stereoselectively interact with the CaSR thus coupling protein digestion to receptor activity. In the present study, we use several complementary approaches including imaging [Ca\(^{2+}\)]\(_{i}\) response in living cells at the cellular level, measuring metal binding affinity, making site-direct mutagenesis, examining conformational changes of purified
extracellular domains with various spectroscopic methods, and using molecular dynamic (MD) simulations at the atomic level to provide important insights into the behavior of the receptor in both normal and disease statuses. We demonstrated that the molecular connectivity between $[\text{Ca}^{2+}]_o$–binding sites is responsible for the functional positive homotropic cooperativity in the CaSR’s response to $[\text{Ca}^{2+}]_o$. Naturally occurring disease mutations near Site 1 disrupted the cooperativity. We further identified an L-Phe-binding pocket adjacent to $\text{Ca}^{2+}$-binding Site 1. This L-Phe-binding pocket is essential for functional positive heterotropic cooperativity by virtue of its having a global impact on all five of the predicted $\text{Ca}^{2+}$-binding sites in the ECD with regards to $[\text{Ca}^{2+}]_o$-evoked $[\text{Ca}^{2+}]_i$ signaling. Furthermore, the CaSR’s ECD has been expressed using both bacteria without glycosylation and mammalian systems with either complex glycans, or high mannose, were characterized using the fluorescence titration spectroscopy, circular dichroism technique as well as the NMR spectroscopy to show calcium and Phe directly bind to the ECD domain directly and interactively. Moreover, we also demonstrated that intracellular trafficking of the CaSR is a complex process, which involves modulation by calmodulin and can possibly be affected by different CaSR isoforms when expressing in various cell lines. The studies on the isolated proteins will pave the way for future protein crystallization and related structural research.

INDEX WORDS: $\text{Ca}^{2+}$, Calcium sensing receptor, L-Phe, Calmodulin, Prediction, Purification, Fluorescence, $\text{Ca}^{2+}$ Signaling, G-protein coupled receptor (GPCR)
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DEDICATION

I dedicate my dissertation work to my family and many friends.

Every single day I was grateful for their support, my father, Shijun Zhang, and my mother, Zhijun Dai, for giving me my life and making me feel special in the world. They prevent me from being arrogant when I achieve small success and they cheered me up when I was feeling blue. My parents traveled a lot with their cameras to different places of historic interest and to scenic beauty scattered across Asia, Europe and North America, recording the daily life of local inhabitants and capturing the marvel of nature. The most important thing is that they keep learning the photography techniques as well as the Photoshop skills to post-process the images they took. They set for me a good example for being a person with passion for life.

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CHAPTER 1. INTRODUCTION

1.1 The role of Ca$^{2+}$ in mammalian cells

Before Sydney Ringer serendipitously discovered calcium (Ca$^{2+}$) to be essential for the contraction of isolated hearts in 1883(1), Ca$^{2+}$ had received little attention for years except the recognition that it was an important element for the formation of bone and teeth. After the mid-20th century, research on Ca$^{2+}$ has grown at an exponential rate and Ca$^{2+}$ became a universal carrier for biological information(2). Ca$^{2+}$ controls the matter of life and death as it modulates the process of fertilization as well as apoptosis.

1.1.1 General principles of calcium signaling

Three major properties of Ca$^{2+}$ are particularly drawing scientists’ attention: the first is that Ca$^{2+}$ can both play a role as a first and as a second messenger; secondly, Ca$^{2+}$ can autoregulate itself through various signals; finally, its ambivalent nature in which it can control a number of essential normal functions of cells and meanwhile could also be a messenger of death(2).

After thousands of years of evolution, eukaryotic cells managed to maintain a free Ca$^{2+}$ concentration at the level of 10$^{-7}$ M in the cytoplasm through various ways while they are surrounded by an environment containing more than 1 mM free Ca$^{2+}$ concentration. The low level of cytosolic Ca$^{2+}$ concentration is necessary in order to prevent the precipitation of Ca$^{2+}$-phosphate salts and prevent the extra energy that would have to be invested to change it. Those various ways are including binding to membrane (acidic) phospholipids, interaction with low molecular weight metabolites, formation of new compounds with inorganic ions, and binding to specific proteins.
The proteins that can interact with Ca\textsuperscript{2+}, and thus regulate the intracellular calcium concentration, can be categorized into two major classes: The first class belongs to intrinsic membrane proteins which transport Ca\textsuperscript{2+} across the membrane to maintain cellular Ca\textsuperscript{2+} homeostasis. The other class is soluble proteins. The minority of the latter is Ca\textsuperscript{2+} buffers, which mainly operate to lower the intracellular calcium concentration. The majority of the aforementioned soluble proteins are defined as calcium sensors as they have specific functional properties besides their Ca\textsuperscript{2+} binding capability. These types of proteins include: the annexins, gelsolin, C-2 domain proteins as well as EF-hand proteins.

1.1.2 Calcium as a first and a second messenger in cells

Before the discovery of the calcium sensing receptor, Ca\textsuperscript{2+} was mainly considered as a crucial second messenger that rapidly yet efficiently regulated the intracellular calcium level and modulated extensive molecular signaling components through calcium channels, exchangers as well as pumps (3).

When Ca\textsuperscript{2+} acts as a second messenger, the Ca\textsuperscript{2+} signaling network can be summarized as four components: A stimulus that induces various signals to mobilize Ca\textsuperscript{2+}, The ON mechanisms that introduce Ca\textsuperscript{2+} into the cytosol, Ca\textsuperscript{2+} functions as a message transducer and lastly, the OFF mechanisms to remove Ca\textsuperscript{2+} via pumps or exchangers (4). When Ca\textsuperscript{2+} plays a role as first messenger, it is also the stimulus in the first component.

The Ca\textsuperscript{2+}-mobilizing second messengers are generated when certain stimuli interact with cell surface receptors. Inositol-1,4,5-trisphosphate (Ins(1,4,5)P\textsubscript{3}) (5) is one of them that can bind to inositol-1,4,5-trisphosphate receptor (InsP3Rs) and release Ca\textsuperscript{2+} from the ER. Sphingosine 1-phosphate (S1P) is another messenger that releases Ca\textsuperscript{2+} from the ER by engaging a sphingolipid
Ca\textsuperscript{2+} release-mediating protein of the ER (SCaMPER)(6). Cyclic ADP ribose (cADPR) (7) is a calcium dependent activator for ryanodine receptors (RYRs).

While the InsP\textsubscript{3}Rs and RYRs are related with Ca\textsuperscript{2+} derived from internal stores, there are other mechanisms contributing to the ON mechanisms via controlling the entry of external Ca\textsuperscript{2+}. The best-known mechanism is voltage-operated channels (VOCs). When a receptor binds to external stimuli such as glutamate, ATP or acetylcholine, the receptor-operated channels (ROCs) will be operated. The depletion of internal calcium stores can trigger the store-operated channels (SOCs) (8).

The Ca\textsuperscript{2+} signal induced by the ON mechanism can then be transferred to cellular responses. Those responses recruit numerous Ca\textsuperscript{2+} binding proteins as Ca\textsuperscript{2+} buffers and sensors. Troponin C (TnC) and calmodulin (CaM) are two calcium sensors that have been extensively studied (4).

The OFF mechanisms will be initiated when Ca\textsuperscript{2+} needs to be removed from the system. There are various ways the extra Ca\textsuperscript{2+} can be sequestered. The plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) pumps and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers can transfer intracellular Ca\textsuperscript{2+} to the extracellular space, the sarco-endoplasmic reticulum ATPase (SERCA) pumps, on the other hand, pump Ca\textsuperscript{2+} back to the internal stores (9,10).

How Ca\textsuperscript{2+} performs as a first messenger will be elucidated in the following introduction to the calcium sensing receptor.

1.1.3 Endoplasmic reticulum dynamics and calcium signaling

A number of cellular functions such as synthesis of protein and lipid, protein folding and post-translational modification as well as maintaining the Ca\textsuperscript{2+} concentration constant take place in the endoplasmic reticulum (ER) (11). The calcium dynamics in the ER influence many cellular pathways and ER functions, from gene regulation to cellular level responses, which include: cell
differentiation and proliferation, apoptosis, from embryo development to the tissue level such as muscle contraction and relaxation; even learning and memory, membrane excitability and energy metabolism (12).

As mentioned in 1.1.2, the InsP3R and RyR (13,14) control the extruding of calcium from ER while SERCA replenishes the calcium store. Besides these “in” and “out” regulations, the Ca^{2+} buffering system in the ER and resident chaperons control the free Ca^{2+} concentration in the range of 50-500 μM (15-17) although the total [Ca^{2+}]_{ER} is in the mM range. A number of ER-located chaperones have been identified that buffer Ca^{2+} including calreticulin, glucose-regulated protein 94 (Grp94), immunoglobulin binding protein (Grp78) (11), the family of PDI-like ER resident proteins (18), calsequestrin (19), and reticulocalbins (11). Some of these chaperones have weak affinity but high capacity, PDI-like proteins, while some of them have high affinity but low capacity, such as Grp94. Proteins with very high affinity (K_d=1μM), for example reticulocalbins, are considered to play a role in maintaining certain structures or participating in specific protein-protein interactions.

In addition to proteins locally regulating the [Ca^{2+}]_{ER} within the calcium store organelle, ER also communicates with the plasma membrane for Ca^{2+} signaling regulation through the machinery named as store-operated Ca^{2+} channel (SOC) (20). In many cell types, the inwardly rectifying current is called I_{CRAC} characterizing as highly Ca^{2+} selective, non-voltage gated current (20). The identification stromal-interacting molecule (STIM1) connects the ER, cytoplasm, and the Orai1 protein resident in the lumen of ER which provides reasonable explanation for the mechanism of SOC (21-23).
Normally, the \([\text{Ca}^{2+}]_{\text{ER}}\) is controlled at 50-400 \(\mu\text{M}\), which is suitable for protein and lipid synthesis as well as protein posttranslational modification (11). Severe consequences will occur if the \([\text{Ca}^{2+}]_{\text{ER}}\) is sustained and prolonged at a low level (below 20 \(\mu\text{M}\)) such as disrupted protein synthesis, accumulation of unfolded proteins, even cell apoptosis (11). On the other hand, the overload of \([\text{Ca}^{2+}]_{\text{ER}}\) stress also has negative impacts on ER function and cellular signaling leading to congenital problems (11).

1.1.4 Spatiotemporal aspects of Ca\(^{2+}\) signaling

Although Ca\(^{2+}\) is considered as a universal signal for the regulation of cells, it could be toxic to cells if it stays at an excessively high concentration. A pulsatile manner of Ca\(^{2+}\) signals has evolved to prevent such toxicity.

Two major mechanisms are involved in the production of the temporal Ca\(^{2+}\) signals that would further lead to more complex downstream signaling. The first one happens in skeletal and cardiac cells when the Ca\(^{2+}\) signal pulses are produced on demand as the cells respond to periodic stimulation that leads to membrane depolarization. This kind of signal can be observed in muscle cells when they contract and also in nerve terminals when neurotransmitters are released upon a brief localized pulse of Ca\(^{2+}\) (Calcium: A Matter of Life or Death p496). The second type appears as an oscillation when the cells receive a continuous stimulation over a period of time. The rhythmical Ca\(^{2+}\) signal has a wide range of frequencies that are tightly related to a variety of cellular responses. The details of which will be discussed in the next paragraph.

The types of oscillations can be categorized by the oscillatory activity: membrane oscillators and cytosolic oscillators. The membrane oscillators are usually the results of K\(^{+}\) outward currents
inducing depolarization and the PMCA (plasma membrane Ca\(^{2+}\) ATPase) activity. The action potentials that are set up by membrane oscillators provide rhythmical pacemaker activity that would be responsible for processes such as cardiac contraction, neuronal activity, secretion, etc. (24,25). The other type, the cytosolic oscillators, cause the periodic release of internal Ca\(^{2+}\) and their mechanism depends on various intracellular stores. For agonist triggered cytosolic Ca\(^{2+}\) oscillation, the InP3/Ca\(^{2+}\) signaling pathway, which requires the participation of endoplasmic reticulum, plays a crucial role in activating the oscillation. This second type of regulation has drawn more attention to researchers since the oscillation frequencies have a large range that varies with the type of stimulation and encode specific information regulating diverse cellular processes. Various hormones and transmitters can induce repetitive cytosolic Ca\(^{2+}\) spikes in hepatocytes in the range of 1-10 min (26-28) and this physiological process is modulated by phorbol esters (29), protein kinase inhibitors (30), extracellular Ca\(^{2+}\) (27,28,31), Na\(^+\), and K\(^+\) (27,31). Ca\(^{2+}\) oscillations are also responsible for initiating embryonic development in both mammalian and ascidian oocytes (32-34). In 2001, Pasti et al. demonstrated that [Ca\(^{2+}\)]\(_i\) oscillations could cause a pulsatile release of glutamate and the calcium signal could be mediated by activation of AMPA/mGluRs (35). Airway epithelial cells utilize the Ca\(^{2+}\) oscillations to control ciliary beat frequency as a response to ATP (36). Figure 1.1 shows a summary of the cytosolic oscillation frequencies and their related biological functions.
Figure 1.1 Biological Processes Regulated By [Ca^{2+}]_i oscillations.

The cytosolic oscillators would cause the periodic release of internal Ca^{2+} (termed [Ca^{2+}]_i oscillation) and their mechanisms depend on various intracellular stores. The oscillation frequencies have a large range varying with the type of stimulation and encoding specific information to diverse cellular processes from fast reactions, such as hypertropy, to slow physiological responses like Interleukin production (37).
1.2 The structure and function of Calcium sensing receptor (CaSR)

1.2.1 The discovery of CaSR

It has been well known that the serum Ca\(^{2+}\) concentration can regulate the secretion of parathyroid hormone (PTH). In 1993, Dr. Edward M. Brown cloned the receptor that is primarily responsible for this type of regulation from bovine parathyroid gland (38). The receptor was given the name “calcium sensing receptor” (CaSR/CaR). The previously observed cytosolic calcium changes in parathyroid cells, as well as in other in vitro expression systems, was triggered by serum calcium concentration change and is greatly attributed to the function of CaSR (39,40). A unique characteristic of CaSR is the high cooperativity of Ca\(^{2+}\) dependent activation, which tightly controls the secretion of parathyroid hormone when the receptor is exposed to serum Ca\(^{2+}\) concentrations within its responsive range (38). The CaSRs expressed in various species are highly conserved (Appendix D), indicating the biological importance of this receptor in regulating physiological functions among living organisms.

1.2.2 Biological roles of CaSR

The major function of the CaSR is to maintain calcium homeostasis through balancing the ingestion and absorption of calcium from the gastrointestinal tract, excretion of calcium by the urinary system and the breakdown and formation of bone (41,42) (Figure 1.2). The CaSR is mainly expressed in the parathyroid and thyroid glands, as well as in other tissues mentioned above. The CaSR regulates PTH secretion from parathyroid glands and calcitonin secretion from thyroidal C-cells upon detecting variations in the calcium concentration in the extracellular fluid (41).
Figure 1.2 Systematic regulation of \( \text{Ca}^{2+} \) homeostasis in human body.

Two major circulating hormones (PTH and CT) regulate \( \text{Ca}^{2+} \) homeostasis via several primary organs. If the \( \text{Ca}^{2+} \) level is too low, PTH is released from parathyroid gland. It then stimulates \( \text{Ca}^{2+} \) uptake from intestines, increases the rate of \( \text{Ca}^{2+} \) release from bone and the rate of \( \text{Ca}^{2+} \) reabsorption in urinary system. If the \( \text{Ca}^{2+} \) level is too high, the opposite regulation will occur to control the blood \( \text{Ca}^{2+} \) level. Thus, the blood \( \text{Ca}^{2+} \) is strictly maintained between 1.1-1.4 mM.

Moreover, CaSR has its specific role in different tissues. As mentioned in the last section, the CaSR was first discovered in parathyroid cells. The main function of the receptor is to mediate the negative feedback of PTH secretion upon the stimulation of various agonists (38). Most of the CaSR expressed in parathyroid cells is located in caveolae, where a significant number of signaling molecules, as well as scaffolding proteins, reside (43). Similarly, one of the critical
roles the CaSR expressed in the kidney plays is maintaining calcium homeostasis (44). However, the CaSR present at different parts of the nephron (e.g. the proximal tubule, thick ascending limb, etc.) may have specific functions (45,46).

The CaSR is also found in the gastrointestinal tract, including the esophagus, stomach, small intestine, and colon (47,48). The activity of CaSR has been reported to be associated with increased phosphorylation of ERK, intracellular calcium mobilization, secretion of IL-8, gastric acid (49-51) and cell proliferation and differentiation (52,53).

The CaSR has been identified in bone, demonstrated by various techniques (54). Both osteoblastic cell line MC3t3-E1 and osteoclasts are reported to express the CaSR. The receptor is involved in osteoblasts proliferation likely via JNK signaling (55,56) and participates in both differentiation and apoptosis of osteoclasts through the PLC pathway (57). The CaSR stimulation is also believed to be inhibiting bone resorbing activity of osteoclasts (58).

Experiments using immunocytochemistry showed that the CaSR is widely distributed in the central nervous system with quite a diverse pattern (59). In the neuron system, the CaSR is also forming heterodimers with other family C GPCRs (60,61). It has been proposed that the CaSR may play a crucial role in regulating currents which mediates the bursting of action potentials and the following depolarization (62). The CaSR in the hippocampus has been reported to regulate both Ca$^{2+}$-permeable, nonselective cation channels as well as Ca$^{2+}$-activated K$^+$ channels (59,63,64).
The CaSR is also detected in cardiac tissue by various techniques including RT-PCR, immunohistochemistry, etc. (65). A few pathological processes have been found to be associated with an increased expression level of the CaSR, for instance the angiotensin II induced cardiac hypertrophy (66), hypoxic-reoxygenation treated cardia myocytes, etc. (67).

1.2.3 The structure of the Calcium Sensing Receptor (CaSR)

The CaSR comprises four major parts in terms of its structure: a large N-terminal extracellular domain (ECD), a cysteine-rich domain, a seven transmembrane domain and the intracellular C-terminal (Figure 1.2). The major function of the CaSR is regulating the calcium concentration in the human blood.

![Figure 1.3 The modeled structure of Ca\(^{2+}\)-sensing receptor.](image)
The modeled CaSR extracellular domain (ECD) is based on mGluR1 (pdb: 1EWT). A number of agonist and antagonists interact with the large ECD that forms a Venus Fly Trap (VFT) structure. The seven transmembrane domain is linked with the ECD via a Cys-rich domain. The C-terminal domain is involved in the interaction with intracellular target proteins (68).

Members in the family C GPCR proteins possess signature large N-terminal ECDs. The low but significant amino acid sequence similarity between the GPCR family C and a group of bacterial periplasmic amino acid-binding proteins suggest evolutionary relevance. Indeed, the crystal structure of the ECD of rat metabotropic glutamate receptor (mGluR1) showed a bi-lobed Venus-flytrap-like structure (VFT) which is fully consistent with the structure of the bacterial periplasmic amino acid-binding proteins, switching between “open” and “closed” conformational status upon releasing or binding their endogenous agonists(69). The alignment of the amino acid sequence of the CaSR ECD (AA36-AA513) with mGluRs and the bacterial periplasmic amino acid-binding proteins results in a proposed VFT model of CaSR ECD with the N-terminal lobe I connecting with C-terminal lobe II(70). However, the alignment also shows four segments in lobe I that do not align with the bacterial periplasmic amino acid-binding protein resulting in four unstructured loops (I–IV) (71). Only a large part of loop III (from residues 365-385) could be deleted without reducing the activity of CaSR while truncation in the other four loops resulted in a low surface expression of the receptor (71).

The VFT structure of mGluR1 forms a homodimers as verified by 3D structure of rat mGluR1 VFT. The intermolecular disulfide requires a conserved cysteine in loop 2, and the equivalent residues in the CaSR are C129 and C131 (70,72). Experiments carrying out mutagenesis of the
two cysteines suggested that disruption resulted in failure to form disulfide bonds, but did not affect the formation of dimer (73). Other cysteines in the ECD of CaSR including C60–C101, C358–C395 and C437–C449 form three intramolecular disulfides, which are critical for CaSR expression and activity(74).

All members of the GPCR family C have a conserved nine-cysteine structure at the end of the C-terminal domain of the ECD and before the seven transmembrane domains except the GABA\textsubscript{B} receptor (75). For CaSR, the region possesses about 84 residues. Mutations on any of the nine Cys to Ser dramatically impair the surface expression and the function of CaSR. Deletion of this Cys-rich domain results in the abrogation of CaSR activity.

The CaSR contains a seven transmembrane domain (7TM) similar to the other proteins in the GPCR superfamily. It is believed that agonist binding to GPCRs will lead to conformational changes of the 7TM $\alpha$-helices, further inducing alterations in intracellular loops as well as the C-terminal domain, thereby triggering the downstream signaling pathways. The crystal structure of rhodopsin (a GPCR Family A protein) with 7TM domain has been reported. However, caution is extremely needed when extrapolating the CaSR 7TM structure from rhodopsin as proteins from family C share low sequence identity with proteins from family A. The 6 prolines in the transmembrane domain are important to maintaining the kinks in the transmembrane helices. Mutations of some prolines (P748R, P823A) (76,77) impair receptor function.

The intracellular domain of the CaSR contains 216 amino acids that do not show high conservation among species (78). Two regions in this intracellular tail are homologous between
species: one from 863-925 located at the proximal of C-tail which is pivotal in surface expression; the other from 960-984 which is involved in interacting downstream proteins (79-81). Three potential protein kinase C (PKC) phosphorylation sites are present in the intracellular domain: T888, S895 and S915 are involved in regulating the receptor activity (82). Especially, T888 plays significant role in the PKC regulation as a negative modulator of CaSR function.

Along with the PKC phosphorylation sites, two other residues participate in protein kinase A phosphorylation: S899 and S900, but studies on these two sites indicate minor effect of PKA phosphorylation in the modulation of CaSR (83).

1.2.4 Contribution of glycosylation to CaSR function

The attachment of sugar residues on a protein is considered to be the most complicated co- or posttranslational modification (84). The glycosylation process is regulated by factors that vary greatly among cell types and species (85). Mature carbohydrate units can be assembled on protein via numerous delicate glycosylation routes and the resultant glycoproteins are secreted out of the cells or become cellular components, including membranes, cytoplasm, or nucleus etc. A variety of carbohydrate-peptide linkages have been found among glycoproteins in nearly all living organisms, ranging from bacteria to eukaryotes (85). Those linkages involve 13 different monosaccharides and 8 amino acid types and the glycopeptide bonds can be further categorized in five distinct groups shown in Figure 1.3(85). One of the common forms of glycopeptides bonds is N-linked glycosylation. This modification is the result of a complex process of trimming and remodeling of the oligosaccharide that takes place in the endoplasmic reticulum (ER) and Golgi as illustrated in Figure 1.4 (86). The synthesis of various glycopeptides bonds takes place within different subcellular organelles (85).
Figure 1.4 Glycan-protein linkages reported in nature.

(Updated and redrawn, with permission of Oxford University Press, from Spiro R.G. 2002. Glycobiology. 12: 43R–56R.) Diagrammatic representation of the five distinct types of sugar-peptide bonds that have been identified in nature. (Ara) arabinose; (Rha) rhamnose; (FucNAc) N-acetylfucosamine (2-acetamido-2,6-dideoxy-D-galactose); (Bac) bacillosamine (2,4-diamino-2,4,6-trideoxy-D-glucose); (Pse) pseudaminic acid (5,7-diacetamido-3,5,7,9-tetraideoxy-L-glycero-L-manno-nonulosonic acid); (Hyl) hydroxylysine; (Hyp) hydroxyproline; (C-term) carboxy-terminal amino acid residue. Glypiation is the process by which a glycosylphosphatidylinositol (GPI) anchor is added to a protein. For other details, including anomeric linkages, please see the Spiro (2002) review cited above.

Endoglycosidases can be categorized into two major types: one for deglycosylation of asparagine-linked (N-linked) oligosaccharides; the other for O-linked oligosaccharides. Endolycosidases F(1,2,3), Endoglycosidases H (Endo H) and peptide-N4-(N-acetyl-beta-glucosaminyl) asparagine amidase (PNGase) cleave N-linked glycosylation. O-Glycosidase
cleaves O-linked glycosylation. Among the first category, Endoglycosidases F1, F2, and F3 are more suitable for removing the glycans of native protein due to their low sensitivity to protein conformation while PNGase F prefers to work on denatured protein. Both Endo F1 and Endo H can cleave asparagine-linked oligosaccharides, however, the later does not cleave sulfated high-mannose form (i.e. containing 5–9 mannose). (http://www.sigmaaldrich.com/technicaldocuments/articles/biology/glycobiology/endoglycosidases.html). The differences between the endoglycosidases are summarized in Figure 1.5.

Figure 1.5 Schematic representation of the generation of N-linked glycosylation.
N-linked oligosaccharide modification takes place in the ER and Golgi. Following glucose trimming in the ER, high mannose glycans are available for further processing and trimming by glycosidase and mannosidases to yield high-mannose, hybrid and complex glycan structures.

Figure 1.6 Schematic representation of different endoglycosidases

CaSR isolated from pcDNA CaSR transfected human embryonic kidney (HEK293) cells exhibited similar expression patterns as the CaSR proteins identified from parathyroid cells (87). The western blot results of CaSR extracted from HEK293 cells showed several immunoreactive
bands using antibodies specific for the CaSR. The minor band at 120 kDa corresponds to the nonglycosylated form of the protein, the major band at 140 kDa stands for the high mannose form while another major band at 160 kDa represents the complex carbohydrates form of CaSR (87,88). Additional bands at higher molecular mass (350 kDa) corresponds to the dimeric form of the receptor. The CaSR that is expressed on the cell membrane is mature receptor that only account for a small portion of the protein (89).

The sequon N-X-S/T is a general position at which N-Linked glycosylation can occur (90). X can be any amino acid but not proline. The glycosylation process is completely blocked if P is at the presence of X position. On the other hand, W, D, E and L at position X leads to inefficient glycosylation (90). The CaSR ECD contains 11 potential N-Linked glycosylation sites and 9 of them (N90, N130, N261, N287, N446, N468, N488, N541, N594) are highly conserved in different species including rat (44), chicken (91), rabbit (45) and bovine (38). The other 2 (N386, N400) are conserved in chicken, rabbit and human (88). A mutagenesis study revealed that N386, N400 and N594 are not efficiently glycosylated in CaSR since the replacement of those sites with glutamine does not lead to the alteration of receptor mass or mobility as shown from western blot, whereas, disruption of other N sites would causes a decrease in receptor mass.

The protein folding, intracellular trafficking, protein secretion as well as cell surface expression of glycoproteins are significantly related with their carbohydrate moieties (92,93). The carbohydrate group also plays a crucial role in protein conformation, enzymatic activity and other structural functions. As K.Ray et al. reported, the cell surface expression of CaSR was disrupted if as few as three glycosylation sites were mutated to glutamine (88). Substitution of
the first five glycosylation sites with Q significantly decreased the surface expression of CaSR (>50%) compared with other mutants. A CaSR functional study using a PI hydrolysis assay further indicated that the mutants that exhibited impaired glycosylation had their responses to extracellular calcium correlated with their surface expression. The maximum responses to calcium were significantly reduced, while the EC50 of the dose response curve did not look altered as judged from the figure, though the authors did not use it as one of the parameters. After compensating for reduction of the surface expression level using higher DNA transfection amount, the calcium-stimulated PI hydrolysis among the mutants were comparable with the wild type.

1.2.5 CaSR induced intracellular calcium oscillation and signaling

The CaSR activators can be defined into several groups: Ca\(^{2+}\) and polycations which interact with the Venus Fly Trap (VFT) domain (94-96) and transmembrane region(97,98) ERK; L-amino acids (12), which bind to the VFT domain; phenylalkylamine type-II calcimimetics whose binding pockets located at transmembrane helices VI and VII (98-100). Upon stimulation with Ca\(^{2+}\) or other polycations, a number of intracellular signal transduction pathways will be activated.

Among them, the phosphoinositide-specific phospholipase C (PI-PLC) and extracellular –signal-regulated kinases (ERK1/2) have been widely applied to the investigation of CaSR activity (101,102). In 1998, McNeil et al first reported that in rat fibroblasts the activation of CaSR could increase c-SRC kinase activity as well as the extracellular signal-regulated kinase 1/mitogen-
activated protein kinase activity and the non-functional mutation R796W inhibited both of the two kinases. Their results linked the activation of the SRC and ERK1 pathway to the modulation of proliferation in different cell types by extracellular calcium (103). One year later, Hoff et al found that, a gain-of-function mutation T151M could enhance ERK1/2 and Jun-N-terminal kinase/stress-activated (JNK/SAPK) pathway at the presence of a physiological extracellular calcium concentration (104). Later on, the ERK1/2 activity has been widely investigated in a number of cells with expression of calcium sensing receptor including ovarian surface epithelial cells (105), proximal tubular OK cells (106), Madin-Darby canine kidney cells (107), prostate cancer cells (108), colon carcinoma cells (109), cytokine-stimulated adult human astrocytes (110), rat neonatal ventricular cardiomyocytes (111), Xenopus melanotrope cells(112), osteoblast (113), etc. The ERK1/2 activity has also been utilized to identify CaSR interacting proteins, for instance, filamin A (114), 14-3-3 (115), etc.

Studies on how the L-amino acids or other type II calcimimetics affect these two major signal pathways were conducted in different labs. It is known that those allosteric modulators require a threshold calcium level or other agonists to active CaSR (100). However, the effect of amino acids on calcium dependent PI-PLC activation is negligible(116). On the other hand, L-Phe induced a significant enhancement on the extracellular calcium triggered ERK1/2 activity (100). It has been reported that the aromatic side chain amino acids (L-Phe and L-Trp) could enhance [Ca^{2+}]_o stimulated intracellular calcium change, while the effect of L-Phe on ERK1/2 signal were comparatively small. Thus it has been suggested that dietary protein intake could fine tune cellular responses to the calcium change.
As constant exposure to serum Ca$^{2+}$ would chronically activate CaSR followed by activation of numerous cellular functions, several key questions need to be addressed, including how single cells that expressing CaSR can adapt to such chronic exposure and what are the kinetic features of intracellular Ca$^{2+}$ response to alterations in extracellular Ca$^{2+}$. In 2001, the heterologous expression system using human embryonic kidney 293 cells was utilized to explore those fundamental mechanisms by Gerda E. Breitwieser. Her group reported several major findings: 1, steady-state intracellular Ca$^{2+}$ concentration change was observed in CaSR expressing cells when they are exposed to steady-state perturbations of the extracellular Ca$^{2+}$ concentration (i.e. stepwise increases of extracellular Ca$^{2+}$ concentration); 2, small changes in extracellular Ca$^{2+}$ could induce intracellular calcium oscillation and the oscillation frequency was constant over the range of 2.5~5 mM [Ca$^{2+}$]$_o$; 3, thapsigargin-sensitive intracellular Ca$^{2+}$ stores contributed to the formation of intracellular Ca$^{2+}$ oscillations; 4, staurosporine, a broad-specificity protein kinase inhibitor (inhibit protein kinases A, C, myosin light chain kinase and calmodulin kinase) did not affect the oscillation frequency but increased the number of oscillating cells (117).

Further study of the regulation of intracellular Ca$^{2+}$ oscillation was carried out by Enrique Rozengurt’s group. Previous models explained [Ca$^{2+}$]$_i$ oscillations occurring following GPCR activation by resorting to negative feedback effects of protein kinase C (PKC) on the production of Ins(1,4,5)P3 or the [Ca$^{2+}$]$_i$ regulation on the Ins(1,4,5)P3 receptor. His group found that the observed [Ca$^{2+}$]$_i$ oscillation triggered by changes in extracellular [Ca$^{2+}$] in HEK293 cells expressing CaSR was also modulated by PKC since addition of PKC specific inhibitor significantly decrease the number of oscillatory cells and converted the intracellular responses to transient, non-oscillatory response. PKC activators could reduce the oscillatory frequency but
didn’t eliminate the oscillations. Their study also demonstrated that the PKC phosphorylation site at T888 of CaSR played crucial role in the negative feedback of [Ca$^{2+}$]$_i$ oscillations.

As early as 2000, Conigrave et.al found that CaSR activity could also be regulated by aromatic amino acids since they shifted the [Ca$^{2+}$]$_o$-[Ca$^{2+}$]$_i$ response curve and lowered the EC50(118). In 2002, the aromatic amino acid induced [Ca$^{2+}$]$_i$ oscillations in CaSR-transfected HEK293 cells was further analyzed by Young et. al. (119). The [Ca$^{2+}$]$_i$ oscillation induced by L-Phe or L-Trp required a normal physiological range of [Ca$^{2+}$]$_o$ (1.8 mM in their study). The average oscillation frequency was 1/min which was much lower than [Ca$^{2+}$]$_o$ induced [Ca$^{2+}$]$_i$ oscillations (~ 4/min). The two different patterns, specifically sinusoidal response induced by [Ca$^{2+}$]$_o$ and transient baseline calcium response elicited by amino acids, allow the CaSR to differentiate which agonist was interacting with the receptor.

The mechanisms of [Ca$^{2+}$]$_i$ oscillation triggered by extracellular calcium and amino acids are considered to be different according to the studies from Dr.Rozengurt’s group(116,120) as well as Dr.Breitwieser’s group(121). By tracking intracellular signaling markers tagged with fluorescent proteins, including GFP-PHD (pleckstrin homology domain of PLC-δ1 linked with GFP in order to detect Ins(1,4,5)P3), PKCα-YFP (for the indirect measurement of [Ca$^{2+}$]$_i$), and PKD-RFP (sensing the generation of DAG), it was found that GFP-PHD and PKCα-YFP oscillatory translocated to the membrane after stimulation by high [Ca$^{2+}$]$_o$ and PKD-RFP was translocated to membrane. It was demonstrated that CaSR mediated activation of Gq and PI-PLC (116). In contrast, [Ca$^{2+}$]$_i$ change stimulated by phenylalanine did not redistribute the location of either GFP-PHD OR PKD-RFP, but PKCα-YFP exhibited cyclically redistributed pattern.
An intact actin cytoskeleton system including the small GTP binding protein RhoA which facilitates actin cytoskeleton organization(122), and filamin A, which is linked to the actin cytoskeleton as a scaffold protein (123) was found to be involved in phenylalanine induced oscillation(116) but not extracellular calcium induced \([\text{Ca}^{2+}]_i\) change. Besides, heterotrimeric G-protein \(\alpha\) subunits G12/13 participated in the phenylalanine regulated oscillations, but it is Gq that mediates the \([\text{Ca}^{2+}]_o\) induced oscillation (116). Since HEK-293 cells express TRPC1 channels (124), it is also suggested by studying of thrombin-mediated \(\text{Ca}^{2+}\) entry in endothelial cells (125) that the \([\text{Ca}^{2+}]_i\) influx stimulated by phenylalanine could due to activation of RhoA, which further caused the translocation of \(\text{Ins}(1,4,5)\text{P3}\) receptors and TRPC1 channels to the cell membrane.

Phenylalkylamine allosteric regulators of CaSR could also change the \([\text{Ca}^{2+}]_i\) oscillation patterns (126).

### 1.2.6 Proteins interacting with CaSR

In addition to heterotrimeric G proteins, the CaSR needs to recruit a number of other proteins to ensure the network is fully functional. Recently, a few of these proteins have been identified using yeast two-hybrid system and/or coimmunoprecipitation studies. The absence of these proteins would influence the CaSR signaling.

Filamin A has been demonstrated to interact with the C-terminal of the CaSR in 2001 by two individual groups (80,81). Later study using truncation and deletion mutants revealed that the binding happened in a two \(\beta\)-strands region from amino acid 962 to 981 of the CaSR. Blocking the interaction between Filamin A and CaSR attenuated CaSR-induced ERK activity(80). The
binding of the two proteins also exhibited importance in the CaSR-mediated Rho signaling (116,127) as well as the JNK activation (128). Studies in a cell line deficient in filamin A suggested Filamin A might protect the CaSR from degradation(114).

Potassium channels Kir4.2 and Kir4.1 have been reported to colocalize with CaSR in HEK293 cells stably expressing the CaSR and in endogenous rat kidney tissues respectively(129). However, how and whether their function can be regulated by the CaSR still needs to be elucidated. It is possible that Filamin A may act as a bridge that links the CaSR and either Kir4.1 or Kir4.2.

The region between amino acids 880-900 of the CaSR has been identified to interact with the region 660-838 of an E3 ubiquitin ligase, dorfin (130). The overexpression of dorfin is believed to increase the ubiquitination of the CaSR, resulting an accelerated degradation process (130). Associated molecule with SH3 domain of STAM (AMSH) is an ubiquitin isopeptidase that regulates the sorting of the receptor EGFR (131). The CaSR C-tail region from 895-1075 was found to bind with AMSH(132). Increasing the AMSH expression level or stimulating the CaSR with calcium could reduce the CaSR expression in HEK293 cells transfected with these two proteins (132,133).

β-Arrestins play an important role in modulating the desensitization and internalization of GPCRs through collaborating with G protein receptor kinases (GRK) (134). Overexpression of β-Arrestins negatively regulates the inositol phosphate signaling via CaSR in HEK 293 cells. A PKC inhibitor or mutation on the CaSR PKC phosphorylation sites reduces the effect of overexpressing β-Arrestins.

RAMP is short for receptor activity-modifying protein which has been reported to influence receptor trafficking, glycosylation as well as second messenger production(135). Experiments in
COS7 cells demonstrated that RAMP1 and RAMP3 can increase the surface expression of transfected CaSR, but not RAMP2 (136). Moreover, RAMP3 had greater influence on modulating the receptor surface expression.

1.2.7 Trafficking of the CaSR in cells

Like many other GPCRs, the trafficking of the CaSR is a complex process that involves interacting with various proteins to determine the final destination of the receptor. Once synthesized at the endoplasmic reticulum (ER), those improperly folded CaSR will fail the quality control and be lead towards the proteasome for degradation. Receptors passing the quality control will traffic to plasma membrane or other cellular compartments via interaction with chaperone and small GTP-binding proteins (137-140).

Several proteins interact with CaSR while the receptor exits from the ER, including p24A (transmembrane emp24 domain trafficking protein 2(TMED2))(141); small GTP-binding protein Sar1(142); receptor-activity-modifying proteins (RAMPs)(136); Rab1(143); calmodulin (CaM) (82); 14-3-3(144) and Dorfin (130). p24A binds predominantly with immature form of CaSR and cycles between the ER, the ER-Golgi intermediate compartment(ERGIC) as well as the cis-Golgi membranes (145,146). Through its interaction with p24A, the stability of CaSR and the plasma membrane targeting will be increased possibly via increasing total cellular CaSR(141).

Sar1 is another protein that is required in releasing the receptor from the ER (141,142). RAMPs and Rab1 are small GTP-binding proteins that localize to the cytoplasmic face of organellar membranes. Both of them are found to facilitate CaSR trafficking from ER to Golgi (136,143). The 14-3-3 is predicted to interact with the arginine-rich domain of CaSR \((^{890}RRxxxxRRKR^{898})\) and may lead to the retention of CaSR in the ER (115,144,147). There is also a calmodulin binding site in the C-tail of CaSR comprising residues 874-895 (148). However, the function of
CaM still needs to be revealed. It is reported that CaM binds to both immature and mature forms of CaSR suggesting its role in modulating anterograde trafficking (82). On the other hand, CaM may stabilize the surface receptor on the cell membrane (148).

Its interactions with different proteins contributes to the sub cellular localization of CaSRs. The association of intracellular loops 1 and 3 with caveolins keeps the CaSR highly enriched in invaginations of plasma membrane called caveolae in certain type of cells, like parathyroid chief cells and cardiac myocytes (43,149-151). Filamin A, an adaptor protein, has also been identified to interact with the C-terminal of CaSR, targeting the receptor to sites of Rho activity (116,127,152). The CaSR also interacts with integrins, the latter may contribute to the regulation of cell migration(153).

The surface expressed CaSRs may undergo endocytosis initiated with phosphorylation by G protein coupled receptor kinase (GRKs) or protein kinase C(154,155), involving β-arrestins and facilitated by Rab7, Rab11a and adaptor protein-2 (AP2)(133,156,157). The endocytosed receptors are either recycled to the cell membrane contributing to the receptor resensitization or translocated to the lysosomes for degradation (158,159).

There are two mechanisms that CaSR can utilize to get degraded. One is mediated by the activity of E3 ubiquitin ligase family member dorfin. CaSR will be targeted to proteasome after being ubiquitinated by dorfin (160). CaSR can also be degraded in lysosomes. A deubiquitinating enzyme specific for k63-likages (AMSH which is short for associated molecule with the SH3 domain of STAM) has been reported to be involved in the process(133). Moreover, the PEST-like sequence in the C-tail of CaSR, which are rich in proline, glutamine, serine and threonine, can also lead the receptor to lysosome (157).
In the continuous presence of agonist stimulation, those secretory pathways as well as the endocytosis mechanisms mentioned above work collaboratively (Figure 1.7), causing a net increase in the plasma membrane expressed CaSR. This phenomenon was named Agonist-Driven Insertional Signaling (ADIS)(144). However, a number of questions raised by the ADIS mechanism remained to be explored.

1.2.8 Disease associated CaSR mutations

The discovery of CaSR and its role in maintaining physiological calcium level led to the identification of disorders of calcium homeostasis that are related to abnormal CaSR activity. There are more than 100 mutations of the CaSR that have been catalogued in the online database at http://www.casrdb.mcgill.ca/. They are the primary attribution for the CaSR related disorders. Autoimmune antibodies against the CaSR are another cause for the receptor dysfunction (161). Mutations of the CaSR may lead to an inactive receptor or an overactive receptor. The former includes cases of familial hypocalciuric hypercalcemia, which is a calcium disorder associated with dominant negative activity of the mutant CaSR (162) as well as a more serious disorder known as neonatal severe hyperparathyroidism (NSHPT) due to inactivating mutations in both copies of the CaSR gene (161). NSHPT can cause severe hypercalcemia, multiple fractures, neurodevelopmental disorders and even death. The latter includes autosomal dominant hypocalcemia (ADH) (163) and Bartter syndrome type V (164). Patients with ADH usually do not show symptoms but have mild reductions in calcium concentration in the blood. Patients with Bartter syndrome type V show symptoms like hypokalemic metabolic alkalosis and hyperaldosteronism along with hypocalcemia (164).
Figure 1.7 The trafficking of CaSR.

Illustrated are the primary compartments during the trafficking of CaSR. The major players are highlighted next to the subcellular organelles where they are expected to interact with CaSR. Abbreviations: AP-2, adaptor protein-2; CaM calmodulin; ER, endoplasmic reticulum; FLNA, filamin A; GRKs, G protein coupled receptor kinases; RAMPs, receptor activity modifying proteins; VCP/p97, valosin-containing protein.
1.3 Physiological condition of the amino acids concentration and the rational for the dosage used in this study

In biological systems, free L-amino acids are essential molecules since they not only serve as the building blocks of protein but are also the metabolic precursors of crucial substances that serve as ligands for receptors (165). Evidence for the presence of amino acid sensing mechanisms in various studies, e.g., regulating insulin secretion from pancreatic B cells, hepatic autophagy, etc., suggest the widespread existence of amino acid sensors, although the identities of these amino acid sensors are in many cases unknown. These findings highlight amino acid-dependent control of cellular signal transduction pathways. Fluctuation of the plasma levels of amino acids can, therefore, regulate the rate of hormone synthesis and secretion as well as Ca\(^{2+}\) metabolism, among other processes (165). CaSR is present throughout the gastrointestinal tract (41,166), including in gastrin-secreting antral G cells and cholecystokinin-secreting I cells, both of which are known to be activated by both \([\text{Ca}^{2+}]_o\) and aromatic amino acids (166). L-amino acids, especially aromatic amino acids, are known to enhance the sensitivity of CaSR to \([\text{Ca}^{2+}]_o\), which could be one potential explanation for how dietary protein modulates \([\text{Ca}^{2+}]_o\) homeostasis in normal individuals as well as in patients with chronic renal failure (165,167).

The amino acids concentration in plasma before and after meal is listed in Table 1.1. All of them are within the range of \(\mu\text{M}\). Even after steak meal, the peak value for certain amino acids (e.g. Gly) is around 0.5 mM (168). However, the reported EC\textsubscript{50} for L-Phe was 3.5 mM as measured by intracellular calcium readout from CaSR transfected HEK293 cells (118). The discrepancy for the phenomena relies on the fact that in physiological conditions, the receptors are stimulated by a mixture of amino acids, small peptides as well as proteins instead a single amino acid. In fact,
small peptides, for instance, \(\gamma\)-glutathione and its variants, have smaller EC_{50}s (in the range of \(\mu\)M) compared with L-Phe or L-Trp (Table 1.2).

Table 1.1 Plasma amino acid concentrations before and during intravenous amino acid infusion and after oral steak meal

Mean (±SE) plasma amino acid concentrations of phenylalanine, tryptophan, glycine, alanine, and histidine before (basal) and in response to intravenous infusion of each individual amino acid and after the steak meal. Each amino acid was infused in 125 ml for 1 h in increasing stepwise manner; doses of Phe, Gly, Ala, and His were 0.0125, 0.025, 0.05, and 0.1 M, respectively, and doses of Trp were 0.005, 0.01, 0.02, and 0.04 M. The steak meal contained 49 g protein and 405 kcal. (Table adopted from J.Clin. Invest. Vol 71, pg. 1254-1262, 1983.)

<table>
<thead>
<tr>
<th>Amino acid infused IV</th>
<th>Basal</th>
<th>0-60 min</th>
<th>61-120 min</th>
<th>121-180 min</th>
<th>181-240 min</th>
<th>Steak meal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu)M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>51±5</td>
<td>68±8</td>
<td>90±7*</td>
<td>103±14*</td>
<td>198±37*</td>
<td>85±7</td>
</tr>
<tr>
<td>Trp</td>
<td>49±5</td>
<td>66±6</td>
<td>97±8*</td>
<td>140±16*</td>
<td>244±38*</td>
<td>94±9</td>
</tr>
<tr>
<td>Gly</td>
<td>283±50</td>
<td>291±49</td>
<td>315±50*</td>
<td>417±75*</td>
<td>559±98</td>
<td>386±53</td>
</tr>
<tr>
<td>Ala</td>
<td>316±16</td>
<td>345±15</td>
<td>353±25</td>
<td>326±10</td>
<td>365±31</td>
<td>549±50</td>
</tr>
<tr>
<td>His</td>
<td>80±14</td>
<td>104±8</td>
<td>130±10</td>
<td>172±19</td>
<td>316±25</td>
<td>100±11</td>
</tr>
</tbody>
</table>
Table 1.2 Potencies of γ-glutamyl peptides for Ca\textsuperscript{2+}\textsubscript{i} mobilization in CaR-expressing HEK-293 cells

HEK-293 cells that stably express the CaR were loaded with fura-2AM and assayed for receptor-dependent intracellular Ca\textsuperscript{2+} mobilization by microfluorimetry. The data were obtained from cells perifused with physiological saline solution in the presence of 2.5 mM Ca\textsuperscript{2+}o. In accompanying experiments, the EC\textsubscript{50} for L-Phe was 1.1 ±0.5 mM (n =4).(Table adopted from JBC vol 286, no. 11, pp. 8786–8797, March 18, 2011)

<table>
<thead>
<tr>
<th>γ-Glutamyl peptide</th>
<th>EC\textsubscript{50} for peptide (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Methylglutathione</td>
<td>1.7 ± 0.5 (n = 4)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>3.9 ± 0.7 (n = 4)</td>
</tr>
<tr>
<td>γ-Glu-Cys</td>
<td>4.7 ± 0.9 (n =3)</td>
</tr>
<tr>
<td>γ-Glu-Ala</td>
<td>4.8 ± 0.7 (n =3)</td>
</tr>
</tbody>
</table>

Conigrave et al. have investigated the physiological relevance of their in vitro findings regarding to the amino acid concentration. They mixed 20 common L-amino acids at similar concentration comparing to those present in fasting human plasma and showed that the mixture could reproduce the effects of high concentrations of single amino acids (Table 1.3). These findings suggest that the CaSR sensing L-amino acid is a universal property and amino acid composition of human plasma has a pronounced effect on the activity of CaSR compared to single amino acid (118).
Table 1.3 Effect of L-amino acids on the potency of Ca\(^{2+}\) ions as agonists of the Ca\(^{2+}\) receptor.

Data are shown ± SEM. All experiments were performed at pH 7.4. Δ EC\(_{50}\) is defined as control minus experimental. Unpaired t tests performed on the data set (individual amino acids vs. control) yielded the following results: L-His, L-Phe, L-Trp, L-Tyr, L-Cys, and L-Thr, P ≤ 0.0001; L-Ala, P ≤ 0.0002; L-Asn, L-Gln, L-Ser, and L-Glu, P ≤ 0.01; Gly, L-Pro, L-Val, L-Met, L-Asp, P < 0.05; L-Lys, P = 0.05; L-Ile, P = 0.31; and L-Leu, P = 0.95. (Data adopted from 2000 PNAS vol.97 no.9 pg 4814-4819)

<table>
<thead>
<tr>
<th>Amino acid (10 mM)</th>
<th>EC(_{50}) for Ca(^{2+}) (n)</th>
<th>ΔEC(_{50}) for Ca(^{2+}) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-His</td>
<td>2.4 ± 0.1 (4)</td>
<td>1.9 ± 0.2 (4)</td>
</tr>
<tr>
<td>L-Phe</td>
<td>2.5 ± 0.1 (7)</td>
<td>1.8 ± 0.2 (7)</td>
</tr>
<tr>
<td>L-Tyr</td>
<td>2.5 ± 0.2 (3)</td>
<td>1.8 ± 0.2 (3)</td>
</tr>
<tr>
<td>L-Trp</td>
<td>2.6 ± 0.3 (3)</td>
<td>1.6 ± 0.2 (3)</td>
</tr>
<tr>
<td>L-Cys</td>
<td>2.8 ± 0.1 (5)</td>
<td>1.6 ± 0.1 (5)</td>
</tr>
<tr>
<td>L-Ala</td>
<td>2.9 ± 0.3 (3)</td>
<td>1.4 ± 0.04 (3)</td>
</tr>
<tr>
<td>L-Thr</td>
<td>3.0 ± 0.1 (3)</td>
<td>1.1 ± 0.3 (3)</td>
</tr>
<tr>
<td>L-Asn</td>
<td>3.1 ± 0.2 (3)</td>
<td>1.1 ± 0.2 (3)</td>
</tr>
<tr>
<td>L-Gln</td>
<td>3.2 ± 0.2 (3)</td>
<td>1.0 ± 0.2 (3)</td>
</tr>
<tr>
<td>L-Ser</td>
<td>3.3 ± 0.2 (3)</td>
<td>1.0 ± 0.2 (3)</td>
</tr>
<tr>
<td>L-Glu</td>
<td>3.5 ± 0.2 (3)</td>
<td>0.9 ± 0.1 (3)</td>
</tr>
<tr>
<td>L-Pro</td>
<td>3.6 ± 0.3 (3)</td>
<td>0.6 ± 0.2 (3)</td>
</tr>
<tr>
<td>L-Val</td>
<td>3.6 ± 0.3 (3)</td>
<td>0.6 ± 0.2 (3)</td>
</tr>
<tr>
<td>L-Met</td>
<td>3.6 ± 0.3 (3)</td>
<td>0.6 ± 0.1 (3)</td>
</tr>
<tr>
<td>L-Asp</td>
<td>3.7 ± 0.2 (3)</td>
<td>0.6 ± 0.3 (3)</td>
</tr>
<tr>
<td>L-Lys</td>
<td>3.7 ± 0.1 (3)</td>
<td>0.6 ± 0.2 (3)</td>
</tr>
<tr>
<td>L-Arg</td>
<td>3.7 ± 0.1 (3)</td>
<td>0.5 ± 0.3 (3)</td>
</tr>
<tr>
<td>L-Ile</td>
<td>3.9 ± 0.5 (3)</td>
<td>0.3 ± 0.1 (3)</td>
</tr>
<tr>
<td>L-Leu</td>
<td>4.2 ± 0.3 (3)</td>
<td>0.6 ± 0.2 (3)</td>
</tr>
<tr>
<td>Control</td>
<td>4.2 ± 0.1 (10)</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>
Our lab utilized computational algorithms HADDOCK to dock amino acids into the CaSR ECD and compared the binding energies among them (Figure 1.8). Similarly, the calculated binding energy correlated well with \textit{in vitro} studies reported by Conigrave’s group.

![Figure 1.8 Calculated binding energy for single amino acids docking into the CaSR ECD using HADDOCK.]

The blue bars show the amino acid that has a calculated binding energy level correlated well with expectation estimated from \textit{in vitro} experiments. The orange bars show the discrepancy.

The metabolism of both Ca$^{2+}$ and amino acids shall be re-appraised in the context of protein ingestion and dietary supplements since L-amino acid and Ca$^{2+}$ can potentiate the sensing capability by CaSR for each other. It is also emphasized by Conigrave \textit{et. al.} that the CaSR should be reassessed as a potential target of aromatic L-amino acids under certain toxic
metabolic conditions. For instance, the CaSR expressed in the CNS might be involved in contributing to the elevated levels of L-Phe in phenylkeonuria or in hepatic encephalopathy (118).

1.4 Challenges in studying the membrane protein with weak metal binding affinities

The challenges in studying the Calcium-sensing-receptor are multifaceted:

First, the CaSR belongs to membrane proteins, so the challenges associated with membrane proteins may also occur to the CaSR. Membrane proteins are difficult to separate from the cells due to their hydrophobic transmembrane domain. The heating process may cause the aggregation of the 7TM region and thus reduce the solubility of the protein.

Although X-ray crystallography has been used as one of the major tools to study the structure of proteins, application of this technique to the crystallization of glycosylated proteins exhibits its own limitations. Like other glycosylated proteins, the complex form of the glycans on the CaSR can prevent the crystallization of this protein because the chemical and conformational heterogeneity of the glycoproteins usually inhibit crystallization.

Due to the limitation of the conditions for crystallization as well as the fast on and off rate of Ca$^{2+}$ when its interaction belongs to weak binding, even some proteins have their crystal structure solved like mGluR1, the detection of Ca$^{2+}$-binding sites in those protein may be still elusive. Moreover, the selectivity of the CaSR for different amino acids is not clear and the interaction between amino acids and the CaSR are relatively weak (in mM range).
Currently, methods for direct measurement of Ca\textsuperscript{2+} and amino acid binding to the CaSR have not yet been well established, nor are there methods for directly monitoring ligand induced conformational changes of the proteins. Approaches for addressing these questions rely on indirect measurements of calcium-induced intracellular signaling changes in living cells or utilizing the Fluorescence Resonance Energy Transfer (FRET) assay for monitoring ligand generated fluorescence changes in isolated proteins.

Last but not least, the large size of the protein (extracellular domain 612 amino acids; whole protein 1078 amino acids) as well as the fact that the CaSR function as a dimer contribute to the difficulties in studying the protein, especially when it possesses a high Hill coefficient which suggests possible multiple binding sites for ligands.

1.5 Approaches and strategies

1.4.1 Prediction of Ca\textsuperscript{2+}-binding sites in proteins and computational simulation

Previous studies on the known Ca\textsuperscript{2+}-binding sites from thousands of proteins with X-ray structures show that Ca\textsuperscript{2+} binding sites usually forms pentagonal or octahedral bipyramidal geometry (169) composed by 4-7 oxygen atoms from ligand residues. The preference of the residues in a Ca\textsuperscript{2+} binding sites falls into the following category: Acidic residues (-COOH) > H2O > main chain carbonyl > Asn > Ser > Thr > Tyr. There are restrictions on forming certain geometries of Ca\textsuperscript{2+} binding pockets as the distances between Ca\textsuperscript{2+} and coordinating oxygen atoms are within the range of 2.3~2.6 Å and the average Ca-O-C angle has been reported to be 93~160°. Two major types of Ca\textsuperscript{2+}-binding sites---continuous and discontinuous---are defined based on whether a short, contiguous primary sequence can form a pocket to chelated Ca\textsuperscript{2+}. The residues composing discontinuous Ca\textsuperscript{2+}-binding sites may be separated in protein sequence but might be close enough to form a pocket in three dimensional structures. Our lab has developed a
series of algorithms, including GG, MUG (169), MUGC (170) etc. to predict Ca\(^{2+}\)-binding sites from discontinuous protein sequences utilizing their 3D structures or modeled structures. While GG algorithm focused on the geometric information of oxygen composition from a protein structure, MUG and MUGC further define calcium center and second shell carbon atoms to minimize the false positive and false negative results. Having a high performance with 90% site sensitivity and 80% site selectivity (171), these approaches have been used to explore the Ca\(^{2+}\)-binding sites in modeled CaSR structure(68,172). Following the initial grafting and subdomain approaches to verify the Ca\(^{2+}\) binding sites on CaSR, the immediate need is to characterize the cooperativity among those Ca\(^{2+}\)-binding sites and how they could be modulated by other allosteric modulators.

1.4.2 Computational docking and molecular dynamic simulation

Molecular docking utilize computational algorithms to calculate the preferred orientation of one molecule to another when the two form a stable complex(173). Since the interaction between proteins and their ligands plays a crucial role in signal transduction, docking is considered as a useful tool to predict the binding orientation of a ligand and its target protein. A scoring function is generally used in docking programs as an attempt to approximate the standard chemical potentials of the system (174). AutoDock Vina developed in Dr. Olson’s group, is a new program for molecular docking. Using a sophisticated gradient optimization approach in its local optimization procedure and the multithreading step, AutoDock Vina exhibits fast execution capability and improved accuracy (174). AutoDock Vina has been widely used in biologically relevant research, including studies probing the target moleculars for GPCR proteins (174). Using this strategy, we are able to predict the L-Phenylalanine binding pocket, and further investigate how this positive allosteric modulator regulates the function of CaSR.
Molecular dynamics (MD) simulation provides an approach complementary to the experiments in live cells for understanding biomolecular structure, dynamics, and function. The crystal structures of mGluR1, a GPCR family C member, with both the ligand-free form and agonist loaded forms available in the Protein Data Bank (PDB) [PDB entries: 1EWT, 1EWK (175), and 1ISR(176)] are considered to be employed as a template in the simulation due to their similarity in structure with that of the CaSR.

1.4.3 Using Ca\(^{2+}\) sensors to monitor intracellular calcium signaling

Monitoring the effects of Ca\(^{2+}\) on numerous cellular processes is an essential step for understanding the complex intracellular signaling pathways regulated by Ca\(^{2+}\). Several endeavors have been made to investigate this problem, characterizing as two major categories: Ca\(^{2+}\)-binding dyes and genetically-encoded fluorescent proteins (177). Fura-2 is one of the representative Ca\(^{2+}\) binding dyes that have been widely applied in many studies. Its advantages, such as high spatiotemporal resolution, good photostability and easy cell membrane permeability via its acetoxyethyl ester has enabled Fura-2 to become one of the most popular cytosolic Ca\(^{2+}\) indicators. However, the drawbacks of these dyes are also apparent since they can not be targeted to subcellular compartments, and the exogenous buffer they introduce during the dye mixing process may perturb endogenous Ca\(^{2+}\) signaling. Moreover, their sensitivity might be reduced in thick tissues and intact organisms (178). On the other hand, the protein-based Ca\(^{2+}\) sensors can be specifically targeted to various organelles in various cell types with grafted signal peptides (179). Aequorin, the first generation of the protein based Ca\(^{2+}\)-sensor, has a wide dynamic range and weak Ca\(^{2+}\) binding affinity, but the requirement for cofactors in order to be applied in living cell imaging has limited its application (180). The second generation was based on the fluorescence resonance energy transfer (FRET) between calmodulin and its binding peptide M13.
linked to two fluorescent proteins (181,182). The disadvantage of the FRET based Ca$^{2+}$ indicators are mainly two folds: the linker between the donor and acceptor might disrupt the endogenous signaling net; and quantitative analysis might be hindered by the distances and orientation of the FRET pair (179,183,184). Our lab has developed a Ca$^{2+}$ sensor named “CATCHER” based on the enhanced green fluorescence protein (EGFP). It can target to the endoplasmic reticulum (ER) and its weak Ca$^{2+}$ binding capability as well as the minimum perturbation of the endogenous signaling pathways provides a new way to explore calcium changes in sub-cellular compartments (185).

1.4.4 Expression and purification of the extracellular domain of CaSR

Various studies including protein structure and function analysis, protein-protein interaction verification, antibody generation and gene regulation exploration, rely on recombinant protein expression technology. Hitherto, six host systems have been used for protein expression, namely cell-free expression, bacterial expression, yeast expression, algal expression, insect expression and mammalian expression. Among these, the bacterial, insect and mammalian systems are widely employed in the study of GPCR proteins. Each system has its own advantages and challenges. The bacterial expression system is best known for its low cost, simple culture conditions and that it can easily be scaled up to generate large amounts of protein. However, it also faces problems like protein solubility, minimal posttranslational modification and is less tolerant to mammalian proteins. On the contrary, the mammalian expression system only generates limited amounts of proteins and the demanding culture conditions usually make the procedure too costly. Nevertheless, mammalian expression systems produce the highest level of correct post-translational modification, and it is more suitable to generate functional human proteins. Our lab routinely utilizes a variety of *E.Coli* strains to express engineered proteins. The
grafted proteins and the subdomain constructs of CaSR have been successfully expressed in bacteria system (68,172). Meanwhile, our collaborator Dr. Kelley Moremen in the University of Georgia has abundant experience of expressing mammalian proteins in suspension culture systems using mammalian cells (HEK293F). It will be worthwhile to study how the proteins expressed from two different systems will vary in various biophysical characteristics (Invitrogen).

1.4.5 Monitoring intracellular signaling pathways using immunoassays

Immunoassays have been widely applied in the exploration of the intracellular molecular signaling networks. They rely on the interaction between a certain antibody and a specific macromolecule or low molecular weight signaling molecule. The other key feature of immunoassays is that they provide a way to quantify the signal in response to the binding. This feature requires a detectable label to be linked to the antibodies. The linked label either can emit radiation, have a color change during the reaction, produce fluorescence, or can be induced to generate light through chemiluminescence detection. Three immunoassays were used in this project: Western Blot, immunocytochemistry (ICC) and ELISA. The Western Blot technique utilizes chemiluminescent substrate for alkaline phosphatase (AP) to generate detectable signals. Those signals can be measured quantitatively by comparing them with an internal control protein such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The secondary antibody for ICC is conjugated with a fluorescent dye that can be excited at various wavelengths and emits lights within a certain measurable range. On the other hand, the secondary antibody for the ELISA assay is conjugated with horseradish peroxidase (HRP) which interacts with the substrate tetramethylbenzidine (TMB) and subsequently generates detectable signals. Those assays can be useful for “visualizing” molecules that play crucial roles in intracellular signaling networks.
1.6 Major questions to be addressed in this dissertation

Several major questions need to be addressed in this dissertation:

1. How CaSR functional cooperativity is regulation by extracellular binding of Ca^{2+} and amino acids?

2. How the disease associated CaSR mutants can affect the functional cooperativity mediated by Ca^{2+} and amino acids?

3. How can we determine the binding of Ca^{2+} and L-Phe to the CaSR ECDs and detect the potential conformational change induced by the interaction?.

4. How the multifunctional intermediate messenger protein--- CaM regulates the CaSR trafficking and signaling?

5. How ER Ca^{2+} signaling regulates CaSR functional cooperativity via modulation of receptor surface expression?

1.7 The objectives and overview of this dissertation

The overall goal of this research is to understand the mechanisms of how CaSR integrates the Ca^{2+}-mediated signaling and to probe the molecular basis for diseases resulting from alterations in Ca^{2+} homeostasis. We will reach the goal via following 5 aims to addressing the above five major questions.

A. Chapter 1 is to overview the background of calcium signaling and calcium sensing receptor.

B. Chapter 2 is to describe methods and materials used in this study.

C. Chapter 3 is to understand the regulation of CaSR functional cooperativity by extracellular binding of Ca^{2+} and amino acids
**A1. Predict Ca\(^{2+}\) and L-Phe binding sites using computational algorithms**

In order to predict the Ca\(^{2+}\) binding sites and potential L-Phe binding site in the ECD, a modeled structure of CaSR was generated based on the known structure of mGluRI\(^ \_\_\_\) GG (171) and MetalFinder (68,186), computational algorithms developed in our lab based on utilizing geometric characteristics of Ca\(^{2+}\) binding sites in proteins to identify the Ca\(^{2+}\) binding pockets in ECD. And the L-Phe binding site will be predicted based on the results of molecular dynamic simulation on the CaSR ECD docked with L-Phe.

**A2 Verify predicted Ca\(^{2+}\) binding pockets and L-Phe binding site by monitoring the intracellular calcium concentration in HEK293 cells transiently transfected with wild type CaSR and its variants using single cell fluorimetric imaging with or without the presence of L-Phe.**

To study the effect of each site-specific Ca\(^{2+}\) binding site on the function of CaSR, the negatively charged residues, mainly Glu and Asp, will be mutated into non-charged residues like Ile or Ala. To confirm the L-Phe binding site, the site-directed mutagenesis strategy was applied. One of the methods to study the effect of calcium binding sites on the function of CaSR is monitoring the intracellular calcium release in mammalian cells. The WT CaSR and its mutants will be transfected into HEK 293 cells and heterogeneously expressed. The intracellular calcium concentration changes will be recorded with the aid of a ratiometric Fura-2 based method upon stepwise increased in extracellular Ca\(^{2+}\). By comparing the responses of WT CaSR and its mutants, the function of mutated calcium binding sites might be revealed. The effect of L-Phe on WT CaSR as well as the mutants was measured by performing the assays described above.

**D. Chapter 3 and 4 are to understand how the disease associated CaSR mutants can affect the functional cooperativity mediated by Ca\(^{2+}\) and amino acids.**
By collaborating with Prof. Thakker’s group at University of Oxford, we will analyze how the disease related mutations can affect the function of CaSR and how L-Phe can modulate the receptor activity.

**E. Chapter 6 and 7 are aimed to probe the Ca^{2+} and L-Phe binding affinity, cooperativity, and related conformational change of wild type ECD and its mutants by various biophysical methods.**

First, we expressed and purified the ECD and disease-related mutants and variants, which were shown to play important role in intracellular calcium responses using bacteria and mammalian expression systems. The native conformation of the bacterial expressed protein was compared to the mammalian expressed protein using by immunoblotting technique and metal binding FRET assay.

Second, The secondary structures of the purified proteins were verified using circular dichroism. Moreover, the agonist induced conformational changes were measured using tryptophan spectral and ANS binding assays. Next, the Tb^{3+} -FRET assay was applied to study the metal binding capability of those proteins. Furthermore, the Ca^{2+}-binding affinities were indirectly acquired using a Ca^{2+} competition assay, in which Ca^{2+} is gradually added to compete with the bound Tb^{3+}.

**F. Chapter 8 is to study the mechanism of receptor internalization upon the stimulation of extracellular calcium.**

The regulation of cytosolic Ca^{2+} signaling and cell surface expression of the receptor by calmodulin binding to the calcium-sensing receptor's proximal C-terminus was investigated in this proposal. The hypothesis to be tested was that extracellular activation triggers the Ca^{2+-}
dependent association of CaM the C-terminus of CaSR in regulating cell surface expression of the receptor and the PKC activity. We will 1) measure the cellular interaction between CaR and CaM and the effect of CaM binding on the phosphorylation at T888 by PKC; 2), investigate the 

\[ [\text{Ca}^{2+}]_i \] oscillation and trafficking changes using CaSR variants with mutations of the calcium and amino acid-binding sites and disease associated mutants and CaM variants with altered calcium binding affinity.

G. Chapter 9 is to investigate how the CaSR regulate its functional cooperativity by modulation of surface expression via \([\text{Ca}^{2+}]_{\text{ER}}\) signaling.

We will use the ER calcium probe developed in our lab --- CatchER as well as the commercial available ratiometric dye Mag-Fura 2 to investigate how \([\text{Ca}^{2+}]_{\text{ER}}\) regulates the expression of the receptor. We will utilize the real-time imaging system to calibrate \([\text{Ca}^{2+}]_{\text{ER}}\) and the surface expression of CaSR will be analyzed using multiple immunoassays (e.g. western blot, immunofluorescence staining).

H. Chapter 10 summarizes major conclusions and the significance of this project.
CHAPTER 2. MATERIALS AND METHODS

2.1 Computational prediction of Ca\textsuperscript{2+}-binding sites in the ECD of CaSR

The structure of the extracellular domain of CaSR (residues 25-530) was modeled based on the crystal structure of mGluR1 (1EWT, 1EWK and 1ISR), and the potential Ca\textsuperscript{2+}-binding sites in the CaSR ECD were predicted using MetalFinder (68,187).

2.2 Computational prediction of L-Phe-binding pocket in the ECD of CaSR

The Prediction of the L-Phe-binding site was performed by AutoDock-Vina (174). In brief, the docking center and grid box of the model structure and the rotatable bonds of L-Phe were defined by AutoDock tools-1.5.4. The resultant L-Phe coordinates were combined back to the pdb file of the model structure for input into the Ligand-Protein Contacts & Contacts of Structural Units (LPC/CSU) server to analyze interatomic contacts between the ligand and receptor (188). The residues within 5 Å around L-Phe were considered as L-Phe-binding residues.

2.3 Computational simulation of CaSR and its mutants

2.3.1 MD simulation and correlation analysis using Amber.

MD simulation provides an approach complementary to the experiments in live cells for understanding biomolecular structure, dynamics, and function. The initial coordinates for all the simulations were taken from a 2.20 Å resolution x-ray crystal structure of mGluR1 with PDB ID 1EWK (175). The AMBER 10 suite of programs (189) was used to carry out all of the simulations in an explicit TIP3P water model (190), using the modified version of the all-atom Cornell et. al.(191) force field and the re-optimized dihedral parameters for the peptide ω-bond (192). An initial 2 ns simulation was performed using NOE restraint during the equilibration in
order to reorient the side chains residues in the Ca$^{2+}$-binding site, but no restraints were used during the actual simulation. A total of three MD simulations were carried out for 50 ns each on apo-form and ligand (Ca$^{2+}$ and L-Phe) loaded forms. During the simulations, an integration time step of 0.002 ps was used to solve the Newton’s equation of motion. The long-range electrostatic interactions were calculated using Particle Mesh Ewald method (193) and a cutoff of 9.0 Å was applied for non-bonded interactions. All bonds involving hydrogen atoms were restrained using the SHAKE algorithm (194). The simulations were carried out at a temperature of 300 K and a pressure of 1 bar. A Langevin thermostat was used to regulate the temperature with a collision frequency of 1.0 ps$^{-1}$. The trajectories were saved every 500 steps (1ps). The trajectories were analyzed using the ptraj module in Amber 10.

2.3.2 Accelerated Molecular Dynamics Simulation.

Accelerated MD (aMD) was carried out on the free CaSR ECD using the RaMD method (195) implemented in a pmemd module of AMBER on the rotatable torsion. A boost energy, E, of 2000kcal/mol was added to the average dihedral energy and a tuning parameter, $\alpha$, of 200 kcal/mol was used. The dual boost was also applied to accelerate the diffusive and solvent dynamics as previously described (196). The simulation conditions were similar to that of the normal MD simulations above. Principal Component Analysis was carried out on the trajectories using the ptraj module in AMBER. The directions of the eigenvectors for the slowest modes were visualized using the Interactive Essential Dynamics (IED) plugin (197).

2.3.3 Docking Studies of Phe, Asp, and Glutathione.
The binding energies for the ligands were calculated using an ensemble-docking method and Autodock vina (198). The ensemble of conformations of CaSR was generated using molecular dynamics simulations as described above. Gasteiger charges were assigned to the ligands and CaSR using the Autodock ADT program. The ligands were flexible during docking to each conformation of CaSR using the following parameters: the grid spacing was 1.0 Å; the box size was 25 Å in each dimension, and the center of the box was chosen as the center of the active site of CaSR, with a large enough space to sample all possible ligand conformations within the box. The maximum number of binding modes saved was set to 10. The conformation with the lowest binding energy was used and assumed to be the best binder. Distributions of the binding energies for each ligand were calculated based on the lowest binding energy of each ligand to each conformation in the ensemble of CaSR conformations.

2.3.4 Principal Component Analysis (PCA)

Using the ptraj module of AMBER 10, the Principal Component Analysis (199,200) was performed on all the atoms of the residues that are 5 Å away from Site 1 of CaSR ECD. The covariance matrix of the x, y, and z coordinates of all the atoms obtained from each snapshot of the combined trajectories of the ligand-free CaSR ECD, the Ca^{2+}-loaded form, the form loaded with only L-Phe, and the form loaded with both Ca^{2+} and L-Phe were calculated. The covariance matrix was further diagonalized to produce orthonormal eigenvectors and their corresponding eigenvalues, ranked on the basis of their corresponding variances. The first three eigenvectors, the Principal Components, which contributed the majority of all the atomic fluctuations, were used to project the conformational space onto them, i.e., along two dimensions.
2.4 Plasmid construction and protein engineering

2.4.1 L-Phe binding site related mutation construction

All of the full length CaSR mutants were generated by site-directed mutagenesis based on the sequence of the human CaSR-pcDNA (a gift from Dr. Edward Brown from Brigham and Women’s Hospital, Boston). Site-directed mutagenesis was performed using the QuikChangeTM kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions. Briefly, a pair of complementary primers of 27–35 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 PCR instrument (TECHNE). After digestion of the template DNA with DpnI (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).

2.4.2 Disease related mutation construction

All of the full length CaSR mutants were generated by site-directed mutagenesis based on the sequence of the human CaSR-pEGFP (a gift from Dr. Rajesh Thakker from Oxford Centre for Diabetes, Endocrinology and Metabolism) as well as human CaSR-pcDNA. Site-directed mutagenesis was performed using the QuikChangeTM kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions as mentioned above.

2.4.3 ECD construction and its mutants

For engineering of the hCaSR-ECD, the sequence Tyr$^{20}$-Phe$^{612}$ was cloned from CaSR-pCDNA 3.1(+) by standard PCR methods using 5’ primer (CCGGATTCTACGGGCCAGACCAGCCCAA) and 3’ primer (CCCAAGCTTTTTAAAGGGCTCCGTCACGACAGAAAAGCCCAA).
The cloned sequence was then inserted into the plasmid pXLG between restriction enzymatic sites EcoRI and HindIII. The engineered protein contains a transmembrane signal (MRLLTALFAYFIVALILAFSVSAKS) followed by His-tag at N-terminal. The sequences were verified through the GENEWIZ Company (www.genewiz.com).

For engineering of the bCaSR-ECD, the ECD CaSR sequence, Met$^1$-Phe$^{612}$ was amplified from CaSR-pCDNA 3.1(+) (2009 Nancy 27) and was further subcloned into the pRSET-A vector between the BamHI and EcoRI restriction sites.

2.4.4 Furin tag construction and its mutants

For engineering of the Furin-CaSR-ECD, the furin cleavage site (ARRRKKRGLDV) was inserted immediately after the FLAG tag in the pcDNA-CaSR plasmid by standard PCR methods using 5’ primer (AAGCGAGGCTTGAGTCACCTTTCTGAGAGGTCACGAAGAAAGTGTCGTCGTGCCTTGTCATCGTCATCCTTGTAGTCCACAGG) and 3’primer (CTTTCGTCGTCGTGCCTTGTCATCGTCATCCTTGTAGTCCACAGG). The sequences were verified through the GENEWIZ Company (www.genewiz.com). All mutations on the Furin site containing construct were introduced using site-directed mutagenesis via the QuikChangeTM kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions as described above.

2.4.5 Construction of the Filamin A interaction region deletion mutant CaSR.

Deletion of the Filamin A interaction region was carried out on the pcDNA-CaSR plasmid. ∆860 refers to the deletion of the whole CaSR C-terminal after residue 860. ∆907-999 refers to the deletion of the region between residue 907 and 999. ∆971 refers to a construct in which the region between residue 971 and 1032 was deleted. All constructs were generated using standard PCR methods.
2.5 Protein expression and purification

2.5.1 CaSR ECD expression in bacteria system

The bacterial expression was carried out in *Escherichia coli* Rosetta-gamiTM pLysS cells in LB medium with 100 mg/L of ampicillin with an initial temperature of 37°C. Isopropyl-β-D-thiogalacto-pyranoside (IPTG) at 300 μM was added when the absorbance at 600 nm reached 0.4~0.5 to induce protein expression for overnight expression at 27°C. The cells were collected by centrifugation at 5000 rpm for 30 min and the cell pellets were lysed using a cell disruptor. After separating the mixture by centrifugation at 17000 rpm for 15 min, the supernatant was filtered via a 0.45 μM filter and further applied to a HisPrep HP column (GE Healthcare) and the protein was purified using AKTA FPLC (GE Healthcare). The bacterial CaSR-ECD concentrations were determined using absorbance at 280 nm with an extinction coefficient of 110,300 M⁻¹ cm⁻¹.

2.5.2 CaSR ECD expression in mammalian system

One liter of Freestyle™ 293 Expression Medium (Life Technologies) was used for HEK293F or Lec1 mutant HEK293 F cell culture. Cells were transfected with pXLG-hCaSR-ECD plasmid using the polyethylenimine (PEI) method when the cell density achieved 8x10⁵. The hCaSR-ECD was secreted into the culture media. When the cell density reached 5x10⁶ cell/mL, the media was collected by centrifugation 3 times at 1500xg for 10 min each. The supernatant was diluted with dilution buffer (Tris NaCl pH=8.0) at a ratio of 1:3 and further filtered through a 0.45 μm filter (Millipore, Billerica, MA). The filtered medium was applied to a 16/10 HisPrep HP column (GE Healthcare) pre-equilibrated with Buffer A (Tris 50 mM, NaCl, 150 mM,
Imidazole 20 mM) and a linear segmented gradient of 0 - 100% Buffer B (50 mM Tris, 500 mM sodium chloride, and 500 mM imidazole, pH 7.4) was run using FPLC to elute the protein. The hCaSR-ECD concentrations were determined using Bio-Rad protein assay.

2.6 Isotopic labeling of ECD CaSR

2.6.1 $^{15}$N labeled CaSR-ECD from bacterial system

For isotopic labeling ($^{15}$N-$^{13}$C-$^{2}$D), $^{15}$NH$_4$Cl and $^{13}$C glucose were supplemented as the sole source of nitrogen and carbon for bacteria growth in the minimal medium (K$_2$HPO$_4$ 45 mM, KH$_2$PO$_4$ 32 mM, Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O 20 µM, MgSO$_4$·7H$_2$O 0.25 mM, glucose 27.7 mM, NH$_4$Cl 9 mM). *Escherichia coli* Tuner™ strain in minimal medium with 100 mg/L of ampicillin was cultured at 37°C at the beginning. Isopropyl-β-D-thiogalacto-pyranoside (IPTG) at 300 µM was added when the A$_{600}$ reached 0.6 to induce protein expression for overnight expression at 27°C. The cells were collected by centrifugation at 5000 rpm for 30 min. The cell lysate was applied to a HisPrep HP column (GE Healthcare) and protein was purified using AKTA FPLC (GE Healthcare). The bCaSR-ECD concentrations were determined using absorption at 280 nm with extinction coefficients of 110,300 M$^{-1}$ cm$^{-1}$.

2.6.2 $^{13}$C Methylation labeling of ECD CaSR

The dimethylation of lysine was performed by reacting with formaldehyde under reducing conditions at 4°C for 16 h. The reaction was optimized to allow complete dimethylation. The formaldehyde was added twice together with DMAB for reductive methylation. For each time, the mole ratio of formaldehyde and Lys was 3:1 and the ratio of DMAB to formaldehyde was 3:1.
The time interval between the two additions was 3 hours. The mono-methylated peak can be easily differentiated because the chemical shift is in the range of 30-35 ppm while the dimethylated lysine has peaks in the range of 42-48 ppm.

2.6.3 \textit{15}N labeled CaSR-ECD from mammalian system

Four–six liters of customized Freestyle™ 293 Expression Medium (Life Technologies) was used for HEK293F cell culture. The normal L-Phe supplement in this medium was substituted by N15 labeled L-Phe. Cells were transfected with pXLG-hCaSR-ECD plasmid using the polyethylenimine (PEI) method when the cell density reached 8x10^5. The protocol for purifying the mammalian expressed protein was the same as purification for the non-labeled mammalian CaSR ECD.

2.7 Circular dichroism spectroscopy

The circular dichroism spectra were recorded from 190 to 260 nm on a Jasco-810 spectropolarimeter purged with N2 and equipped with a temperature control system CTC-345. Spectral and temperature dependent measurements were performed at a bandwidth of 2 nm using a U-type quartz cell of path length 0.1 mm with protein concentrations ranging from 8 to 10 \( \mu \text{M} \) in 10 mM Tris-HCl, pH 7.4. Five spectra were recorded at a scan rate of 50 nm/min and response time of 1s. The continuous temperature dependence of the ellipticity at 222 nm was measured using a scan rate of 50 °C/h and a delay time of 100 s. The cuvette was sealed with parafilm to prevent solvent evaporation.

The spectra were deconvoluted using the selcon method after subtracting the spectrum of the buffer as the blank and the CD data was depicted in units of molar ellipticity per residue. The melting curve was fitted using equation “\( \Delta S = \Delta S_{\text{max}} / (1 + e^{(T_m-T)/k}) \)” to obtain the thermal
transition point, where $\Delta S$ and $\Delta S_{\text{max}}$ are the signal changes at each data point and final point, $T_m$ and $T$ are the transition temperature and experimental temperature, respectively. $k$ is a transition rate that defines how fast the temperature-induced change occurs, which is represented by the slope of the transition phase in the fitting curve, a smaller $k$ results in a sharper transition (201).

2.8 Fluorescence spectroscopy

2.8.1 Intrinsic tryptophan fluorescence

A PTI lifetime fluorimeter was used to record the fluorescence spectra at room temperature using a 1 cm path length cell. Intrinsic tryptophan emission spectra were recorded from 300 to 400 nm with the excitation wavelength at 282 nm. The slit widths were set at 1~4 nm for excitation and 3~8 nm for emission respectively. The calcium titration was performed in 10 mM Tris with the protein concentration of 1.5~2.0$\mu$M at pH 7.4 by adding known amount of CaCl$_2$.

2.8.2 Tb$^{3+}$-FRET assay

For the Tyr/Trp-sensitized Tb$^{3+}$ fluorescence energy transfer (Tb$^{3+}$-FRET) assay, protein samples (1.5~2.0$\mu$M) were in 20 mM PIPES-10 mM KCl at pH 6.8. The emission spectra was collected from 500~600 nm with the excitation at 282 nm. The slit widths for excitation and emission were set at 1 nm and 3 nm, respectively. Secondary Rayleigh scattering was circumvented by using a glass filter with a cutoff of 320 nm. The Tb$^{3+}$ titration was performed by adding various volumes of Tb$^{3+}$ stock solutions (1 mM) stepwise into the cuvettes. The Ca$^{2+}$-Tb$^{3+}$ competition experiments were performed in solutions containing 30~50 $\mu$M Tb$^{3+}$ and 2 $\mu$M proteins as the starting point. The stock solution of 100 mM ~1 M CaCl$_2$ with the same concentration of Tb$^{3+}$ was gradually added into the mixture. The background fluorescence intensity was subtracted...
using logarithmic fitting and the Tb\(^{3+}\) binding affinity of the protein was calculated by fitting normalized fluorescence intensity data using the Hill equation \(\Delta S = \frac{[M]^n}{K_d^n + [M]^n} \) (Eq.2.1) 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.

The Ca\(^{2+}\) competition data was first analyzed to derive the apparent dissociation constant by Equation 1. By assuming that the samples were saturated with Tb\(^{3+}\) at the starting point of the competition, the Ca\(^{2+}\)-binding affinity is further obtained by using the equation,

\[
K_{d,Ca} = K_{app} \frac{K_{d,Tb}}{K_{d,Tb} + [Tb]} \quad \text{(Eq.2.2)}
\]

where \(K_{d,Ca}\) and \(K_{d,Tb}\) are the dissociation constants of Ca\(^{2+}\) and Tb\(^{3+}\), respectively. \(K_{app}\) is the apparent dissociation constant.

2.8.3 ANS-binding measurement

For the ANS (8-anilino-1-naphthalenesulfonic acid) binding assay, protein samples were incubated with 40 \(\mu\)M ANS in 50 mM Tris-HCl and 100 mM KCl (pH 7.4) with either 5 mM EGTA or 3 mM Ca\(^{2+}\) at room temperature for 1 h prior to measurement. The excitation wavelength was set at 370 nm, and the emission spectra was acquired from 400 to 600 nm. For the Ca\(^{2+}\) titration, the protein concentration was 1.5 \(\mu\)M and the Ca\(^{2+}\) concentration was varied from 0 to 30 mM in 20 mM Tris-HCl and 50 mM KCl (pH 7.4).
2.8.4 Measurement of [Ca\textsuperscript{2+}]\textsubscript{i} in cell population by fluorimetry

The [Ca\textsuperscript{2+}]\textsubscript{i} responses of wild type CaSR and its mutants were measured as described by Huang, et al. (68). Briefly, CaSR-transfected HEK293 cells were grown on 13.5 × 20 mm coverslips. After the cells reached 90% confluence, they were loaded by incubation with 4 μM Fura-2 AM in 20 mM HEPES, containing 125 mM NaCl, 5 mM KCl, 1.25 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 1% glucose, and 1% BSA (pH 7.4) for 1 h at 37°C and then washed once with 20 mM HEPES, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 0.5 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 1% glucose, and 1% BSA (bath buffer). The coverslips with transfected Fura-2-loaded HEK293 cells were placed diagonally in 3 ml quartz cuvettes containing bath buffer. The fluorescence spectra at 510 nm were measured during stepwise increases in [Ca\textsuperscript{2+}]\textsubscript{o} with alternating excitation at 340 or 380 nm. The ratio of the intensities of the emitted light at 510 nm when excited at 340 or 380 nm was used to monitor changes in [Ca\textsuperscript{2+}]\textsubscript{i}. The EC\textsubscript{50} and Hill constants were fitted using the Hill equation

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

(Eq. 2.3) where \( \Delta S \) is the total signal change in the equation.

2.9 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were collected on a 900 MHz NMR spectrometer. Two-dimensional total correlation spectra were collected using a heteronuclear single quantum coherence spectroscopy pulse sequence with an isotropic mixing time of 75 ms at 37°C. The spectrum width was 13.3 ppm at both dimensions with a complex data point of 4096 at the first dimension and 400 increments at the second dimension. Two dimensional \((^{15}\text{N},^{1}\text{H})\)-heteronuclear single quantum correlation (HSQC) NMR spectra were collected with 4096 complex data points at the \(^1\text{H}\) dimension and 128 increments at the \(^{15}\text{N}\) dimension. Samples contained the protein at 10 mM
Tris buffer, 10% D2O at pH 7.4. All of the NMR data was processed using FELIX (Accelrys) on a Silicon Graphics computer.

2.10 Cell culture and transfection

For measuring cytosolic free [Ca\(^{2+}\)], HEK293 cells were seeded on 22 × 40 mm coverslips in 60 mm culture dishes and cultured in 5% CO\(_2\) at 37°C in High Glucose Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) containing 10% fetal bovine serum with 100 μg/ml penicillin-streptomycin. For fluorimetric assays, the cells were grown on 13.5×20 mm coverslips that were placed in 6-well plates one day before the transfection. CaSR-pcDNA and its mutants were transfected using Lipofectamine 2000TM (Invitrogen) according to the manufacturer’s instructions. Cells were incubated for 48 h in high glucose DMEM after transfection.

2.11 Measurement of [Ca\(^{2+}\)]\(_i\) responses in single cells

Measurement of intracellular free Ca\(^{2+}\) was assessed as described by Huang, et al.(148). Briefly, wild type CaSR or its mutants were transiently transfected into HEK293 cells grown on coverslips and cultured for 48 h. The cells were subsequently loaded for 15 min using 4 μM Fura-2 AM in 2 mL physiological saline buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1.0 mM MgCl\(_2\), 1 mM CaCl\(_2\) and pH 7.4). The coverslips were mounted in 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 510 nm was recorded in real time as the concentration of extracellular Ca\(^{2+}\) was increased in a stepwise manner in the presence or absence of 5 mM L-Phe. The ratio of the emitted fluorescence intensities resulting from excitation at both wavelengths was utilized as a surrogate for changes in [Ca\(^{2+}\)]\(_i\) and was further plotted and analyzed as a function of [Ca\(^{2+}\)]\(_o\). All experiments were performed at room
temperature. The signals from 30 to 60 single cells were recorded for each measurement. Oscillations were identified as three successive fluctuations in $[\Ca^{2+}]_i$, after the initial peak.

2.12 Immunoassays

2.12.1 Western Blot

2.12.1.1 Detection of membrane protein

The transfected cells grown on 60 mm plates were washed with PBS and lysed with 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, and 1X protease inhibitor cocktail on ice for 30 min. Cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatants were denatured in SDS sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromphenol blue) at 55°C for 10 min under reducing conditions and subjected to 7.5% SDS-PAGE. The proteins resolved by SDS-PAGE were subsequently electrotransferred to a nitrocellulose membrane (Thermo Scientific). The membrane was blocked using 2% non-fat milk (Bio-Rad). The CaSR on the blot was detected by incubation with a 1:3000 dilution of monoclonal anti-CaR antibody (ADD, Abcam) followed by horseradish peroxidase-conjugated, goat anti-mouse secondary antibody. CaSR was visualized with an enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce Biotechnology).

2.12.1.2 ERK activity detection

Thirty-six hours post transfection of monolayers of HEK293 cells with CaSR or its mutants, cells were incubated in serum-free high glucose DMEM medium supplemented with 0.2% w/v BSA at 37°C overnight. On the following day, cells were first incubated with HBSS for 30 min, followed
by stimulation with varying levels of CaCl₂ (0-20 mM) with or without L-Phe (5 mM) for 10 min. At the end of the [Ca²⁺]₀ stimulation, cells were lysed with RIPA lysis buffer (Millipore, CA, USA). In total, 150 μg aliquots of lysate protein were loaded into either a 12.5% SDS-gel or a 4%-12.5% gradient gel for PAGE and analyzed by western blotting with an anti-phospho-p44/42 ERK polyclonal antibody (Cell signaling Technology, Beverly, MA, USA) diluted (1:2000). A chemiluminescent method (AP Conjugate Substrate Kit) was employed to detect the phospho-p44/42 proteins. Quantitative analysis of the results was performed using ImageJ software (National Institutes of Health). The responses were normalized to the maximal effect observed with [Ca²⁺]₀ alone. The EC₅₀ of [Ca²⁺]₀-dependent responses was calculated by fitting the [Ca²⁺]₀ concentration-response curves with 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.

2.12.2 Immunofluorescence staining

2.12.2.1 Immunostaining of membrane protein

CaSR-transfected HEK293 cells were grown on poly(L-lysine)-coated glass coverslips for immunofluorescence microscopy. After treatment with polyoxymethylene for 15 minutes, non-specific antigens on the cells were blocked by incubating with 2% BSA/PBS at 37°C for 1 hr. Anti-CaSR, mouse monoclonal antibody, ADD, was diluted 1:3000 in 1% BSA/PBS and incubated with the coverslips overnight at 4°C. Cells were then washed with PBS for 10 minutes at room temperature before incubating with a 1:3000 dilution of a FITC-labeled goat anti-mouse IgG antibody (Invitrogen) at 37°C for 1 hr. Coverslips were then washed with PBS followed by
incubation with 0.1% Triton X-100 for 15 minutes. Cells were further stained with propidium iodide (PI) (0.5 μg/mL) for 15 minutes at room temperature before they were mounted with anti-fade reagent (Invitrogen). Assessment of plasma membrane staining was carried out using a 63× objective (Zeiss 700).

2.12.2.2 ADIS experiment
HEK293 cells were transfected with WT CaSR and variant mutations. After 48 hours, cells were first incubated with rabbit anti-flag monoclonal antibody for 1 hour at 4 °C, then stimulated with various levels of CaCl2 (0-20 mM) for 10 min (37 °C). Cells were subsequently fixed with 3.7% formaldehyde for 15 mins at room temperature and labeled with mouse anti-Flag monoclonal antibody for 1 hour (room temperature). Cells were further permeabilized with 0.2% triton x-100 for 5 mins and stained with goat anti-rabbit Alexa488 and goat anti-mouse Alexa 568 conjugated secondary antibodies. Images were collected on Zeiss LSM700 confocal microscope (Carl Zeiss, Germany).

2.12.3 Immunoprecipitation
2.12.3.1 Biotin assay
HEK293 cells transfected with different CaSR mutants were incubated with 30 μg/ml disulfide-cleavable biotin for 30 min at 4°C. Cells were subsequently washed with TBS and incubated in Ca²⁺-free DMEM at 37°C for 15 min. Cells were washed three times with TBS and stimulated with buffers containing various [Ca²⁺]₀ for 10 minutes. Next, additional calcium was removed by washing with TBS and the cells were incubated with reducing buffer (containing 50 mM glutathione, 75 mM NaCl, 75 mM NaOH, and 1% FBS) for 20 min at 4°C. Iodoactaminde (50 mM with 1% BSA in PBS) was added to quench residual unreacted glutathione. Cells were washed three times with TBS and lysed in lysate buffer (10 mM Tris-HCl (pH 7.4), 150 mM
NaCl, 0.5% Nonidet P-40, 1% Triton-X100 and a 1×protease inhibitor cocktail (EDTA-Free; Roche)). CaSR was subsequently immunoprecipitated with anti-FLAG M2 antibody and protein A agarose. Samples were denatured in 2× sample loading buffer and subjected to SDS-PAGE. Biotinylated CaSR was visualized using Vectastain ABC immunoperoxidase reagent (Vector Laboratories, Burlingame, CA).

2.12.3.2 Pull-down assay

HEK293 cells were lysed 48 hours after transient transfection with the wild type CaSR or its truncated constructs. The lysis buffer contains 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% Triton-X100. The supernatant of the cell lysates (total about 0.4mg of protein) were collected and incubated with 5 μl of anti-flag monoclonal antibodies (Sigma) for half an hour at 4 ºC. About 30 μl of the protein A-agarose (Santa Cruz Biotechnology, Inc) was subsequently added, and the mixture was gently rotated for overnight at 4 ºC. After washing three times with the respective buffers, the agarose beads were finally resuspended in 45 μl 2X SDS sample buffer and heated at 42°C for 15 min and subjected to 7.5% SDS-PAGE. The standard western-blotting procedure was performed to detect the CaSR, filamin or CaM using anti-FLAG antibody (Sigma) and agarose A.

2.12.4 IP-1 ELISA assay

HEK293 cells were seeded in 24-well plates at 3 x 10^5 cells per well in 500 μl of culture medium. After transfection with WT CaSR, or its various mutants, cells were further cultured for 24 hours at 37°C. Cell monolayers were first washed with Ringer’s buffer without calcium (121 mM NaCl, 2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 10 mM HEPES, 5.5 mM glucose, 1.2 mM MgCl_2) and then
incubated for 1 hour at 37°C in stimulation buffer (140 mM NaCl, 5 mM KCl, 10 mM LiCl$_2$, 0.55 mM MgCl$_2$, 10 mM HEPES) containing varying concentrations of CaCl$_2$. After treatment, cells were lysed for 30 min at 37°C with 50 µl of 2.5% IP1 ELISA Kit Lysis Reagent (CIS Bio International, Gif-sur-Yvette, France). The accumulation of IP1 was measured using an immunoassay based on competition between free IP1 and horseradish peroxidase (HRP) conjugated IP1 for binding to monoclonal anti-IP1 antibody. The results for IP1 were expressed as percentage inhibition of IP1-HRP binding = $\left[1 - \text{IP1-HRP binding in stimulated cells}/\text{IP1-HRP binding in unstimulated cells}\right]$ x 100.

![Figure 2.1 Schematic representation of IP1 competition assay.](image)

**Figure 2.1 Schematic representation of IP1 competition assay.**

IP-One ELISA is a competitive immunoassay which uses IP1-HRP and an anti-IP1 monoclonal antibody. The kit comes with 96-well microplates pre-coated with an anti-mouse antibody. Cells are stimulated in the presence of LiCl, causing the accumulation of IP1 upon receptor activation. The protocol consists of two steps following cell stimulation:
addition of ELISA components and addition of TMB, the HRP substrate. The HRP reaction is stopped and the optical density (OD) read at 450nm (http://www.htrf.com/usa/ip-one-elisa-assay).

2.13 Lectin Agarose Binding Assay

The ECD containing fractions after His-tag purification were mixed with 30 µL of RCA-1 lectin agarose (Vector Lab, Burlingame, CA) for overnight incubation at 4°C followed by a washing step with PBS. The ECD protein was eluted using 0.2 M galactose. The elution fractions were analyzed by Western blot, using an anti-flag antibody.

2.14 Deglycosylation reactions

Triton X-100 was added to heat- and SDS-denatured, purified ECD protein (20 µg) at a final concentration of 2% before the protein was subjected to the PNGaseF deglycosylation procedure. Around 1.4 units of PNGase F were added per microgram of purified protein and incubated for 2 h at 37°C. The samples subjected to deglycosylation were further analyzed by Western blot using an anti–flag antibody. For Endo F1 enzyme treatment, purified Lec1-hCaSR-ECD protein was directly taken from FPLC fraction and incubated with Endo F1 at mass ratio 1:3 in 10 mM Tris buffer, pH 7.4 for overnight incubation at 4 °C.
CHAPTER 3. IDENTIFICATION OF AN L PHENYLALANINE BINDING SITE 
ENHANCING THE COOPERATIVE RESPONSES OF THE CALCIUM SENSING 
RECEPTOR TO CALCIUM 

3.1 Introduction 

It has long been recognized that Ca\(^{2+}\) acts as a second messenger that is released from intracellular stores and/or taken up from the extracellular environment in response to external stimuli to regulate diverse cellular processes. The discovery of the parathyroid Ca\(^{2+}\)-sensing receptor (CaSR) by Brown et al. has established a new paradigm of Ca\(^{2+}\) signaling (38). In addition to its known role as a second messenger, extracellular Ca\(^{2+}\) can function as a first messenger by CaSR-mediated triggering of multiple intracellular signaling pathways, including activation of phospholipases C, A\(_2\), and D, and various mitogen-activated protein kinases (MAPKs), as well as inhibition of cAMP production (121,202-206). This receptor is present in the key tissues involved in [Ca\(^{2+}\)]\(_o\) homeostasis (e.g., parathyroid, kidney, bone) and diverse other non-homeostatic tissues (e.g., brain, skin, etc.) (41,49,101,207). CaSR consists of a large N-terminal extracellular domain (ECD) (~600 residues) folded into a Venus Fly Trap (VFT) motif, followed by a 7-pass transmembrane region (7TM) and a cytosolic C-terminus. The ECD has been shown to play an important role in the cooperative response of the CaSR to [Ca\(^{2+}\)]\(_o\). Elevations in [Ca\(^{2+}\)]\(_o\) activate the CaSR, evoking increases in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), producing [Ca\(^{2+}\)]\(_i\) oscillations, modulating the rate of parathyroid hormone (PTH) secretion, and regulating gene expression (24,119,121,126). The pattern of [Ca\(^{2+}\)]\(_i\) oscillations is one of the most important signatures reflecting the state of CaSR activity.
More than two hundred naturally-occurring mutations have been identified in the CaSR that either inactivate the receptor (reducing sensitivity to \([Ca^{2+}]_o\)), leading to familial hypocalciuric hypercalcemia (FHH) or neonatal severe hyperparathyroidism (NSHPT), or activate it (increasing sensitivity to \([Ca^{2+}]_o\)), thereby causing autosomal dominant hypoparathyroidism (ADH) (208-210). Several of these naturally-occurring mutations of CaSR exhibit altered functional cooperativity (208).

Functional cooperativity of CaSR (i.e., based on biological activity determined using functional assays rather than a direct binding assay), particularly the positive homotropic cooperative response to \([Ca^{2+}]_o\), is essential for the receptor’s ability to respond over a narrow physiological range of \([Ca^{2+}]_o\) (1.1-1.3 mM). CaSR has an estimated Hill coefficient of 3-4 for its regulation of processes such as activating intracellular Ca\(^{2+}\) signaling and inhibiting PTH release. Under physiological conditions, L-amino acids, especially aromatic amino acids (e.g., L-Phe), as well as short aliphatic and small polar amino acids (211), are able to potentiate the high \([Ca^{2+}]_o\)-elicited activation of the CaSR by decreasing the EC\(_{50}\) values required for \([Ca^{2+}]_o\)-evoked \([Ca^{2+}]_i\) responses and its functional cooperativity (118,212). The levels of amino acids in human serum are close to those activating the CaSR in vitro (118,213) and can further enhance functional cooperativity via positive heterotropic cooperativity. Recently, several groups have reported that the CaSR in cells within the lumen of the gastrointestinal (GI) tract is activated by L-Phe and other amino acids, which have long been recognized as activators of key digestive processes. Hence, the CaSR enables the GI tract to monitor events relevant to both mineral ion and protein/amino acid metabolism in addition to the CaSR’s sensing capability in blood and other extracellular fluids (118,166,214). Glutathione and its \(\gamma\)-glutamylpeptides also allosterically
modulate the CaSR at a site similar to the L-amino acid-binding pocket but with over 1,000-fold higher potencies (212,215). Thus, CaSR is essential for monitoring and integrating information from both mineral ions/nutrients/polyamines in blood and related extracellular fluids. Nevertheless, we still lack a thorough understanding of the molecular mechanisms by which CaSR is activated by \([\text{Ca}^{2+}]_o\) and amino acids, which, in turn, regulate CaSR functional cooperativity. In addition, in a clinical setting, the molecular basis for the alterations in this cooperativity caused by disease-associated mutations is largely unknown due to the lack of knowledge of this receptor’s structure and its weak binding affinities for \([\text{Ca}^{2+}]_o\) and amino acids (68,126,172,208).

In the present study, we use two complementary approaches--monitoring \([\text{Ca}^{2+}]_i\) oscillations in living cells and molecular dynamic (MD) simulations--to provide important insights into how the CaSR functions and the behavior of the receptor at the atomic level. We first demonstrate that the molecular connectivity between \([\text{Ca}^{2+}]_o\)-binding sites that is encoded within the key \(\text{Ca}^{2+}\)-binding Site 1 in the hinge region of the CaSR’s ECD is responsible for the functional positive homotropic cooperativity in the CaSR’s response to \([\text{Ca}^{2+}]_o\). We further identify an L-Phe-binding pocket adjacent to \(\text{Ca}^{2+}\)-binding Site 1. We show that this L-Phe-binding pocket is essential for functional positive heterotropic cooperativity by virtue of its having a marked impact on all five of the predicted \(\text{Ca}^{2+}\)-binding sites in the ECD with regard to \([\text{Ca}^{2+}]_o\)-evoked \([\text{Ca}^{2+}]_i\) signaling. Furthermore, with molecular dynamics (MD) simulations we show that the motions of \(\text{Ca}^{2+}\)-binding Site 1 are correlated with those of the other predicted \(\text{Ca}^{2+}\)-binding sites. Finally, the dynamic communication of L-Phe at its predicted binding site in
the hinge region with the CaSR’s Ca^{2+}-binding sites globally enhances cooperative activation of
the receptor in response to alterations in [Ca^{2+}]_o.

3.2 Results

3.2.1 Molecular connectivity among predicted calcium-binding sites is required for functional cooperativity of CaSR

It has been documented that in several regions of the CaSR and mGluRs, the amino acid residues are highly conserved (216). Those conserved elements provide a structural framework for the modeling of the CaSR ECD. Among all the available crystal structures of the mGluRs, studies on mGluR1 give concrete structural information about ligand-free as well as various ligand-bound forms of the receptor. Moreover, CaSR and mGluR1 share similar signaling pathways and can form heterodimers either in vivo or in vitro (61). Thus, the crystal structures of mGluR1 were employed for modeling the CaSR ECD. By using our own computational algorithms, we previously identified five putative Ca^{2+}-binding sites in the modeled CaSR ECD (Figure 3.1) (68,169,172). Among those, Site 1 is located in the hinge region of the two subdomains in the VFT motif. Among 34 newly found naturally-occurring missense mutations within the ECD, 18 are located within 10 Å of one or more of the predicted Ca^{2+}-binding sites (208). Interestingly, a few disease-associated human mutations severely alter the functional cooperativity of CaSR (161).

Functional positive homotropic cooperativity here refers to [Ca^{2+}]_o-induced changes in CaSR activity that can be ascribed to interactions between the five predicted Ca^{2+}-binding sites, which are located in different regions of the ECD (217-219). To understand the observed
cooperativity and the origin of changes in cooperativity caused by disease-associated mutations at the atomic level, we have carried out MD simulations on the modeled CaSR ECD to predict correlated motions. MD simulation provides an approach complementary to experiments in live cells for understanding biomolecular structure, dynamics, and function (220). We calculated the cross-correlation coefficients of each residue with all of the other residues of the CaSR ECD from the simulations. Figure 3.1 (lower-right panel) shows the normalized correlation matrix map of both negative (blue) and positive (red) correlated motions between each pair of residues that indicate movements in the opposite direction or in the same direction, respectively. Positive correlations occur between groups of residues if they are within the same domain or directly interact with each other. Figure 3.1 shows strong correlations amongst residues from S169 to A324. Notably, the negative correlation motions between residues K47-L125 and residues S240-A300 suggest that the two lobes undergo a dynamic change similar to that of mGluR1 upon interactions with its ligands. Closer analysis indicates that residues involved in Ca\(^{2+}\)-binding Site 1 exhibit negative correlations with residues in Sites 2, 3, 4 and 5 (Table 3.1).
Figure 3.1 Delineating the molecular connectivity associated with functional positive homotropic and positive heterotropic cooperativity of the CaSR’s ECD by molecular modeling of L-Phe- and Ca\(^{2+}\)-binding sites.

The hinge region is defined as locations near calcium binding site 1. Upper Left corner: The location of the predicted calcium binding site 1 and the potential residues involved in the L-Phe interaction. Residues in cyan: residues which are both involved in calcium and L-Phe interaction. Residues in purple: residues are predicted to interact with L-Phe
Table 3.1 Analysis of correlated motions of WT CaSR model structure.

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<tr>
<th>Residue Pairs</th>
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<tr>
<td></td>
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<tr>
<td><strong>Negative Correlation</strong></td>
</tr>
<tr>
<td>S170 (Site 1): D398 (Site 5)</td>
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<tr>
<td>D190 (Site 1): S244 (Site 2)</td>
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<tr>
<td>D190 (Site 1): D248 (Site 2)</td>
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<td>E297 (Site 1): D248 (Site 2)</td>
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<tr>
<td>D190 (Site 1): Q253 (Site 2)</td>
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<tr>
<td>E218 (Site 1): E350 (Site 4)</td>
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<tr>
<td>E297 (Site 1): E378 (Site 5)</td>
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<tr>
<td><strong>Positive Correlation</strong></td>
</tr>
<tr>
<td>E224 (Site 3): E228 (Site 3)</td>
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<tr>
<td>E228 (Site 3): E229 (Site 3)</td>
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<tr>
<td>S244 (Site 2): D248 (Site 2)</td>
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<td>S244 (Site 2): Q253 (Site 2)</td>
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<td>E350 (Site 4): E353 (Site 4)</td>
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<td>E354 (Site 4): E353 (Site 4)</td>
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<tr>
<td>E378 (Site 5): E379 (Site 5)</td>
</tr>
<tr>
<td>D398 (Site 5): E399 (Site 5)</td>
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The cross-correlation coefficients of each residue to all of the other residues of the modeled CaSR ECD structure after docking with calcium were calculated from the simulation. The strongest positive correlation of a residue with itself is given the value 1; the strongest negative correlation between two residues is given the value -1. A cut off at > 0.7 and < -0.4 is considered as a strong correlation between residues. Strongly correlated residues in predicted calcium-binding sites are listed in the table.

We then predicted a putative amino acid-binding site in the modeled CaSR based on its sequence homology to mGluR1 and its ligand-loaded form using AutoDock-Vina (174). As
shown in Figure 3.1 (upper-left panel), this potential amino acid-binding pocket, formed by residues K47, L51, W70, T145, G146, S169, S170, I187, Y218, S272, H413 and R415, partially overlaps Ca$^{2+}$-binding Site 1 in the modeled CaSR ECD. Its predicted location is consistent with previous functional studies, suggesting important roles for S170 and T145 in amino acid-potentiated intracellular calcium responses (221-223). This L-Phe-binding site is also located within the hinge region of the ECD of CaSR with a relatively localized configuration. Other Ca$^{2+}$-binding Sites (Sites 2-5) are more than 10 Å away from the L-Phe-binding site.(172). This partial colocalization of the predicted Ca$^{2+}$- and amino acid-binding sites at the hinge domain in the ECD of the CaSR is also observed in other members of the family C GPCRs, including mGluRs and taste receptors, which share some degree of sequence similarity Figure 3.2a (38,96,101,204,209,224,225). The calculated binding free energy of CaSR for various ligands (in the order of glutathione>L-Phe>L-Asp) is in excellent agreement with previous reported experimental results obtained by determining the EC$_{50}$ of intracellular Ca$^{2+}$ responses to the same ligands Figure 3.2b (118).
Figure 3.2 Sequence alignment and binding energy calculations based on the modeled structure of CaSR ECD.

(a) Sequence alignment of the orthosteric binding site for Glu in mGluR1 with CaSR and ten other GPCRs of family C. Residues involved in the predicted CaSR Ca\textsuperscript{2+}-binding Site 1 are labeled at the top and corresponding residues in other group members are highlighted in yellow. (b) The binding energies were calculated after molecular dynamics simulations. Red line: CaSR-ECD docking with glutathione (GLUT); Black line: CaSR-ECD docking with phenylalanine (PHE); Blue line: CaSR-ECD docking with aspartic acid (ASP).
We define functional positive heterotropic cooperativity as that which occurs when the functional positive cooperative effect of interaction with one ligand (e.g., Ca\(^{2+}\)) affects the functional response resulting from interaction of a different ligand with the protein (e.g., an aromatic amino acid) (226). This term can be applied in the case of CaSR when it simultaneously senses Ca\(^{2+}\) and L-Phe. We have also observed greater correlated motions among the multiple Ca\(^{2+}\)-binding sites after docking both Ca\(^{2+}\) and L-Phe compared with docking of L-Phe alone to the ECD domain of the CaSR (Figure 3.3). Taking these results together, we propose that there is molecular connectivity centered at predicted calcium-binding Site 1 that plays an essential role in regulating the correlated motions among the multiple Ca\(^{2+}\)-binding sites. Further communication of this site with the amino acid-binding site is likely to mediate functional heterotropic cooperativity of CaSR-mediated signaling, as shown later.

![Figure 3.3](image_url)

**Figure 3.3** The correlation map of the modeled CaSR ECD structure with the L-Phe-loaded form, calcium-loaded form and the form loaded with both calcium and L-Phe.

The cross correlation matrices show the movements of residues during MD simulation. Positive values (in red) show residues moving in the same direction while negative values (in blue) indicate residues moving away from one another.
3.2.2 Functional positive homotropic cooperativity among Ca\textsuperscript{2+}-binding sites.

Given that it is not readily feasible to perform radioligand binding assays on CaSR due to its low affinities for its ligands, especially Ca\textsuperscript{2+} (e.g., mM K\textsubscript{d}) as well as difficulty in purification of CaSR, we monitored intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) responses using both a cuvette population assay and by monitoring [Ca\textsuperscript{2+}]\textsubscript{i} oscillations using single cell imaging to determine functional cooperativity of CaSR. HEK293 cells transfected with WT CaSR exhibited sigmoidal concentration response curves for the [Ca\textsuperscript{2+}]\textsubscript{i} responses (as monitored by changes in the ratio of fluorescence at 510 nm when excited at 340 or 380 nm) elicited by changes in [Ca\textsuperscript{2+}]\textsubscript{o} with a Hill coefficient of 3.0 ± 0.1 and a EC\textsubscript{50} of 2.9 ± 0.2 mM, suggesting strong positive homotropic cooperativity within its five predicted Ca\textsuperscript{2+}-binding sites. The sensitivity to agonist was assessed using the [Ca\textsuperscript{2+}]\textsubscript{o} at which cells began to show [Ca\textsuperscript{2+}]\textsubscript{i} oscillations and the frequency of the oscillations at the respective levels of [Ca\textsuperscript{2+}]\textsubscript{o} at which more than 50% of the cells started to oscillate.

To seek the key determinants underlying the observed functional positive homotropic cooperativity, mutations were introduced into the various predicted Ca\textsuperscript{2+}-binding sites of the CaSR by site-directed mutagenesis, which resulted in impaired Ca\textsuperscript{2+}-sensing with altered oscillation patterns in single cell studies and higher EC\textsubscript{50} values compared with WT CaSR in population studies (Figure 3.4, 3.5) (Table 3.2 & 3.3). Such population studies were also reported in our previous studies (68,172). Results from western blot and immunocytochemistry utilizing immunofluorescence to detect the CaSR indicated essentially equivalent expression of WT CaSR and its variants on the cell surface (Figure 3.6). As shown in Figure 3.4a, the level of [Ca\textsuperscript{2+}]\textsubscript{o} required to initiate oscillations in mutant E297I at predicted Ca\textsuperscript{2+}-binding Site 1 or D215I at Site 2 increased markedly from 3.0 ± 0.1 mM to 17.0 ± 0.4 mM and 13.9 ± 0.2 mM, respectively,
Correlating well with these results, the two mutants had significantly impaired responses to $[\text{Ca}^{2+}]_o$ in the population assay with increased EC$_{50}$ values (Table 3.3). The Hill coefficients in Table 3.3 & Figure 3.4b indicate that the cooperativity among the various Ca$^{2+}$-binding sites was impaired by mutating each of them separately. Strikingly, removal of Ca$^{2+}$-binding ligand residues, such as E297I and Y218Q at Site 1, converted the single process for functional activation of the WT CaSR by $[\text{Ca}^{2+}]_o$ to biphasic functional processes, suggesting that the underlying cooperative binding mechanism had been substantially perturbed Figure 3.4b&Figure 3.7d.
Figure 3.4 Intracellular $\text{Ca}^{2+}$ responses of CaSR mutants involving various $\text{Ca}^{2+}$-binding sites following simulation with increases in $[\text{Ca}^{2+}]_o$.

a. Statistical analysis of the starting points for $[\text{Ca}^{2+}]_i$ oscillation in HEK-293 cells transfected with WT, E297I or D215I, respectively. The $[\text{Ca}^{2+}]_o$ was recorded at the level of $[\text{Ca}^{2+}]_i$ at which single cells began to oscillate. Around 30–60 cells were analyzed and further plotted as a bar chart. b. Population assays of WT and $\text{Ca}^{2+}$-binding site-related
mutations. HEK293 cells transfected with CaSR or its mutants were loaded with Fura-2 AM. The intracellular \( \text{Ca}^{2+} \) level was assessed by monitoring emission at 510 nm with excitation alternately at 340 or 380 nm as described previously (148). The \([\text{Ca}^{2+}]_i\) changes in the transfected cells were monitored using fluorimetry during stepwise increases in \([\text{Ca}^{2+}]_o\). The \([\text{Ca}^{2+}]_i\) responses at various levels of \([\text{Ca}^{2+}]_o\) were plotted and further fitted using the Hill equation.
Figure 3.5 Individual cellular responses of mutants in calcium-binding sites to the indicated increments of [Ca^{2+}]_o in the presence or absence of L-Phe.

(a) Representative intracellular calcium response from a single cell. Fura-2 loaded HEK293 cells expressing CaSRs with mutations in calcium-binding sites were prepared for single cell experiments. In each experiment, with or without 5 mM L-Phe in non-calcium-containing Ringer’s buffer, stepwise increases in [Ca^{2+}]_o in non-calcium containing Ringer’s buffer, in the presence or absence of L-Phe, were carried out until [Ca^{2+}]_i reached a plateau (up to 30 mM). At least 30 cells were analyzed for each mutant. (b) Statistical analysis of the [Ca^{2+}]_o at which CaSR-transfected single HEK293 cells started to oscillate. Empty bar: in the absence of L-Phe; Black bar: in the presence of 5 mM L-Phe. (c) The frequency distribution of the oscillation patterns in single cells was investigated as described before. For experiments without L-Phe, the peaks per minute were recorded at the level of [Ca^{2+}]_o at which the majority of cells (>50%) started to oscillate; for experiments with 5.0 mM L-Phe, the frequency was analyzed at the same [Ca^{2+}]_o that was used in the absence of L-Phe. Specifically, the frequency of oscillations observed with mutant E353I was analyzed at 5.0 mM [Ca^{2+}]_o; for D398A/E399I it was investigated at 10.0 mM [Ca^{2+}]_o and E224I it was studied at 4 mM [Ca^{2+}]_o. Empty bar: in the absence of L-Phe; black bar: in the presence of 5 mM L-Phe.
Table 3.2 Summary of individual cellular responses to the indicated increments of \([\text{Ca}^{2+}]_o\) in HEK293 cells transiently transfected with WT CaSR or mutations in the indicated \(\text{Ca}^{2+}\)-binding sites.

<table>
<thead>
<tr>
<th>Predicted Sites</th>
<th>Residues</th>
<th>Mutants</th>
<th>Starting Point (mM)</th>
<th>Ending Point (mM)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>w/o L-Phe w L-Phe</td>
<td>w/o L-Phe w L-Phe</td>
<td>w/o L-Phe w L-Phe</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>WT</td>
<td>3.0 ± 0.1 2.0 ± 0.2 (^b)</td>
<td>6.4 ± 0.3 4.3 ± 0.2</td>
<td>1.5 ± 0.1 2.2 ± 0.2 (^b)</td>
</tr>
<tr>
<td>Site 1</td>
<td>S147, S170, D190, Y218, E297</td>
<td>E297I</td>
<td>17.0 ± 0.4 (^a) 7.3 ± 0.2 (^b)</td>
<td>N/A N/A</td>
<td>1.6 ± 0.1 2.9 ± 0.1 (^b)</td>
</tr>
<tr>
<td>Site 2</td>
<td>D215, L242, S244, D248, Q253</td>
<td>D215I</td>
<td>13.9 ± 0.2 (^a) 6.7 ± 0.3 (^b)</td>
<td>N/A 17.7 ± 0.3</td>
<td>1.8 ± 0.1 2.5 ± 0.2 (^b)</td>
</tr>
<tr>
<td>Site 3</td>
<td>E224, E228, E229, E231, E232</td>
<td>E224I</td>
<td>5.0 ± 0.2 (^a) 3.2 ± 0.1 (^b)</td>
<td>16.8 ± 0.3 11.0 ± 0.1 (^b)</td>
<td>1.4 ± 0.2 2.2 ± 0.1 (^b)</td>
</tr>
<tr>
<td>Site 4</td>
<td>E350, E353, E354, N386, S388</td>
<td>E353I</td>
<td>3.4 ± 0.1 (^a) 2.4 ± 0.1 (^b)</td>
<td>10.8 ± 0.2 4.9 ± 0.2 (^b)</td>
<td>1.2 ± 0.2 2.0 ± 0.2 (^b)</td>
</tr>
<tr>
<td>Site 5</td>
<td>E378, E379, T396, D398, E399</td>
<td>D398A/E399I</td>
<td>9.3 ± 0.1 (^a) 5.5 ± 0.2 (^b)</td>
<td>N/A N/A</td>
<td>1.4 ± 0.2 2.2 ± 0.2 (^b)</td>
</tr>
</tbody>
</table>

The average \([\text{Ca}^{2+}]_o\) was recorded at which \([\text{Ca}^{2+}]_i\) oscillations started or terminated. Values are means ± S.E. N/A= not available. \(^a\) indicates significance with respect to wild type CaSR without L-Phe, \(p < 0.05\); \(^b\) indicates significance with respect to the corresponding experiment in the same mutant without L-Phe, \(p < 0.05\).
Table 3.3 Summary of EC$_{50}$ values and Hill coefficients predicted using Hill equation for the WT and mutant CaSRs.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Mutants</th>
<th>Response at 30 mM [Ca$^{2+}$]</th>
<th>EC$_{50}$[Ca$^{2+}$]$_o$</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>w/o L-Phe</td>
<td>w  L-Phe</td>
<td>w/o L-Phe</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>100.0 ± 2.0</td>
<td>104.6 ± 2.9</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Site 1</td>
<td>E297I</td>
<td>78.0 ± 6.9$^a$</td>
<td>132.8 ± 1.9$^b$</td>
<td>Phase 1: 3.2 ± 0.4</td>
</tr>
<tr>
<td>Site 2</td>
<td>D215I</td>
<td>88.0 ± 2.5$^a$</td>
<td>147.5 ± 6.0$^b$</td>
<td>14.7 ± 1.9$^a$</td>
</tr>
<tr>
<td>Site 3</td>
<td>E224I</td>
<td>83.2 ± 5.2$^a$</td>
<td>104.2 ± 4.5$^b$</td>
<td>5.3 ± 0.4$^a$</td>
</tr>
<tr>
<td>Site 4</td>
<td>E353I</td>
<td>80.7 ± 1.6$^a$</td>
<td>88.9 ± 1.8$^b$</td>
<td>4.0 ± 0.1$^a$</td>
</tr>
<tr>
<td>Site 5</td>
<td>D398A/E399I</td>
<td>80.9 ± 7.0$^a$</td>
<td>78.3 ± 6.5</td>
<td>4.7 ± 0.7$^a$</td>
</tr>
</tbody>
</table>

HEK293 cells were transiently transfected with the WT CaSR or CaSRs with mutations in Ca$^{2+}$-binding sites, and after 48 h the cells were loaded with fura-2 as described under “Materials and Methods”. The cells on glass coverslips were then transferred into the cuvette for measurement of [Ca$^{2+}$]$_i$ by fluorimetry and exposed to various increases in [Ca$^{2+}$]$_o$ in the absence or presence of 5 mM L-Phe. The data were obtained from three experiments for each construct. The maximum response for each mutant was subtracted from the baseline and normalized to the maximal cumulative [Ca$^{2+}$]$_i$ response of the WT receptor. Curve-fitting was performed using the Hill equation.$^a$ indicates significance with respect to wild type CaSR in the absence of L-Phe, p < 0.05; $^b$ indicates significance with respect to the corresponding mutants in the absence of L-Phe, p < 0.05.
Figure 3.6 Expression of WT CaSR and its mutants in HEK293 cells.

(a) Western blot analyses of CaSR and its mutants in transiently transfected HEK293 cells. 40 µg of total protein from cellular lysates were subjected to 8.5% SDS-PAGE. Three characteristic bands are shown in the upper panel, including the top band representing the dimeric receptor, the middle band showing mature glycosylated CaSR monomer (150 kDa), and the lowest band indicating immature glycosylated CaSR monomer (130 kDa). The non-glycosylated CaSR monomer form is about 110 kDa. (b) Quantification of the expression of WT CaSR and its mutants in HEK293 cells. All the bands are shown, including the one indicating dimeric receptor, mature glycosylated monomer and the one showing immature CaSR monomer. The internal control GAPDH was used to standardize CaSR expression and the mutants were further normalized to the WT CaSR expression. (c)
Immunofluorescence analyses of surface expressed WT CaSR and its mutants in HEK293 cells. Immunostaining was done with anti-CaSR monoclonal antibody ADD (227), and detection was carried out with Alex Fluor 488-conjugated, goat anti-mouse secondary antibody. Red, PI staining of cell nuclei; Green, CaSR. Equivalent expression of WT CaSR as well as its variants on the cell surface suggests that the difference in the Ca$^{2+}$-sensing capabilities among the WT and mutant receptors are due to perturbation of the cell surface receptors’ functions, rather than, for example, impaired trafficking of the receptor proteins to the cell surface.

3.2.3 Functional positive heterotropic cooperativity contributed by the identified L-Phe-binding site.

Figure 3.7a shows the effect of 5 mM L-Phe on the [Ca$^{2+}$]$_i$ responses at different levels of [Ca$^{2+}$]$_o$ in HEK293 cells transiently transfected with the WT CaSR or its variants with mutations around the predicted L-Phe-sensing site. L-Phe lowered the threshold for [Ca$^{2+}$]$_o$–induced oscillations in the WT CaSR from 3.0 ± 0.1 mM to 2.0 ± 0.2 mM, about a 1.5-fold shift (Figure 3.7b, Table 3.2). Concurrently, L-Phe also increased the oscillation frequency from 1.5 ± 0.1 to 2.2 ± 0.2 peaks/min (p<0.05) in the presence of 3.0 mM [Ca$^{2+}$]$_o$ in the single cell assay (Figure 3.7b). Meanwhile, L-Phe produced functional positive heterotropic cooperativity of the receptor, as it facilitated the response of the WT CaSR to [Ca$^{2+}$]$_o$ by significantly decreasing the EC$_{50}$ from 2.9 ± 0.2 mM to 1.9 ± 0.2 mM (p<0.05) and increasing the Hill coefficient from 3.0 to 4.0 in the cell population assay (Figure 3.7b, Table 3.3).
We then performed detailed analyses to understand the role of residues in the modeled L-Phe-binding site in the functional positive heterotropic cooperativity contributed by L-Phe (Figure 3.7, Table 3.4). Five out of 12 residues located within 5 Å of the modeled L-Phe-binding site exhibited impaired L-Phe-sensing ability. Mutants L51A and S170T exhibited impaired L-Phe-sensing capability as indicated by the absence of any change in the starting point (Figure 3.7b) as well as constant oscillatory frequencies (~1.7 and 1.4 peaks/min) in the presence of L-Phe (Figure 3.7c), while they maintained relatively unaltered calcium-sensing functions. Consistent with the single cell assay results, cell population studies revealed that the EC$_{50}$ values of L51A, S170T and Y218Q remained the same with or without L-Phe (Figure 3.7d). The effect of S170T on the sensing of L-Phe has previously been reported by Zhang et. al. in a cell population assay (221). Addition of 5 mM L-Phe lowered the [Ca$^{2+}$]$_o$ required to initiate oscillations in cells transfected with mutations S272A or T145A but failed to increase the oscillatory frequency at 2.5 mM [Ca$^{2+}$]$_o$ (the level at which the majority of the cells began to oscillate), nor did it reduce the EC$_{50}$ (Table 3.4, Figure 3.8). Y218 is predicted to be involved in binding of both L-Phe in its binding pocket and of Ca$^{2+}$ in Site 1. Indeed, the mutation Y218Q largely disrupted the functional positive homotropic cooperativity with transformation of the single cooperative response to [Ca$^{2+}$]$_o$ of the WT CaSR to a biphasic process in the cell population assay. Y218Q also exhibited less sensitivity to [Ca$^{2+}$]$_o$, as [Ca$^{2+}$]$_i$ oscillations did not start until [Ca$^{2+}$]$_o$ was increased to more than 10 mM, reflecting its role in this Ca$^{2+}$-binding site. Of note, however, addition of 5 mM L-Phe failed to restore the calcium sensitivity of this mutant as manifested by an unchanged oscillation pattern. An oscillatory frequency of ~1.5 peaks/min was observed at 20 mM [Ca$^{2+}$]$_o$ both with and without L-Phe for this mutant. In contrast, mutations such as K47A, Y63I, W70L, G146A, I162A, S169A, I187A, H413L, and R415A did
not abrogate the positive allosteric effect of 5 mM L-Phe (Table 3.5). Taken together, these results suggest that residues located at the predicted L-Phe-binding site, including L51, T145, S170, S272, and Y218, play key roles in sensing L-Phe.

Figure 3.7 Functional studies of receptors with mutations in L-Phe-binding site in HEK293 cells.
(a). Representative oscillation pattern from a single cell. Each experiment with or without 5 mM L-Phe began in Ca^{2+}-free Ringer’s buffer followed by stepwise increases in [Ca^{2+}]_o until [Ca^{2+}]_i reached a plateau (up to 30 mM [Ca^{2+}]_o). (b). The pattern of [Ca^{2+}]_i responses in each cell (minimum of 30 cells) was analyzed, and the [Ca^{2+}]_o at which individual cells started to oscillate was recorded and plotted as a bar chart. (c). The frequency of the oscillation patterns of the individual cells was investigated. For experiments without L-Phe, the peaks per minute were recorded at the level of [Ca^{2+}]_o at which the majority of the cells (>50%) started oscillating; while for experiments with 5 mM L-Phe, the frequency was analyzed at the same levels of [Ca^{2+}]_o as in the corresponding experiments carried out without L-Phe. (d). Population assay for [Ca^{2+}]_i responses of HEK293 cells transiently overexpressing WT CaSR or CaSR mutants L51A or Y218Q using Fura-2 AM during stepwise increases in [Ca^{2+}]_o from 0.5 to 30 mM. The ratio of the intensity of light emitted at 510 nm upon excitation with 340 or 380 nm was normalized to its maximum response. The [Ca^{2+}]_o concentration response curves were fitted using the Hill equation.
Table 3.4 Summary of cellular responses of HEK293 cells transiently transfected with WT CaSR or mutants in the predicted L-Phe-binding site.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Starting Point (mM) w/o L-Phe</th>
<th>Frequency (Peaks/Min) w/o L-Phe</th>
<th>Frequency (Peaks/Min) w L-Phe</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mM) w/o L-Phe</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mM) w L-Phe</th>
<th>Hill Number w/o L-Phe</th>
<th>Hill Number w L-Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.0 ± 0.1 a</td>
<td>1.5 ± 0.1 a</td>
<td>2.2 ± 0.2 b</td>
<td>2.9 ± 0.1 a</td>
<td>1.8 ± 0.2 b</td>
<td>3.2 ± 0.3 a</td>
<td>4.1 ± 0.1 b</td>
</tr>
<tr>
<td>L51A</td>
<td>2.9 ± 0.1 a</td>
<td>2.8 ± 0.2 b</td>
<td>1.5 ± 0.1 a</td>
<td>3.5 ± 0.2 a</td>
<td>3.2 ± 0.1 a</td>
<td>2.4 ± 0.1 a</td>
<td>2.8 ± 0.2 a</td>
</tr>
<tr>
<td>T145A</td>
<td>2.8 ± 0.1 a</td>
<td>2.4 ± 0.2 b</td>
<td>1.7 ± 0.3 b</td>
<td>3.0 ± 0.1 a</td>
<td>3.0 ± 0.1 a</td>
<td>3.3 ± 0.3 b</td>
<td>3.7 ± 0.4 b</td>
</tr>
<tr>
<td>S170T</td>
<td>2.1 ± 0.2 a</td>
<td>1.5 ± 0.1 a</td>
<td>1.4 ± 0.2 a</td>
<td>2.7 ± 0.2 a</td>
<td>2.9 ± 0.1 a</td>
<td>2.4 ± 0.3 a</td>
<td>2.2 ± 0.1 a</td>
</tr>
<tr>
<td>Y218Q</td>
<td>17.7 ± 1.0 a</td>
<td>16.7 ± 2.2 a</td>
<td>1.5 ± 0.1 a</td>
<td>3.7 ± 0.4 a</td>
<td>3.6 ± 0.2 a</td>
<td>3.8 ± 1.0 a</td>
<td>3.9 ± 0.4 a</td>
</tr>
<tr>
<td>S272A</td>
<td>2.7 ± 0.2 a</td>
<td>2.1 ± 0.1 b</td>
<td>2.3 ± 0.1 b</td>
<td>2.4 ± 0.1 a</td>
<td>2.2 ± 0.1 a</td>
<td>1.9 ± 0.1 a</td>
<td>3.0 ± 0.1 a</td>
</tr>
</tbody>
</table>

The average [Ca<sup>2+</sup>]<sub>o</sub> at which cells started to exhibit [Ca<sup>2+</sup>]<sub>i</sub> oscillations was recorded for WT or each mutant CaSR. For the oscillation frequency in the absence of L-Phe, peaks per minute were measured at the level of [Ca<sup>2+</sup>]<sub>o</sub> at which more than 50% cells started to oscillate; when L-Phe was added, frequencies were recorded at the same [Ca<sup>2+</sup>]<sub>o</sub> as their counterparts without L-Phe. Specifically, the frequencies of WT and L51A was measured at 3.0 mM [Ca<sup>2+</sup>]<sub>o</sub> S272A, T145A, and S170T were measured at 2.5 mM [Ca<sup>2+</sup>]<sub>o</sub>; and Y218Q was analyzed at 15.0 mM [Ca<sup>2+</sup>]<sub>o</sub>. Values are means ± S.E. EC<sub>50</sub> and Hill numbers obtained from the cell population assay by fitting plots using the Hill equation. a significant difference between mutant receptor and WT CaSR without L-Phe, p<0.05; b significant difference between cases without L-Phe and with 5 mM L-Phe, p<0.05.
Figure 3.8 Individual cellular responses of mutants in the L-Phe-sensitive site to the indicated increments of \([Ca^{2+}]_o\) in the presence or absence of L-Phe.

HEK-293 cells transfected with wild type CaSR or mutants were loaded with Fura-2 AM for 15 min. Each experiment with or without 5 mM L-Phe began in the same non-calcium-containing Ringer’s buffer followed by stepwise increases in \([Ca^{2+}]_o\) until \([Ca^{2+}]_i\) reached a plateau (up to 30 mM) as monitored by changes in the ratio of light emitted at 510 nm following excitation at 340 or 380 nm. At least 30 cells were analyzed for mutants S272A and T145A. Representative cellular responses from a single cell are shown.
Table 3.5 Summary of cellular responses from HEK293 cells transiently transfected with WT CaSR or mutants in the predicted L-Phe-sensing site.

The intracellular calcium responses of HEK293 cells transiently overexpressing WT CaSR or various mutants were measured using Fura-2AM during stepwise increases in \([\text{Ca}^{2+}]_o\). The pattern of \([\text{Ca}^{2+}]_i\) responses in each cell (minimum of 30 cells) was analyzed, and the \([\text{Ca}^{2+}]_o\) at which individual cells started to oscillate was recorded. For experiments without L-Phe, the peaks per minute were recorded at the level of \([\text{Ca}^{2+}]_o\) at which the majority of the cells (>50%) started oscillating; while for experiments with 5 mM L-Phe, the frequency was analyzed at the same levels of \([\text{Ca}^{2+}]_o\) as in the corresponding experiments carried out without L-Phe. Specifically, WT, K47A, G146A, S169A and H413L were measured at 3.0 mM \([\text{Ca}^{2+}]_o\); Y63I and R415A were measured at 3.5 mM \([\text{Ca}^{2+}]_o\); I187A was measured at 10.0 mM \([\text{Ca}^{2+}]_o\); and W70L and I162A were measured at 12.5 mM \([\text{Ca}^{2+}]_o\). The average fluorescence intensity ratio at each increase in the level of \([\text{Ca}^{2+}]_o\) was plotted against \([\text{Ca}^{2+}]_o\) and fitted using the Hill equation, which gave EC50 and Hill numbers. Values are means ± S.E.
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Starting Point (mM)</th>
<th>Frequency (Peaks/Min)</th>
<th>EC(_{50}) (mM)</th>
<th>Hill Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w/o L-Phe</td>
<td>w L-Phe</td>
<td>w/o L-Phe</td>
<td>w L-Phe</td>
</tr>
<tr>
<td>WT</td>
<td>3.0 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>K47A</td>
<td>2.4 ± 0.1(a)</td>
<td>1.9 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>Y63I</td>
<td>3.3 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>W70L</td>
<td>13.9 ± 1.0(a)</td>
<td>9.2 ± 0.3</td>
<td>2.7 ± 0.2</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>G146A</td>
<td>2.5 ± 0.1(a)</td>
<td>1.9 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>I162A</td>
<td>13.0 ± 1.2(a)</td>
<td>9.6 ± 0.3</td>
<td>1.4 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>S169A</td>
<td>3.0 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>I187A</td>
<td>8.1 ± 0.4(a)</td>
<td>3.4 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>H413L</td>
<td>2.8 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>R415A</td>
<td>3.5 ± 0.3</td>
<td>2.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>2.6 ± 0.3</td>
</tr>
</tbody>
</table>

\(a\) significant difference between mutant receptor and WT CaSR without L-Phe, p < 0.05; \(b\) significant difference between cases without L-Phe and with 5 mM L-Phe, p<0.05
3.2.4 *Global functional positive heterotropic cooperative tuning by L-Phe of the positive homotropic cooperative response of CaSR to* $[\text{Ca}^{2+}]_o$.

The sensing of L-Phe at the hinge region adjacent to Site 1 has marked global effects on the five predicted Ca$^{2+}$-binding sites predicted earlier that are spread over several different locations in the CaSR’s ECD. Interestingly, addition of 5 mM L-Phe significantly rescued the $[\text{Ca}^{2+}]_i$ responses of the two mutants, E297I and D215I, in Sites 1 and 2, respectively, that exhibited disrupted cooperativity. Notably, L-Phe converted the biphasic Ca$^{2+}$-response curve for E297I back to a uniphasic curve (Figure 3.9). Figure 3.9 shows that their starting points for $[\text{Ca}^{2+}]_o$-initiated oscillations were reduced from 17.0 ± 0.4 mM to 7.3 ± 0.2 mM (E297I) and from 13.9 ± 0.2 mM to 6.7 ± 0.3 mM (D215I), respectively, in the presence of L-Phe (Table 1). Both of the mutants exhibited more than 2-fold-shifts in their starting points in the presence of L-Phe. The frequencies of their oscillations increased to more than 2 peaks/min in both cases compared to their respective frequencies without L-Phe (Figure 3.9c and Table 3.2). Similarly, addition of L-Phe decreased their EC$_{50}$s in the cell population assay (Table 3.3).

Mutant E224I at Site 3 exhibited increases in the $[\text{Ca}^{2+}]_o$ required to initiate $[\text{Ca}^{2+}]_i$ oscillations but manifested smaller changes (from 3.0 ± 0.1 mM to 5.0 ± 0.2 mM $[\text{Ca}^{2+}]_o$ (P<0.05), compared with the previous two mutants. Moreover, its elevated EC$_{50}$ obtained in the population study correlated well with results from the single cell study (Table 3.2&3.3) suggesting an impaired ability of this mutant to sense $[\text{Ca}^{2+}]_o$. But addition of L-Phe not only significantly decreased the level of $[\text{Ca}^{2+}]_o$ required for initiating oscillations but also increased the oscillation frequency measured at the same level of $[\text{Ca}^{2+}]_o$. Furthermore, the population
assay showed that the EC<sub>50</sub> was reduced and the maximum response was rescued by 5 mM L-Phe.

Figure 3.9 [Ca<sup>2+</sup>]<sub>i</sub> responses of receptors with mutations in the Ca<sup>2+</sup>-binding sites stimulated by increasing [Ca<sup>2+</sup>]<sub>o</sub> in the presence or absence of 5 mM L-Phe.

(a). [Ca<sup>2+</sup>]<sub>i</sub> was monitored in mutants E297I and D215I in the absence or presence of L-Phe. [Ca<sup>2+</sup>]<sub>o</sub> was increased stepwise up to 30 mM or until [Ca<sup>2+</sup>]<sub>i</sub> reached a plateau. (b). Statistical analysis of the [Ca<sup>2+</sup>]<sub>o</sub> at which CaSR-transfected single HEK293 cells started to
exhibit $[\text{Ca}^{2+}]_i$ oscillations. (c). In the single cell experiments, the frequency of the oscillation patterns was investigated in more than 30 cells. For experiments without L-Phe, the peaks per minute were recorded at the level of $[\text{Ca}^{2+}]_o$ at which the majority of the cells (>50%) started to oscillate, while for experiments with 5 mM L-Phe, the frequency was analyzed at the same level of $[\text{Ca}^{2+}]_o$ that was utilized in the absence of L-Phe. Empty bar: in the absence of L-Phe; Black bar: in the presence of 5 mM L-Phe. (d). Population assay for $[\text{Ca}^{2+}]_i$ responses of HEK293 cells transiently transfected with CaSRs with mutanted Ca$^{2+}$-binding site-related using Fura-2 AM to measure $[\text{Ca}^{2+}]_i$ during stepwise increases in $[\text{Ca}^{2+}]_o$ from 0.5 to 30 mM. $[\text{Ca}^{2+}]_i$ responses were then fitted using the Hill equation.

E353 is predicted to be a $[\text{Ca}^{2+}]_o$-binding residue in Site 4 in lobe 2, while D398 and E399 are part of Site 5 (172). Table 3.3 and Figure 3.5 show that removal of these negatively charged residues at Sites 4 and 5 increased the level of $[\text{Ca}^{2+}]_o$ required to initiate $[\text{Ca}^{2+}]_i$ oscillations and also their EC$_{50}$S. L-Phe at 5 mM enhanced these mutants’ sensitivities to $[\text{Ca}^{2+}]_o$ toward, albeit not to normal and decreased their EC$_{50}$s. Therefore, as shown in Table 3.3, mutations not only in those predicted Ca$^{2+}$-binding residues adjacent to the potential L-Phe-binding site (e.g., in Sites 1 and 2) exhibited L-Phe-induced increases in their sensitivities to $[\text{Ca}^{2+}]_o$ as well as in their Hill coefficients, but also other in $[\text{Ca}^{2+}]_o$-binding residues in sites farther away from the hinge region. These results support the concept that there are global functional positive heterotropic cooperative interactions between L-Phe and the bindings sites for $[\text{Ca}^{2+}]_o$ in the CaSR ECD.
3.2.5 The ensemble of conformations of calcium- and L-Phe-loaded CaSR ECD is distinguishable from the non-loaded form.

To provide a more detailed description of the CaSR’s mechanism of action, including the interactions between the binding sites for $[\text{Ca}^{2+}]_o$ and L-Phe at the atomic level, we analyzed the trajectories of molecular dynamics simulations using principal component analysis (PCA), which separates out the protein motions into principal modes ranked according to their relative contributions[199]. Projection of the trajectories of the different states of CaSR onto the first three modes that accounted for the majority of the total fluctuations is shown in Figure 3.10. The conformations sampled by the $\text{Ca}^{2+}$-free and L-Phe-free forms of the CaSR are distinctly different from those sampled by the $\text{Ca}^{2+}$-loaded and $\text{Ca}^{2+}$- and L-Phe-loaded forms of the receptor. Interestingly, the conformations of the L-Phe-loaded and the free form of CaSR are essentially indistinguishable as can be seen in Figure 3.10a. The results suggest that $[\text{Ca}^{2+}]_o$ shifts the population of conformational ensembles of CaSR to a semi-active ensemble that can subsequently be shifted to an ensemble of more active conformations upon interaction of the receptor with L-Phe. These results are consistent with the experiments described above and suggest that the unbound CaSR does not respond to L-Phe alone, because L-Phe cannot effectively shift the inactive conformations to an active ensemble of conformations. The above results do not rule out the role of conformational selection, which implies the existence of all relevant active and inactive conformations of the receptor before binding, in the mechanism of activation of CaSR, but clearly indicate that the ensembles of active and inactive conformations are distinctly different. Moreover $[\text{Ca}^{2+}]_o$ alone and/or $[\text{Ca}^{2+}]_o$ and L-Phe together are required to shift the population, in agreement with previously well documented experimental results that L-
Phe could not activate CaSR at sub-threshold levels of $[\text{Ca}^{2+}]_o$ (below about 1.0 mM in CaSR-transfected HEK-293 cells) (165).

Based on all the results described above, we propose a model to illustrate the possible mechanism by which $\text{Ca}^{2+}$ and L-Phe regulate the function of the CaSR mainly through the molecular connectivity encoded at the hinge region of the ECD of the protein. Our model (Figure 3.10b) suggests that a local conformational change upon interaction of the CaSR with L-Phe might affect the overall conformation of the receptor, thereby influencing the cooperativity between multiple $\text{Ca}^{2+}$-binding sites and enhancing the receptor’s overall response to $\text{Ca}^{2+}$. 
Figure 3.10 Principal component analysis (PCA) of CaSR ECD with experimental data suggesting a model for the mechanism underlying activation of the CaSR by extracellular Ca\(^{2+}\) and L-Phe.
(a). Principal component analysis (PCA) of CaSR ECD. The trajectories of the molecular
dynamics simulations were analyzed using principal component analysis (PCA), which
separates out the motions of the CaSR ECD into principal modes ranked according to their
relative contributions. The first three principal modes were included in the present study to
analyze four different states of the protein: Ligand-free (black), presence of L-Phe-only
(Green), presence of Ca$^{2+}$-only (Red) or presence of both Ca$^{2+}$ and L-Phe (Blue). (b). Model
for the mechanism underlying the activation of the CaSR by extracellular Ca$^{2+}$ and L-Phe.
Ca$^{2+}$ and L-Phe modulate the activity as well as the cooperativity of CaSR. Higher [Ca$^{2+}$]o,
around 3.0 mM, could change the conformation of CaSR into an active form (second stair
level) in a positive homotropic cooperative manner (as indicated by the change of the color
of the Ca$^{2+}$-binding sites from yellow to orange) and further trigger [Ca$^{2+}$]i oscillations. L-
Phe binds to the hinge region between Lobe 1 and Lobe 2, modulating the receptor together
with Ca$^{2+}$ in a positive heterotropic cooperative way (as indicated by the change in the color
from orange to red). This could produce a conformation of the receptor that is a “super-
activated” form (third stair level), associated with a higher frequency of [Ca$^{2+}$]i oscillations
and a reduced EC$_{50}$. CaSR mutants (lower activity stair level), especially those containing
mutations close to the hinge region between lobe 1 (LB$_1$) and lobe 2 (LB$_2$), could cause a
disruption of the cooperativity among the various Ca$^{2+}$-binding sites (lower activity second
stair level). The impaired receptor function and the cross talk between Ca$^{2+}$-binding sites
can be rescued, at least in part, by introducing L-Phe into the extracellular buffer. Red
Arrow: receptor activity.
Figure 3.11 Docking Glutathione into the CaSR ECD.

Glutathione was docked into the CaSR ECD using Audodock Vina. The listed residue numbers are the real sequence residue number minus 24. Yellow highlighted residues are overlapping with Phe binding sites; Blue highlighted residues are predicted to interact with Glutathione only.
Aspartate was docked into the CaSR ECD using Audodock Vina. The listed residue numbers are the real sequence residue number minus 24. Yellow highlighted residues are overlapping with Phe binding sites; Blue highlighted residues are predicted to interact with Aspartate only.

3.3 Discussion

Several major barriers have hampered our understanding of the long-standing question of how the CaSR integrates its activation by two different classes of nutrients, divalent cations and amino acids as well as, to regulate the functional cooperativity of the receptor and the alterations of this cooperativity caused by naturally occurring mutations in human diseases. These include
“invisible” binding pockets for these two key physiological agonists of the CaSR, namely Ca\textsuperscript{2+} and amino acids, challenges in obtaining structural information associated with membrane proteins, and the lack of direct binding methods for determining the mechanisms underlying the cooperative activation of the CaSR by Ca\textsuperscript{2+} and amino acids (118,126,228). To overcome these limitations, we have applied several computer algorithms and grafting approaches for identifying and predicting Ca\textsuperscript{2+}-binding sites in proteins and we have verified the intrinsic Ca\textsuperscript{2+}-binding capabilities of predicted Ca\textsuperscript{2+}-binding sites in the CaSR and mGluR1\textalpha (68,124,172,229).

Our studies, shown in Figures. 3.1, 3.4 and 3.7 suggest that mutations in Ca\textsuperscript{2+}-binding Site 1, such as E297I and Y218Q, not only disrupt the CaSR’s Ca\textsuperscript{2+}-sensing capacity but also have an impact on the positive homotropic cooperative interactions of Ca\textsuperscript{2+} with the other Ca\textsuperscript{2+}-binding sites. Such biphasic behavior with a large disruption of cooperativity, is very similar to our previously reported metal-binding concentration response curves of subdomain 1 and its variants with increases in [Ca\textsuperscript{2+}]\textsubscript{o} (172). Subdomain 1 of CaSR contains a protein sequence encompassing Ca\textsuperscript{2+}-binding Sites 1, 2 and 3, but without Ca\textsuperscript{2+}-binding Sites 4 and 5. It also exhibits both a strong and a weak metal-binding component. This strong metal-binding process can be removed by further mutating Site 1 (E297I). In contrast, mutations at Sites 2 and 3 have less impact on the first binding process (172). These molecular dynamics simulation studies carried out here are consistent with the experimental results showing that residues located within Site 1 have strong correlated motions with other residues in Sites 2, 3, 4 and 5 (Table 3.1). These results suggest that the dynamics of Site 1 is intricately coupled to those of the other binding sites; therefore, any changes in the dynamics of Site 1 could affect that of the other sites. The observation of this molecular connectivity and its relationship to positive cooperativity from the molecular
dynamics simulations provide a description at the atomic level of the crosstalk between the different sites of the CaSR suggested by the experimental results in live cells.

Here, we have also computationally identified an L-Phe-binding pocket formed by residues L51, S170, T145, Y218 and S272, which is adjacent to and partially overlaps the key Ca\(^{2+}\)-binding Site 1 at the hinge region of the Venus Fly Trap (VFT) of the CaSR. This Ca\(^{2+}\)-binding Site is also conserved in other family C GPCRs, including the mGluR1 VFT (Figure 3.1) (96,175,229). Y218 is involved in sensing both Ca\(^{2+}\) and L-Phe. The aromatic ring from residue Y218 could form delocalized pi bonds with the side chain of L-Phe and the hydrophobic interaction between L51 and L-Phe would further stabilize this interaction. Mutating S170 might interfere with hydrogen-bonding of the ligated amino acid to the \(\alpha\)-amino group of S170 based on the structure of mGluR1\(\alpha\) (223). S170T has been reported by several groups to interfere with the CaSR’s L-Phe-sensing ability(221,223). Consideration of the crystal structure of the glutamate-bound form of mGluR1 (175), together with our docking analysis, implies that residues T145 and S272 may not directly participate in the interaction with L-Phe but could possibly interact with L-Phe by ligation of water molecules, which is a relatively weaker type of interaction.

We have observed in this study essentially equivalent expression of WT CaSR and its variants on the cell surface (Figure 3.6). These lines of evidence suggest that the difference in the Ca\(^{2+}\)-sensing capacities among the WT and mutant receptors are due to perturbation of the cell surface receptors’ functions, rather than, for example, impaired trafficking of the receptor proteins to the cell surface. L-Phe restored the \([Ca^{2+}]_i\) responses of the tested mutants located in all five
predicted Ca\(^{2+}\)-binding sites, and it had more dramatic rescuing effects on mutants E297I and D215I compared with the other mutants (Figure 3.9). Thus, the importance of the hinge region, where L-Phe likely interacts with the CaSR ECD, is once again highlighted, although we only used [Ca\(^{2+}\)]\(_i\) responses as a read out, and the results could possibly differ if other second messenger pathways were examined.

The PCA results suggest that the need for Ca\(^{2+}\) in initially activating CaSR, as suggested by the experiments, is related to shifting the ensemble of conformations of CaSR from inactive states to active states. The activity of the Ca\(^{2+}\)-loaded form of CaSR is then further enhanced by the binding of L-Phe, which produces additional changes in the ensembles of conformations of CaSR. Therefore, the global modulation of receptor activity by Ca\(^{2+}\) and L-Phe might be explained by a combination of an induced fit and population shift models (230) in which the receptor’s overall structure could vary in the equilibrium distributions of conformations of the receptor that can interchange dynamically in the absence of Ca\(^{2+}\) and L-Phe. Our experimental results suggest that binding of Ca\(^{2+}\) at its various sites is associated with motions of these sites that are highly correlated with one another. Consequently, the shift in the ensemble of conformations of CaSR induced by the initial binding of Ca\(^{2+}\) at Site 1 will alter the equilibrium population of the unbound conformations of other Ca\(^{2+}\)-binding sites due to their crosstalk with Site 1. The binding of Ca\(^{2+}\) to Site 1 and the subsequent interaction of CaSR with L-Phe can further shift the conformations of the ECD from one part of the free energy landscape to another in such a way that Ca\(^{2+}\)-binding to other sites is more readily favorable. Our findings here also enhance our understanding of the role of Ca\(^{2+}\) in modulating key Ca\(^{2+}\)-binding proteins such as calmodulin to mediate signal transduction via correlated motions among their multiple Ca\(^{2+}\)-
binding sites, thereby generating cooperative responses with critical biological consequences (231,232).

The CaSR’s co-activation by these two classes of ligands may be particularly important in the gastrointestinal tract, where high concentrations of amino acids resulting from protein digestion ensure activation of the CaSR and its stimulation of digestive processes even when there are relatively low levels of $[\text{Ca}^{2+}]_o$. Moreover, as reported in clinical studies, there are 33 disease-related mutations near Ca$^{2+}$-binding Site 1 associated with receptor activation or inactivation and, in some cases, with reduced cooperativity (208). Our work suggests that it is likely that such mutations disrupt the molecular connectivity encoded in the receptor and provide a better understanding of the molecular basis of some of the CaSR-related clinical disorders. Our finding of the capacity of L-Phe to rescue disease-linked mutations suggests the possibility of rescuing such mutant receptors using calcimimetics of various types as pharmacotherapy, thereby increasing cell surface expression or intrinsic activity of the mutant CaSRs. These results, therefore, provide insights into key factors regulating the receptor’s overall activity, which could lay the foundation for a new generation of therapeutics.

In addition to the Ca$^{2+}$-sensing receptor, $[\text{Ca}^{2+}]_o$ regulates 14 of the other members of the family C G protein-coupled receptors (GPCRs), including the metabotropic glutamate receptors (mGluR), $\gamma$-aminobutyric acid GABA$\beta$ receptors and receptors for pheromones, amino acids and sweet substances (38,96,101,204,209,224,225,233). The observed molecular connectivity centered at predicted calcium-binding Site 1 of the CaSR, which is adjacent to an amino acid-binding pocket at the hinge region of the receptor, may be shared by other members of the family
C GPCRs (234,235). Besides the strong conservation of the predicted calcium-binding site 1 and amino acid-binding pocket, several lines of evidence support this suggestion (Figure 3.2) (68,96,172,236). We have predicted a $\text{Ca}^{2+}$-binding site partially sharing the Glu-binding site in the ECD of mGluR1$\alpha$, and both of these agonists co-activate the receptor (229). A $\text{Ca}^{2+}$-binding pocket was proposed to be present in the ligand-binding site of the $\text{GABA}_B$ receptor (225). Many animals and humans can detect the taste of calcium via a calcium taste receptor that is modulated by an allosteric mechanism (237).

In summary, our present study provides a mechanistic view of the interplay among extracellular $\text{Ca}^{2+}$, amino acids and the CaSR via molecular connectivity that modulates the positive homotropic and heterotropic cooperativity of CaSR-mediated intracellular $\text{Ca}^{2+}$ signaling. The positive cooperative co-activation of the CaSR by $\text{Ca}^{2+}$ and L-Phe and the importance of the positive homotropic and heterotropic cooperativity, respectively, exhibited by the two agonists may be further extended to other members of the family C GPCRs to facilitate an understanding of the molecular basis for related human disorders and the development of new therapeutic strategies.
CHAPTER 4. IDENTIFICATION OF 71 CALCIUM-SENSING RECEPTOR MUTATIONS IN HYPERCALCAEMIC AND HYPOCALCAEMIC PATIENTS:
EVIDENCE FOR CLUSTERING OF EXTRACELLULAR DOMAIN MUTATIONS AT CALCIUM BINDING SITES

4.1 Introduction

The human calcium sensing receptor (CaSR) is a 1078 amino-acid cell-surface protein (Figure. 4.1) that is expressed predominantly in the parathyroids and kidneys, where it regulates PTH secretion and renal calcium (Ca\(^{2+}\)) reabsorption appropriate to the prevailing extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) (38,238). The CaSR is a member of family C of the superfamily of G-protein coupled receptors (GPCRs) that includes the metabotropic glutamate receptors, vomeronasal pheromone receptors V2R, taste receptors TAS1R, and the gamma-aminobutyric acid (GABA-B) receptors (239). The CaSR consists of a 612 amino-acid extracellular domain (ECD) that is critical for co-translational processing (240), receptor dimerisation (241), binding ligands (95,234,242), and transmitting activation signals through the 7 transmembrane domains (TMDs), which comprises residues 613 to 862, and the intracellular domain (ICD), which comprises residues 863 to 1078 (Figure 4.1) (243). Ligand binding by the CaSR ECD results in the activation of multiple signalling cascades including \(G_{q/11}\)-protein dependent stimulation of phospholipase C (PLC) activity, causing accumulation of inositol 1,4,5-triphosphate (IP3) and a rapid increase of cytosolic calcium ([Ca\(^{2+}\)]\(_i\)) concentrations (101). These intracellular events mediate a decrease in the rate of PTH secretion from the parathyroid chief cell and a reduction in renal tubular Ca\(^{2+}\) reabsorption, as illustrated by CaSR mutations which result in alterations in plasma calcium and urinary calcium excretion. For example, inactivating (i.e. loss-of-function) CaSR mutations result in familial hypocalciuric hypercalcaemia (FHH) and neonatal severe
primary hyperparathyroidism (NSHPT) (161,238); whereas activating (i.e. gain-of-function) CaSR mutations result in autosomal dominant hypocalcaemia with hypercalciuria (ADHH) (244). In addition, CaSR mutations have been reported to be associated with primary hyperparathyroidism (PHPT) and a form of Bartter syndrome, designated type V, in rare patients (164,245). Moreover, in vitro studies which have functionally expressed these mutant CaSRs and determined their dose–response curves and the [Ca$^{2+}$]o needed to produce a half-maximal (EC$_{50}$) response in total [Ca$^{2+}$]i, have demonstrated that the inactivating CaSR mutations associated with FHH, PHPT and NSHPT result in a rightward shift of the dose–response curve and a significantly higher EC$_{50}$; whereas the activating CaSR mutations associated with ADHH and Bartter syndrome type V result in a leftward shift of the dose–response curve and a significantly lower EC$_{50}$ when compared to the wild-type CaSR (87,246). The identification of these disease-causing CaSR mutations has highlighted the importance of the ECD for CaSR function, as many mutations (i.e. 40%) which result in FHH, NSHPT or ADHH cluster in the first 300 ECD residues (163).

The CaSR ECD three-dimensional structure has been deduced based on its homology to the metabotropic glutamate receptor type 1 (mGluR1) whose crystal structure has been determined (175). The CaSR ECD is considered to adopt a bilobed venus flytrap conformation. The venus flytrap domain (VFTD), comprises residues 20 to 540, and consists of amino- and carboxyl-terminal regions that are designated lobe 1 (residues 20 to 205 and residues 324 to 477) and lobe 2 (residues 206 to 323 and residues 478 to 540), respectively (175). These lobes form a large cleft, and consist of alpha helices and beta sheets that are connected by multiple loops (175). The CaSR ECD has been proposed to be the major region for Ca$^{2+}$ binding (68,172), and an analysis of the common structural properties of Ca$^{2+}$ binding sites in proteins, as well as
studies of a CaSR model, has identified 5 putative Ca$^{2+}$ binding sites (calcium binding sites-1 to -5) within the CaSR VFTD (68,172). This is consistent with the finding that the CaSR has a Hill coefficient of around 3 (87) and binds Ca$^{2+}$ in a cooperative manner. These 5 calcium binding sites are located at flexible loops or helical domains near the protein surface and consist of clusters of negatively charged or neutral amino acids (68,172). Calcium binding sites-1, which is the principal site, is located in the cleft between lobes 1 and 2 (172). To further define these structure-function relationships of the CaSR ECD, we undertook studies to characterise additional CaSR mutations in patients with FHH, NSHPT and ADHH and assessed the functional consequences of those that would increase understanding of the ECD residues within the VFTD and their relationship to the 5 Calcium binding sites.
Figure 4.1 Schematic representation of the location of the 72 CaSR variants.
The CaSR comprises an extracellular domain (ECD), which is formed by residues 1-612; seven transmembrane domains (TMDs) formed by residues 613 to 862; and an intracellular domain (ICD), formed by residues 863 to 1078. Every 50th amino acid is indicated. The 612 amino acid ECD is consists of the following: an N-terminal signal peptide (amino acids 1-19); a venus fly trap domain (VFTD) (amino acids 20-540); a nine cysteine domain (NCD) (amino acids 542-598); and a peptide linker (PL) (amino acids 599-612) that connects the ECD to the TMD. The residues involved in forming the 5 VFTD Ca$^{2+}$ binding sites (CaBSs) are indicated by asterisks. The number preceding the asterisk indicates the respective Calcium binding sites. The 72 CaSR variants reported in this study (Table 1) are shown in black. The amino acid change is detailed above the mutated residue. The bracketed letter following the amino acid change indicates whether this variant was identified in a patient with FHH (F), NSHPT (N), ADHH (A), or in both FHH and ADHH (A/F). Boxes indicate the mutations associated with either loss- or gain of function at codons 173 and 221. The location of the splice site mutation (Table 1), which is predicted to lead to either a 2 amino acid missense peptide followed by a stop at codon 167, or an in-frame deletion of exon 4 is shown as S.S. The location of the previously reported Pro339Thr mutation (245) in relation to calcium binding sites-4 residues is also shown.

4.2 Results

4.2.1 Overview of CaSR abnormalities and variants

DNA sequence analysis of the entire 3,236 bp coding region and 12 intron-exon boundaries of the CASR in the 290 unrelated probands revealed the occurrence of a CASR sequence abnormality in 82 patients (Table 4.1), thereby representing a 28% CaSR variant detection rate in
this group of patients with hypercalcaemia and hypocalcaemia. Seven abnormalities (Pro55Leu, Arg172Gly, Arg220Pro, Arg227Gln, Glu250Lys, Leu461Pro, Lys831Thr) occurred in more than one patient thereby giving a total of 72 abnormalities (Table 4.1). The absence of all of these DNA sequence abnormalities in 110 alleles from 55 unrelated normal individuals indicated that these abnormalities were mutations or rare polymorphisms. Fifty-five CaSR abnormalities were found in the 224 hypercalcaemic patients, representing a 25% detection rate, and 45 of these CaSR abnormalities were in FHH patients and 10 were in NSHPT patients (Figure. 4.2) The remaining 27 abnormalities of the CaSR were found in the 66 hypocalcaemic patients, representing a 41% detection rate, thereby confirming ADHH as the aetiology for the hypocalcaemia. Amongst the 72 different CaSR abnormalities, 30 (42%) had not been reported in previous studies, and the remaining 42 (58%) had been reported to occur in other, presumably unrelated patients (247). One variant (Glu250Lys) was found to occur in FHH and ADHH patients, raising the possibility that this may be a polymorphic variant. Indeed, in vitro functional expression studies demonstrated that the Glu250Lys variant represented a functionally neutral polymorphism (Figure 4.3). Forty-five different CaSR mutations (63%) affected ECD residues (Figure 4.1), while 22 (31%) mutations affected TMD residues and 4 (6%) mutations affected ICD residues (Table 4.1). An analysis of the location of FHH, NSHPT and ADHH mutations identified in this study, revealed that the majority (> 50%) of mutations associated with these hyper- and hypocalcaemic disorders were located in the ECD (Figure 4.4A), and a similar analysis of the 200 different previously reported CaSR mutations (248) also revealed that > 50% of the abnormalities associated with such disorders were located in the ECD (Figure 4.4A). The phenotypic features of FHH, NSHPT and ADHH in patients with CaSR mutations were similar to those patients without CaSR mutations (data not shown), thereby indicating that phenotypic
features alone are unlikely to be useful predictors for the presence or absence of a CaSR mutation.

Table 4.1 Details of 82 CaSR abnormalities/variants identified in patients with FHH (n = 45), NSHPT (n = 10), and ADHH (n = 27).

<table>
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<tr>
<th>Disorder</th>
<th>Codon</th>
<th>Nucleotide</th>
<th>Base</th>
<th>Amino</th>
<th>Exon</th>
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<td>Arg→Stop</td>
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<td>c.164</td>
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<td>Pro→Leu&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
<td>ECD</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>c.164</td>
<td>CCG→CT</td>
<td>Pro→Leu&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
<td>ECD</td>
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<tr>
<td></td>
<td>55</td>
<td>c.164</td>
<td>CCG→CT</td>
<td>Pro→Leu&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
<td>ECD</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>c.164</td>
<td>CCG→CT</td>
<td>Pro→Leu&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>ECD</td>
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<td>ECD</td>
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<td>ECD</td>
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<td>c.515</td>
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Nucleotide is numbered in relation to CaSR cDNA reference sequence (GenBank accession number NM_000388) whereby nucleotide +1 corresponds to the A of the ATG – translation initiation codon; ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain; recurrent mutation; novel mutation; predicted to abolish acceptor splice site (S.S.) and lead to either a 2 amino acid missense peptide followed by a stop at codon 167, or an in-frame deletion of exon 4; IVS, intervening sequence; demonstrated to be a functionally neutral polymorphism (Fig. 3); deletion/frameshift, 13 missense peptide followed by a stop at codon 626; homozygous mutation; compound heterozygous mutation; deletion/frameshift, 65 amino acid missense peptide followed by a stop at codon 256; deletion/frameshift, 27 amino acid missense peptide followed by a stop at codon 938.
Figure 4.2 Detection of a CaSR mutation in exon 2 in a family with neonatal severe primary hyperparathyroidism (NSHPT).

A, DNA sequence analysis of the affected individual (II.1) revealed a C to T transition at codon 25, thus altering the wild-type (WT) sequence CGA, encoding an arginine to the mutant (m) sequence TGA, which is a termination (Stop) codon. This nonsense mutation
also resulted in the loss of the wild-type \textit{Cac8I} restriction enzyme (GCN/NGC), and this facilitated the confirmation of the mutation. B, PCR amplification and \textit{Cac8I} digestion would result in two products of 186 and 114 bp from the wild-type (WT) sequence, but a 300 bp band would be expected from the mutant (m) sequence as is illustrated in the restriction map. This Arg25stop mutation was demonstrated to be homozygous in the siblings affected with NSHPT (individuals II.1 and II.2), while their consanguineous parents were both heterozygous for this mutation. The absence of this Arg25stop mutation in 110 alleles from 55 unrelated normal individuals (N1-N3 shown) indicates that it is not a common DNA sequence polymorphism. Individuals are represented as: male (squares), female (circles), unaffected (open circle), affected with NSHPT (filled symbols), affected with hypercalcaemia (half filled square).
Figure 4.3 Functional expression of wild-type and variant codon 173, 221 and 250 CaSRs in transfected HEK293 cells.

The $[Ca^{2+}]_o$-evoked increases in $[Ca^{2+}]_i$ were measured in HEK293 cells that had been transiently transfected with wild-type (WT) or variant codon 173, 221 and 250 CaSRs (Wild-type sequences - Leu173 (L173), Pro221 (P221) and Glu250 (E250); variant sequences – Phe173 (F173), Pro173 (P173), Leu221(L221), Gln221 (Q221) and Lys250 (K250). A, The mutant (m) CaSRs, F173, which occurred in an ADHH kindred (Table 1), and P173, which occurred in an FHH kindred, resulted in a leftward and rightward shift of the concentration-response curve, respectively, when compared to that of the wild-type CaSR. Thus, the EC$_{50}$ values of the mutant CaSRs F173 and P173 were significantly ($p < 0.001$) lower and higher, respectively when compared to that of the wild-type CaSR. These findings indicate that the mutant CaSRs F173 and P173 result in a gain-of-function and a loss-of-function of the CaSR, respectively, and this is consistent with their associated phenotypes of ADHH and FHH in man. B, The mutant CaSRs, L221, which occurred in an ADHH kindred (Table 1), and Q221, which occurred in an FHH kindred, resulted in a leftward and rightward shift of the concentration-response curve, respectively, when compared to that of the wild-type CaSR. Thus, the EC$_{50}$ values of the mutant CaSRs L221 and Q221 were significantly ($p < 0.01$) lower and higher, respectively when compared to that of the wild-type CaSR. These findings indicate that the mutant CaSRs L221 and Q221 result in a gain-of-function and a loss-of-function of the CaSR, respectively, and this is consistent with their associated phenotypes of ADHH and FHH in man; C, The variant CaSR, K250, which occurred in both ADHH and FHH kindreds, did not significantly alter...
the EC$_{50}$ or concentration-response curve when compared to that of the wild-type CaSR. These findings indicate that K250 is a functionally neutral polymorphism (poly) and not a pathogenic mutation.
Figure 4.4 Analysis of CaSR mutations by disease association, location and type of mutation.

A, frequencies of ECD, TMD and ICD CaSR mutations in FHH, NSHPT and ADHH patients observed in this study (n = 71), and in an analysis of 200 previously reported CaSR mutations. The majority (>50%) of mutations associated with FHH, NSHPT and ADHH in this study and amongst the previously reported CaSR mutations were located in the ECD. Significant differences between this study and the previously reported mutations (248) were not observed. In addition, no significant differences in the location of mutations were observed between FHH, NSHPT and ADHH. B, frequencies of missense, truncating and deletion/insertion CaSR mutations in FHH, NSHPT and ADHH patients observed in this study (n = 71) and in an analysis of 200 previously reported CaSR mutations. Greater than 90% of mutations associated with FHH and ADHH were missense substitutions. However, NSHPT was associated with a significantly (p < 0.05) higher proportion of nonsense and frameshift mutations when compared to either FHH or ADHH. No significant difference in the frequency of mutation types was observed between this study and all the previously reported mutations (248).

4.2.2 NSHPT is associated with a high proportion of truncating CaSR mutations

Four of the ten NSHPT associated CaSR mutations (40%) were nonsense or frameshift mutations that were predicted to lead to a truncated CaSR (Table 4.1, Figure 4.2). This high proportion of truncating mutations in NSHPT differed significantly (p = 0.01) from that of FHH, of which three (6%) mutations were predicted to lead to a truncated CaSR (Table 1, Figure 4.4B).
An analysis of previously reported NSHPT mutations also revealed a high proportion of truncating mutations, with >40% being nonsense or frameshift mutations (Figure 4.4B), and this differed significantly ($p < 0.01$) from the previously reported FHH mutations, of which 20% were predicted to be truncating (Figure 4.4B). All of the 10 mutations found in NSHPT patients occurred in the homozygous (n=8) or compound heterozygous (n=2) state. The truncating mutation located closest to the CaSR N-terminus in this study was a homozygous Arg25Stop mutation that was present in two neonatal siblings who both presented with NSHPT and serum calcium concentrations > 4.0 mmol/l (Figure 4.2). Restriction endonuclease analysis revealed that the consanguineous parents were both heterozygous for the Arg25Stop mutation (Figure 4.2). The homozygous Arg25Stop mutation was predicted to lead to a peptide that was truncated six amino acids after the signal peptide cleavage site, and hence was not suitable for \textit{in vitro} expression and functional characterisation.

4.2.3 Functional characterisation of CaSR variants at codons 173, 221 and 250 detected in FHH and ADHH patients

Our DNA sequence analysis of the \textit{CASR} revealed that variants that would be consistent with missense mutations at codons 173, 221 and 250 were associated with both FHH and ADHH (Table 4.1, Figure 4.1). Thus, at codon 173, we identified a Leu173Phe variant associated with ADHH, while, a Leu173Pro variant has been previously reported in association with FHH (249); at codon 221, we identified Pro221Gln and Pro221Leu variants that were associated with FHH and ADHH, respectively; at codon 250 we identified a Glu250Lys variant in a FHH proband and an ADHH proband, and therefore the pathogenic nature of this mutation was uncertain. To investigate the functional effects of these variants at codons 173, 221 and 250 we transiently transfected wild-type and variant pEGFP-CaSRs into HEK293 cells and assessed the $[\text{Ca}^{2+}]_i$. 


responses to changes in $[\text{Ca}^{2+}]_o$. Expression of both monomeric and dimeric forms of the wild-type and variant EGFP-CaSRs was confirmed by Western blot analysis (data not shown). An analysis of the response curves of the wild-type and variant CaSRs (Figure 4.3) revealed that the Leu173Phe and Pro221Leu variant CaSRs lead to a leftward shift in the concentration–response curves and significantly ($p < 0.01$) reduced EC$_{50}$ values (Leu173Phe versus wild-type = 1.8 ± 0.07 mM versus 2.49 ± 0.06 mM; and Pro221Leu versus wild-type = 0.87 ± 0.15 mM versus 2.74 ± 0.29 mM); and the Leu173Pro and Pro221Gln mutant CaSRs lead to a rightward shift of the concentration–response curve and significantly ($p < 0.01$) raised EC$_{50}$ values (Leu173Pro versus wild-type = 11.0 ± 0.92 mM versus 2.49 ± 0.06 mM; and Pro221Leu versus wild-type = 4.3 ± 0.18 mM versus 2.74 ± 0.29 mM). In contrast, an analysis of the concentration-response curve and EC$_{50}$ of the Glu250Lys CaSR variant (Figure 4.3) revealed this not to significantly differ ($p = \text{NS}$) from that of the wild-type receptor (Glu250Lys versus wild-type = 2.56 ± 0.02 mM versus 2.49 ± 0.06 mM). Thus, the Glu250Lys missense substitution, which involved substitution of the negatively charged Glu residue for a positively charged Lys residue, did not alter CaSR function and therefore likely represents a functionally neutral polymorphism. However, the Leu173Phe and Pro221Leu mutations, which involved substitutions of a non-polar residue for another non-polar residue, led to a gain of CaSR function; while the Leu173Pro, which involved substitution of a non-polar residue for another non-polar residue, and Pro221Gln, which involved substitution of the non-polar Pro for a polar Gln, mutations were associated with a loss of CaSR function. This represents a very unusual situation whereby mutations of a residue can result in two opposing functional effects on CaSR responses, such that it appears that the residues at codons 173 and 221 when mutated act as “toggles” which either activate or inactivate the CaSR.
4.2.4 Three dimensional modelling of VFTD mutations

The two “toggle” residues at Leu173 and Pro221, which are associated with FHH and ADHH, are located in the VFTD (Figure 4.1). Moreover, another 30 CaSR missense mutations identified by our study (Table 4.1) and 93 mutations identified by previous studies (248), i.e. 47% and 47% of all the CaSR mutations, are also located in the VFTD (Figure 4.1). We therefore performed homology modelling of the VFTD to determine its three dimensional structure and the functional relationships between the VFTD 5 calcium binding sites and the 34 mutations that included the Leu173Phe, Leu173Pro, Pro221Gln and Pro221Leu mutations (Figure 4.5). This revealed that: nine of the mutated residues represented by 12 mutations (Thr151Met, Arg172Gly, Leu173Phe, Leu173Pro, Leu174Arg, Arg185Gln, Tyr218His, Arg220Pro, Arg220Trp, Pro221Leu, Pro221Gln and Ser296Asn) were located in the cleft between lobes 1 and 2 that contained calcium binding sites-1 (Figure 4.5A); two mutations (Ile212Ser and Glu241Lys) were adjacent to site 2 (Figure 4.5B); and six residues represented by eight mutations (Arg172Gly, Trp208Cys, Trp208Ser, Pro221Leu, Pro221Gln, Arg227Gln, Glu228Lys and Cys236Gly) were located within or near to the alpha helix that contained site 3 (Figure 4.5C), with three of these (Arg172Gly, Pro221Leu, Pro221Gln) also being in close proximity to site 1. None of the VFTD mutations in our study were present at either calcium binding sites-4 or -5, whereas, a Pro339Thr CaSR mutation, that we have previously reported in association with primary hyperparathyroidism in adulthood (245), was shown by our analysis to be located in a short flexible loop that is linked to an alpha helix containing calcium binding sites-4 Ca²⁺ binding residues (Figure 4.5D). Thus, 18 of the 34 CaSR missense mutations (i.e. > 50%) characterised by our study were located near one or more sites, with the majority (> 50%) of Calcium binding
sites mutations located at site 1 (Table 4.2). A further analysis of the 93 previously reported missense mutations (248) located within the CaSR VFTD revealed that 42 (27 from FHH patients, 5 from NSHPT patients, and 10 from ADHH patients) mutations (i.e. 45%) were predicted to be located at or near one or more of the 5 VFTD calcium binding sites (Table 4.2) with >50% located at calcium binding sites -1; these findings are in agreement with our observations of the 71 CaSR mutations (Table 4.1 and 4.2). In addition, FHH calcium binding sites mutations most commonly involved the substitution of an Arginine residue, indicating a key role for this residue in Ca\(^{2+}\) binding, whereas, the majority of ADHH mutations clustering around calcium binding sites involved the loss of a glutamate residue, thereby highlighting a role for glutamate in Ca\(^{2+}\) binding (Figure 4.1). A detailed homology modelling analysis of the locations of residues 173 and 221 at the VTFD cleft, revealed that both of these amino acid residues are situated at the entrance to calcium binding sites at the cleft region (Figure 4.5F), and hence mutations of these residues would be predicted to influence the entry and binding of Ca\(^{2+}\) into the VTFD cleft binding site. This location is consistent with the “toggle” role that these residues were found to have in the functional expression studies (Figure 4.3) and in their clinical phenotypic expression in being associated with both FHH and ADHH (Table 4.1).

### 4.3 Discussion

We report, 71 different CaSR mutations that have been identified in 80 patients with FHH, NSHPT and ADHH (Table 4.1, Figure 4.1). The majority (>50%) of these CaSR mutations are missense substitutions located within the ECD (Figure 4.1 and Figure 4.4). These and other previously reported disease-causing mutations have highlighted the importance of the ECD in CaSR function. The ECD has key roles in co-translational processing of the CaSR (240),
receptor dimerisation (241), binding different ligands that include Ca$^{2+}$ and amino acids (95,234,242), and transmitting activation signals to the TMD and ICD (243). The CaSR ECD has a bilobed VFTD that is predicted to contain 5 calcium binding sites, which are located at flexible loops or helical regions close to the protein surface (172). The presence of these multiple binding regions explains the ability of the CaSR to bind Ca$^{2+}$ in a cooperative fashion. Homology modelling of the 34 missense mutations (Table 4.1, Figure 4.1) characterised by our study and which affect VFTD residues revealed that >50% of these mutations are situated within 10 Å of one or more calcium binding sites (Figure 4.5). Thus, our study of disease-causing mutations emphasises the key role of the extracellular VFTD in binding Ca$^{2+}$. The VFTD calcium binding sites have been previously demonstrated to exhibit differing affinities for Ca$^{2+}$ (172). In particular, VFTD calcium binding sites-2 to -5, when compared to calcium binding sites-1 exhibit a lower affinity for Ca$^{2+}$, and are considered to be involved only in binding Ca$^{2+}$ in hypercalcaemic states (172). Given the auxiliary role of sites outside the VFTD cleft, in Ca$^{2+}$ binding, mutations at these calcium binding sites would be expected to induce a milder alteration in CaSR function than calcium binding sites-1 mutations. Homology modelling in this study revealed that a Pro339Thr mutation (245) was situated in the proximity of Ca$^{2+}$ binding site 4 that is located in lobe 1. Consistent with binding sites outside the VFTD cleft having a minor role in Ca$^{2+}$ binding, the Pro339Thr mutation has been reported to lead to a mild loss of function and was associated with normocalcaemia in the heterozygous state (245).

Over 70% of these calcium binding sites CaSR mutations are associated with FHH or NSHPT (Figure 4.1), and it therefore seems likely that the residues, which are within the VFTD either participate in the binding of Ca$^{2+}$, or indirectly enhance this process. Interestingly, the most commonly mutated residue within the VFTD was found to be arginine, which is able to
form multiple hydrogen bonds to carbonyl groups of the peptide backbone and be involved in maintaining tertiary protein structure (250). Mutated arginine residues were located at calcium binding sites-1 and -3, and of the 23 arginine residues, which comprise 4% of the VFTD residues, 5 (i.e. 22%) were involved in mutations that resulted in FHH or NSHPT, but not ADHH. This observation was confirmed by our analysis of the previously reported 200 different CaSR mutations (248) which revealed the occurrence of mutations at 30% of the VFTD arginine residues that were associated with FHH or NSHPT, and never ADHH. Hence it is possible that the arginine residues located at calcium binding sites-1 and -3 (Figure 4.5) promote CaSR activation by providing a favourable VFTD conformation for Ca$^{2+}$ binding. In contrast, 2 out of 5 (i.e. 40%) of the ADHH associated CaSR mutations in this study and 6 out of 10 (i.e. 60%) of the previously reported ADHH mutations located near to a calcium binding sites involved the substitution of a glutamate residue (Table 4.1). The mechanism of CaSR activation associated with the loss of a glutamate binding site residue may involve a reduction in the overall negative charge of that binding site, leading to a reduced repulsion between binding site residues that facilitates Ca$^{2+}$ binding (172).

Our identification and functional characterisation of mutations involving codons 173 and 221, which are located at the entrance to the VFTD cleft, and lead to either loss- or gain of CaSR function, help to illustrate further the important role of this region. Indeed, the VFTD cleft is the principal region for the binding of orthosteric ligands such as Ca$^{2+}$ and amino acids, and it has been previously demonstrated that this site has the greatest binding affinity for Ca$^{2+}$ and facilitates the cooperative binding of Ca$^{2+}$ to the other four calcium binding sites (172). Such a role for the VFTD cleft is consistent with many of the VFTD mutations being located near to this critical region and the observation that mutations of Glu297, which is located in the calcium
binding sites-1 and VFTD cleft, are also associated with opposing effects on CaSR function (96). Our analysis of the mutations at Leu173 predicted that the introduction of a mutant phenylalanine residue would enhance CaSR activation by increasing the area of interaction with Ca\(^{2+}\) at the VFTD cleft. The importance of phenylalanine for Ca\(^{2+}\) binding by the CaSR has previously been demonstrated by studies of Phe270, which is required to complete the coordination sphere for Ca\(^{2+}\) at calcium binding sites-1 (96). The introduction of the mutant Pro173 residue was predicted to disrupt an alpha helix at calcium binding sites-1 and thereby result in a loss of CaSR function. This alpha helix is adjacent to Ser170, which is directly involved in Ca\(^{2+}\) coordination, and loss of this secondary structure at calcium binding sites-1 may reduce the proximity of Ser170 to Ca\(^{2+}\). The predicted derangement of the architecture of calcium binding sites-1, in the mutant Pro173 receptor, is in keeping with the severe loss of function observed in our in vitro studies of this mutant receptor (Figure 4.3). Our analysis mutations at Pro221 predicted that the activating Leu221 mutation would enhance Ca\(^{2+}\) entry into calcium binding sites-1 due to the substitution of the rigid side chain of the wild-type proline residue at the entrance to the VFTD cleft with the more flexible leucine side chain. In contrast, the side chain of the mutant glutamine residue, associated with the Gln221 mutation, would be predicted to extend across the entrance of calcium binding sites-1, thereby impairing ligand entry and leading to a loss of CaSR function.

The value of undertaking such functional characterisation studies is demonstrated by our analysis of the Glu250Lys variant. The Glu250Lys variant has previously been reported by several studies to be a causative mutation in patients with FHH and PHPT (251,252), and is also listed in the calcium sensing receptor database (CASRdb) (248) as a recurrent mutation. The finding of this Glu250Lys variant in both FHH and ADHH patients in our study (Table 4.1),
suggested that this variant, which involves an evolutionary conserved Glu250 residue, may not be a mutation but instead a functionally neutral polymorphism. We therefore undertook a functional characterisation of the wild-type CaSR and Glu250Lys CaSRs which demonstrated that they each had an EC50 that was similar (Figure 4.3), thereby indicating that Glu250Lys was not a CaSR mutation but instead a polymorphism. Thus, our finding emphasises the value of undertaking functional characterisation of CaSR variants, which is not generally performed because of time and cost constraints. Instead variants are considered to be pathogenic mutations on the basis of co-segregation with the disease, absence in a panel of normal individuals, and alteration of an evolutionary conserved residue. Our findings indicate that this approach should be used with caution for recurrent variants and especially if they are observed in FHH and ADHH patients. Indeed, such variants should be evaluated by a functional assay as they may represent functionally neutral polymorphisms.

The NSHPT mutations described in this study included an Arg25Stop mutation (Figure 4.2) which represents the most N-terminal truncating mutation described to date. The mutant 25Stop allele would be predicted to produce a transcript that would undergo nonsense mediated decay (162), or result in the expression of a non-functional peptide that comprises the 19 amino acid signal peptide and an additional 6 amino acids. However, the Arg25Stop CaSR mutation was associated with severe hypercalcaemia in the homozygous state, while the heterozygous affected parents had differing phenotypes, with the mother being normocalcaemic and the father having mild hypercalcaemia. This finding of hypercalcaemia in the heterozygous father suggests that the effects of the 25Stop mutant CaSR maybe due to haploinsufficiency rather than a dominant-negative action on receptor dimerisation as previously reported (162,246,253). Indeed, such an effect due to CaSR haploinsufficiency would be consistent with the observations in Casr
mice, which have an ~50% reduction in CaSR protein expression, and exhibit mild (i.e. ~ 10%) increases in serum calcium concentrations (227). Thus, it seems likely in the Casr +/− mice and in humans with heterozygous null CaSR mutations that the hypercalcaemia results from a reduction in the expression of the wild-type receptor from the normal allele, consistent with a gene dosage effect or haploinsufficiency. The disparity in the phenotypes of individuals with the same CaSR mutation as observed in the hypercalcaemic father and normocalcaemic mother (Figure 4.2), which has been previously reported (254,255), may be due to the differences in genetic background and gene modifiers.

Mutations of the CaSR gene were not identified in >70% of the probands, and the phenotypes of these patients without CaSR mutations were similar to those with CaSR mutations. Therefore it seems likely that other mutations involving the non-coding regions of the CaSR, or mutations involving other mediators of calcium regulation may underlie the hyper- and hypocalcaemic phenotypes of these patients. Indeed, the occurrence of such genetic heterogeneity is supported by the identification of two other FHH loci located on chromosome 19p and 19q13 (256-258).

In conclusion, our study reports 71 CaSR mutations identified in patients with FHH, NSHPT or ADHH, and demonstrates the value of functional characterisation studies by showing that the Glu250Lys variant, which had been previously considered to be a recurrent mutation, is instead a functionally neutral polymorphism. Our study also highlights that NSHPT is associated with a high proportion of truncating CaSR mutations, such as Arg25Stop, which is one of the most N-terminal truncating mutations described to date, and that haploinsufficiency of the CaSR may have a role in the etiology of NSHPT. Furthermore, our results reveal that missense mutations of the CaSR ECD cluster at multiple calcium binding sites, and particularly in the
vicinity of the cleft region formed by the two lobes of the VFTD. Indeed, mutations of residues 173 and 221, located at the entrance to the VFTD cleft, were demonstrated to have opposing effects on receptor function and likely influence receptor function through their effects on the entry and binding of Ca\(^{2+}\) in the cleft region. Thus, our findings, which help to further elucidate the structure-function relationships of the CaSR extracellular domain, provide support for the VFTD cleft in having a major role in the binding of extracellular Ca\(^{2+}\) and regulation of CaSR function.
CHAPTER 5. DISEASE-ASSOCIATED “TOGGLE” MUTATIONS ON THE CALCIUM-SENSING RECEPTOR CHANGE THE CA$^{2+}$ AND L-PHE REGULATED FUNCTIONAL COOPERATIVITY

5.1 Introduction

The human calcium (Ca$^{2+}$)-sensing receptor (CaSR) is a seven transmembrane G protein-coupled receptor (GPCR) that is expressed at the highest levels in the parathyroid glands and kidneys (38). The principal role of CaSR is to sense alterations of the extracellular calcium concentration ([Ca$^{2+}$]$_o$) and to maintain [Ca$^{2+}$]$_o$ homeostasis by regulating parathyroid hormone (PTH) secretion as well as renal Ca$^{2+}$ reabsorption. Like other members of family C in the GPCR superfamily, including the metabotropic glutamate receptors, the V2R vomeronasal pheromone receptors, the TAS1R taste receptors, and the gamma-aminobutyric acid (GABA-B) receptors (241), CaSR possesses a large extracellular domain (ECD) consisting of more than 600 amino acids (95). Ca$^{2+}$, as the principal physiological agonist for CaSR, is thought to bind to the ECD resulting in activation of phospholipase C (PLC), with an attendant accumulation of inositol 1,4,5-triphosphate (IP$_3$) followed by a resultant increase in the cytosolic calcium ([Ca$^{2+}$]$_i$) concentration (101).

The downstream signaling pathways triggered by CaSR generally involve the interactions of intracellular loops 2 and 3 of the receptor with heterotrimeric G-proteins, including $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ (259). The $G_{q/11}$ proteins are linked to receptor-mediated activation of phospholipase-C$_{\beta}$ which plays critical roles in Ca$^{2+}$$_o$-mediated PTH secretion. The $G_{i/o}$ proteins inhibit adenylyl cyclase, while the $G_{12/13}$ proteins have been shown to activate monomeric G-proteins, for instance, Rho (259). Studies have shown that CaSR stimulates phosphorylation and concomitant activation of the extracellular signal-regulated kinases (ERK$_{1/2}$) through both a pertussis toxin-
sensitive pathway involving G\textsubscript{i} protein as well as a pertussis toxin-insensitive pathway utilizing G\textsubscript{q/11}. ERK\textsubscript{1/2} are known to modulate many cellular processes, including cell proliferation, differentiation, apoptosis, oncogenic transformation as well as enzymatic activity (260,261).

In biological systems, free L-amino acids are essential molecules since they not only serve as the building blocks of protein but are also the metabolic precursors of crucial substances that serve as ligands for receptors (165). Evidence for the presence of amino acid sensing mechanisms in various studies, e.g., regulating insulin secretion from pancreatic beta cells, hepatic autophagy, etc., suggest the widespread existence of amino acid sensors, although the identities of these amino acid sensors are in many cases unknown. These findings highlight amino acid-dependent control of cellular signal transduction pathways. Fluctuation of the plasma levels of amino acids can, therefore, regulate the rate of hormone synthesis and secretion as well as Ca\textsuperscript{2+} metabolism, among other processes (165). CaSR is present throughout the gastrointestinal tract (41,166), including in gastrin-secreting antral G cells and cholecystokinin-secreting I cells, both of which are known to be activated by both [Ca\textsuperscript{2+}]\textsubscript{o} and aromatic amino acids (166). L-amino acids, especially aromatic amino acids, are known to enhance the sensitivity of CaSR to [Ca\textsuperscript{2+}]\textsubscript{o}, which could be one potential explanation for how dietary protein modulates [Ca\textsuperscript{2+}]\textsubscript{o} homeostasis in normal individuals as well as in patients with chronic renal failure (165,167).

Mutations of the CaSR can perturb intracellular signaling events (e.g., intracellular calcium response) and disrupt the regulation of PTH secretion from the parathyroid chief cells and of Ca\textsuperscript{2+} reabsorption in the renal tubule. Mutations that inactivate (i.e., cause loss-of-function)
CaSR result in familial hypocalciuric hypercalcemia (FHH) and neonatal severe primary hyperparathyroidism (NSHPT) (161,240) by producing a rightward shift in the CaSR’s concentration–response curve, with a significantly higher EC$_{50}$ in $in vitro$ studies. On the other hand, activating mutations of the CaSR lead to autosomal dominant hypocalcemia with hypercalciuria (ADHH) (245), and produce a leftward shift of the concentration–response curve and lower EC$_{50}$ (163). These mutations, including the four studied here and described below that involve the two “toggle” residues, L173 and P221, where mutations of the same residue can either activate or inactivate the CaSR, serve as a rich source of structure-function information and have provided important insights into how the receptor functions.

Molecular dynamics (MD) simulation provides an approach complementary to the experiments in live cells for understanding biomolecular structure, dynamics and function (220) and computational modeling of the GPCR 3D structures have become increasingly indispensable tools for studying these receptors as well as discovery of the drugs which effect their behavior. Using our previously reported computational algorithm, we have predicted five potential Ca$^{2+}$-binding sites in the CaSR ECD based on the charge distribution on the surface of the Venus flytrap (VFT) domain and on the known structural properties of Ca$^{2+}$-binding sites (68,172). The CaSR ECD was modeled from the known structure of the ECD of metabotropic glutamate receptor type 1 (mGluR1) (175),Ca$^{2+}$-binding site 1 in the CaSR ECD is located in the hinge region between lobes 1 and 2, and is proposed to be the principle site for Ca$^{2+}$-binding during receptor activation. Moreover, the putative L-Phe binding site is also predicted to be located nearby in the hinge region (221). Interestingly, among 34 identified ECD missense mutations in patients with FHH, NSHPT and ADHH, 18 are located within 10 Å of one or more of the predicted Ca$^{2+}$-binding sites, particularly site 1(208). Four disease-related mutations involving
residues 173 and 221, which are located at the entrance to the Ca\(^{2+}\)-binding site in the VFT cleft, have been identified. Two of these are activating mutations (L173F, P221L) and two are inactivating mutations (L173P, P221Q). These four so-called “toggle” mutations, defined as mutations involving the same residue that lead to opposite effects on receptor function, are near Ca\(^{2+}\)-binding site 1 in our predicted ECD model structure, thereby highlighting the importance of these residues for entry and binding of Ca\(^{2+}\) by the CaSR ECD. (161).

In the present studies, we extended Dr. Hannan’s studies to investigate whether residues L173 and P221 are important for the CaSR’s positive homotropic cooperativity and for the coupling of the receptor to several intracellular signaling cascades. We also utilized these four mutations to further probe the structure-function relationships of the CaSR as they relate to the activation of the CaSR by Ca\(^{2+}\) and L-Phe and how L-Phe interacts with Ca\(^{2+}\) to modulate intracellular signaling. These results provide important insights into how Ca\(^{2+}\) and L-Phe interact at their respective binding sites in the cleft of the VFT.

5.2 Results

5.2.1 The disease-related mutations show altered [Ca\(^{2+}\)]\(i\) oscillation patterns compared with WT CaSR.

The two residues involved in the four disease-associated mutations (L173, P221) are located in the hinge region near predicted calcium-binding site 1, which is between lobes 1 and 2 as shown in Figure 4.1a. The immunostaining results suggest that all of these mutant CaSRs can be expressed at the cell membrane (Figure 5.1b). We investigated the impact of these four disease-associated mutations on [Ca\(^{2+}\)]\(_o\)-induced changes in [Ca\(^{2+}\)]\(i\) to examine their effects on the pattern of [Ca\(^{2+}\)]\(i\) oscillations, apparent affinity and positive homotropic cooperativity of the CaSR (Figure 5.1c). Three parameters were employed to analyze the [Ca\(^{2+}\)]\(i\) oscillation patterns:
the starting point, which refers to the \([\text{Ca}^{2+}]_o\) at which a given cell starts to show at least three continuous, sequential peaks; the ending point, which considers the \([\text{Ca}^{2+}]_o\) at which the \([\text{Ca}^{2+}]_i\) reaches to a plateau, and the frequency, which is defined as the number of peaks of \([\text{Ca}^{2+}]_i\) per minute. A stepwise increase in \([\text{Ca}^{2+}]_o\) induced oscillations in \([\text{Ca}^{2+}]_i\) in some HEK293 cells transfected with WT CaSR (Figure 5.2, WT). Frequency distribution showed that about 53% of the cells showing oscillatory responses started to show \([\text{Ca}^{2+}]_i\) oscillations at 2.5 mM \([\text{Ca}^{2+}]_o\) (Figure 5.3a). The frequency of oscillations at 2.5 mM \([\text{Ca}^{2+}]_o\) in these cells exhibited a normal distribution with an average frequency in the range of 1.0~1.5 peaks/min (Figure 5.3b). About 50% of the oscillatory cells reached a \([\text{Ca}^{2+}]_i\) plateau at 2.5 mM \([\text{Ca}^{2+}]_o\) (Figure 5.3c). The gain-of-function mutations have an impact on the responsiveness of the cells to changes of \([\text{Ca}^{2+}]_o\). This behavior was reflected in the following ways: firstly, the majority of the cells, 54% for L173F and 40% for P221L, started to oscillate at lower levels of \([\text{Ca}^{2+}]_o\), 1.5 mM and 2.0 mM, respectively, than observed with the WT CaSR. Secondly, the average frequencies at 2.5 mM \([\text{Ca}^{2+}]_o\) increased to 1.7 ± 0.1 peaks/min (p<0.05) and 1.6 ± 0.1 peaks/min, respectively (Table 4.1). The majority of the cells transfected with the gain-of-function mutations had oscillations ending points at lower \([\text{Ca}^{2+}]_o\) level (≤ 3.0 mM) than for WT CaSR. Conversely, the loss-of-function mutations had opposite effects on the respective oscillation patterns and apparent affinities. The majority of cells transfected with the mutant containing P221Q did not oscillate until \([\text{Ca}^{2+}]_o\) reached 4.0 mM, and the threshold for \([\text{Ca}^{2+}]_i\) oscillations in the cells transfected with L173P was even higher at 15.0 mM. It was difficult to compare the \([\text{Ca}^{2+}]_i\) oscillation frequencies of these two mutants at any given level of \([\text{Ca}^{2+}]_o\), because they responded to changes in \([\text{Ca}^{2+}]_o\) over a substantially different range. Therefore, the frequencies were measured at the individual EC\(_{50}\)s of their \([\text{Ca}^{2+}]_o\)- concentration response curves. Notably, these two
mutations also disrupted the cooperativity of the receptor as reflected by the decreased Hill number (P221Q) or an alteration of the monophasic $[\text{Ca}^{2+}]_o$-induced $[\text{Ca}^{2+}]_i$ concentration response curve to a biphasic one (L173P) (Table 4.1).

Figure 5.1 The disease-related mutations are located in the hinge region near $\text{Ca}^{2+}$-binding site 1.
(a) The model structure of the CaSR ECD, which was based on the mGluR1 crystal structure (PDB entry: 1ISR) was generated using MODELER 9v4 and Swiss-Model. The global view of the ECD is shown in the left panel. Spheres highlighted in red: Ca\textsuperscript{2+}; residues involved in predicted Ca\textsuperscript{2+}-binding sites: purple. L-phenylalanine (in yellow) was positioned at the hinge region between the two lobes by Autodock vina. Right panel: A zoomed in view of the site 1 Ca\textsuperscript{2+}-binding pocket. Labels highlighted in black represent residues involved in the Ca\textsuperscript{2+}-binding site 1; residues with disease-related mutations are highlighted in purple. (b) Surface expression of CaSR and its four disease related toggle mutants. Green: anti-flag staining of the surface expressed CaSR. (c) Illustrated are intracellular responses induced by Ca\textsuperscript{2+}-and L-Phe, and the principle underlying the IP\textsubscript{1} accumulation assay. The addition of Li\textsuperscript{+} impeded the degradation of IP\textsubscript{1}. 

Figure 5.2 Functional studies of disease-related CaSR mutations in HEK293 cells.

Representative oscillation pattern from a single cell. HEK-293 cells transiently transfected with CaSR or one of its mutants were loaded with Fura-2 AM for 15 min. \( [\text{Ca}^{2+}]_i \) was assessed by monitoring emission at 510 nm with excitation alternately at 340 or 380 nm as described in methods. Each experiment was carried out with or without 5 mM L-Phe began in \( \text{Ca}^{2+} \)-free Ringer’s buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, and 1.0 mM MgCl\(_2\), pH 7.4), which was followed by stepwise increases in \( [\text{Ca}^{2+}]_o \) until \( [\text{Ca}^{2+}]_i \) reached a plateau (up to 30 mM \([\text{Ca}^{2+}]_o\)).
Figure 5.3 The frequency distribution of the starting points, frequencies and the ending points extracted from the \([\text{Ca}^{2+}]_i\) oscillation patterns in HEK293 cells transfected with CaSR or its mutants.

(a). Frequency distribution of the starting points of \([\text{Ca}^{2+}]_i\) oscillations in HEK-293 cells transfected with WT CaSR, or the L173F, L173P, P221L or P221Q mutants, respectively, in the presence or absence of L-Phe. \([\text{Ca}^{2+}]_o\) was recorded at the point when single cells started to oscillate. Around 30~60 cells were analyzed and further plotted as a bar chart.

(b). The frequencies of the individual cell oscillation patterns were investigated. For experiments without L-Phe, the peaks per minute were recorded at the level of \([\text{Ca}^{2+}]_o\) at
which the majority of the cells (>50%) started oscillating. Specifically, for the gain-of-function mutants, the peaks per minute were recorded at 2.5 mM [Ca$^{2+}$]$_o$; while for loss-of-function mutants, the frequency was analyzed at their individual EC$_{50}$s calculated from the concentration response curves for [Ca$^{2+}$]$_o$. For experiments with 5.0 mM L-Phe, the frequency was analyzed at the same EC$_{50}$s for [Ca$^{2+}$]$_o$ that were used in the absence of L-Phe. Empty bar: in the absence of L-Phe; black bar: in the presence of 5 mM L-Phe. (c). Frequency distributions of the ending points for [Ca$^{2+}$]$_i$ oscillations.
Table 5.1 Summary of [Ca\(^{2+}\)]_o-evoked [Ca\(^{2+}\)]_i concentration response data in HEK293 cells transiently transfected with WT CaSR and its mutants.

The cells were exposed to stepwise increases in [Ca\(^{2+}\)]_o in the absence or presence of 5 mM L-Phe. For WT, L173F and P221L, the frequency were recorded at 2.5 mM [Ca\(^{2+}\)]_o, while for L173P, the frequency was measured at 15.0 mM [Ca\(^{2+}\)]_o; and for P221Q the frequency at 5.0 mM [Ca\(^{2+}\)]_o was chosen for comparison. The WT oscillation frequency measured at 3.0 mM [Ca\(^{2+}\)]_o is indicated by the brackets. Curve-fitting was performed using the Hill equation. The data were obtained from three experiments for each construct. # indicates significance with respect to wild type CaSR in the absence of L-Phe, p < 0.05; * indicates significance with respect to the corresponding mutants in the absence of L-Phe, p < 0.05.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>EC(_{50})[Ca(^{2+})]_o</th>
<th>Hill coefficient</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w/o L-Phe</td>
<td>with 5 mM L-Phe</td>
<td>w/o L-Phe</td>
</tr>
<tr>
<td>WT</td>
<td>3.0 ± 0.2</td>
<td>1.9 ± 0.3*</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>L173F</td>
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<td>1.6 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>P221L</td>
<td>2.0 ± 0.1#</td>
<td>1.7 ± 0.1</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>L173P (Phase 1)</td>
<td>3.8± 0.3</td>
<td>3.1 ± 0.2</td>
<td>3.3 ± 0.6</td>
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<tr>
<td>L173P (Phase 2)</td>
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<td>12.2 ± 0.2*</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>P221Q</td>
<td>5.2 ± 0.4#</td>
<td>3.7 ± 0.4*</td>
<td>2.4 ± 0.4*</td>
</tr>
</tbody>
</table>
5.2.2 L-Phe selectively potentiates the $[\text{Ca}^{2+}]_o$-evoked $[\text{Ca}^{2+}]_i$ responses among the four mutants.

Since the potential L-Phe binding pocket, similar to that for $\text{Ca}^{2+}$, is also located in the cleft of the VFT (Figure 5.1), we next assessed the impact of L-Phe on $[\text{Ca}^{2+}]_o$-induced changes in $[\text{Ca}^{2+}]_i$ in order to study the interactions between the functional effects of $[\text{Ca}^{2+}]_o$ and L-Phe and the impact of the four mutations. For the majority of HEK293 cells transfected with the WT CaSR, $[\text{Ca}^{2+}]_i$ oscillations began at 2.0 mM $[\text{Ca}^{2+}]_o$ and ended at 3.0 mM $[\text{Ca}^{2+}]_o$ in the presence of 5 mM L-Phe, and the oscillation frequency at 2.5 mM $[\text{Ca}^{2+}]_o$ increased to $2.1 \pm 0.1$ peaks/min, which was significantly higher than the $1.3 \pm 0.1$ peaks/min observed in the absence of L-Phe (Figure 5.3). As shown in Figure 5.3, this allosteric activator exhibited different effects among the four mutant receptors. For the gain-of-function mutations (L173F, P221L), L-Phe lowered the threshold for the initiation and the termination of $[\text{Ca}^{2+}]_i$ oscillations, but barely increased their frequency at 2.5 mM $[\text{Ca}^{2+}]_o$ (Figure 5.3 middle panel & Figure 5.3 right panel). In terms of the loss-of-function mutants, cells transfected with mutant L173P started oscillating at 10.0 mM $[\text{Ca}^{2+}]_o$ in the presence of 5.0 mM L-Phe and in most cells the $[\text{Ca}^{2+}]_i$ oscillations stopped at 25.0 mM $[\text{Ca}^{2+}]_o$; in contrast, without L-Phe, 80% of the cells were still oscillating at 30 mM $[\text{Ca}^{2+}]_o$. Regarding the effect of L-Phe on the frequency parameter, the level of $[\text{Ca}^{2+}]_o$ at which the majority of the cells (>50%) started to oscillate in the absence of L-Phe was chosen to assess the influence of the amino acid. The L173P mutant had a frequency of $1.8 \pm 0.2$ peaks/min at 15.0 mM $[\text{Ca}^{2+}]_o$ in the presence of L-Phe, which was significantly higher than the rate in the absence of this allosteric agonist observed at the same level of $[\text{Ca}^{2+}]_o$ ($1.3 \pm 0.1$) (Figure 5.3b & Table 5.1). For the P221Q mutant, the threshold for initiation of $[\text{Ca}^{2+}]_i$ oscillations decreased from 5.0 mM to 3.5 mM $[\text{Ca}^{2+}]_o$, while the ending point also decreased in the presence of L-Phe, There was an increase in oscillation frequency for this mutant from $1.6 \pm 0.1$ peaks/min to $2.2 \pm$
0.1 peaks/min with L-Phe at 5.0 mM [Ca^{2+}]_o.

To evaluate the impact of the mutations on the apparent affinities and the cooperativity of the responses of the receptor to [Ca^{2+}]_o and L-Phe, the averaged [Ca^{2+}]_i response at each level of [Ca^{2+}]_o were plotted to construct [Ca^{2+}]_o concentration-response curves for [Ca^{2+}]_i as shown in Figure 5.4. L-Phe shifted the sigmoid curve to the left producing a lower EC_{50} for the [Ca^{2+}]_o-evoked increase in [Ca^{2+}]_i in WT CaSR-transfected HEK293 cells as well as in cells transfected with the P221Q mutant. Interestingly, although considered an inactivating mutation, L-Phe did not left-shift the activation of the CaSR mutant L173P by [Ca^{2+}]_o to the same extent that it did with the other loss-of-function mutant, P221Q, potentially because of the proximity of this residue to the binding site for L-Phe, which might interfere with L-Phe binding. The disruption of the positive homotropic cooperativity induced by [Ca^{2+}]_o as a result of the Leu to Pro change, which was reflected as a biphasic concentration response curve, was also not corrected by the addition of L-Phe (Table 4.1). Meanwhile, the addition of L-Phe barely further left-shifted the concentration-response curves of the two gain-of-function CaSR mutants (p>0.05).
Figure 5.4  L-Phe modulates the $[Ca^{2+}]_o$ concentration response curves for $[Ca^{2+}]_o$ in CaSR-transfected HEK293 cells.

The $[Ca^{2+}]_i$ responses of HEK293 cells transiently overexpressing WT CaSR or disease-related mutations were measured using Fura-2AM during stepwise increases in $[Ca^{2+}]_o$ from 0.5 to 30 mM with or without L-Phe as above. The ratio of light emitted at 510 nm upon alternate excitation with 340 or 380 nm was normalized to its maximum response. And the $[Ca^{2+}]_i$ responses at various $[Ca^{2+}]_o$ were plotted and further fitted using the Hill equation. Open circle: in the absence of L-Phe; Closed circles: in the presence of 5 mM L-Phe.
5.2.3 L-Phenylalanine potentiates $[Ca^{2+}]_o$-induced IP$_1$ accumulation.

To determine whether the functional impact of the four mutations on $[Ca^{2+}]_i$ extended to other functional read-outs, we examined the effects of $[Ca^{2+}]_o$ and L-Phe on the activation of PLC as assessed by IP$_1$ accumulation and, in the following section, on ERK activity. Activation of the G$_{q/11}$ pathway by GPCRs activates phospholipase C (PLC), causing accumulation of inositol phosphates (IPs) (101). The responses to $[Ca^{2+}]_o$ in the absence and presence of L-Phe in cells transiently transfected with WT CaSR or the four mutants were assessed by measuring the accumulation of intracellular IP$_1$ (Figure 5.5). The EC$_{50}$ for the stimulation of IP$_1$ accumulation was 2.9 ± 0.3 mM for the WT CaSR, which agrees well with the EC$_{50}$ calculated from the $[Ca^{2+}]_i$ responses (3.0 ± 0.2). The addition of 5 mM L-Phe significantly elevated the level of IP$_1$ accumulation at 2.0 and 3.0 mM $[Ca^{2+}]_o$ resulting in a left-shifted $[Ca^{2+}]_o$-IP$_1$ concentration response curve. In contrast, the two gain-of-function mutants, L173F and P221L, showed higher levels of IP$_1$ accumulation at 1.0, 2.0 and 3.0 mM $[Ca^{2+}]_o$ compared with the WT CaSR, leading to lower EC$_{50}$s for the IP$_1$ responses (1.3 ± 0.1 and 1.1 ± 0.1 respectively) (Table 4.2). On the other hand, the loss-of-function mutations, L173P and P221Q, exhibited impaired $[Ca^{2+}]_o$-stimulated IP$_1$ accumulation as the EC$_{50}$s for the $[Ca^{2+}]_o$-IP$_1$ concentration response curves were higher than with WT (10.8 ± 1.9 and 6.5 ± 0.4 respectively, vs. 2.9 ± 0.3 mM for the WT CaSR). In the presence of L-Phe, the EC$_{50}$ was reduced to 4.3 ± 0.1 (p<0.05) in the case of P221Q, 8.9 ± 1.7 (p>0.05) in the case of L173P. On the contrary, L-Phe failed to reduce the EC$_{50}$ of L173P significantly. The EC$_{50}$s of the IP$_1$ responses for the two receptors with activating mutations, L173F and P221L, were not altered by L-Phe either. The outcome of IP1 experiments were consistent, therefore, with the $[Ca^{2+}]_o$ experiments in terms of EC$_{50}$. However, it seems that the alteration in the $[Ca^{2+}]_o$ oscillation pattern for L173P was not dramatic enough to change the
overall population response.

Figure 5.5. L-Phenylalanine potentiates $[Ca^{2+}]_o$-induced IP$_1$ accumulation.

IP accumulation was measured in the IP-One ELISA Kit as detailed in Methods. Changes in IP$_1$ accumulation were measured in response to $[Ca^{2+}]_o$ at 0, 1.0, 2.0, 3.0, 5.0 and 7.5 mM in HEK293 cells transfected with WT CaSR, or with mutant L173F or P221L. For mutant L173P, IP$_1$ accumulation was measured at 0, 3.0, 5.0, 7.5, 10.0, 15.0, 20.0 and 25.0 mM $[Ca^{2+}]_o$; for mutant P221L, the IP$_1$ responses to $[Ca^{2+}]_o$ at 0, 3.0, 5.0, 7.5, 10.0, 15.0 mM $[Ca^{2+}]_o$ were recorded. The IP$_1$ response-$[Ca^{2+}]_o$ concentration responses were fitted using Hill equation as mentioned in method.
Table 5.2 Summary of EC$_{50}$s from experiments measuring IP$_1$ accumulation and ERK$_{1/2}$ phosphorylation.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>EC$_{50}$ (IP$_1$-Elisa)</th>
<th>EC$<em>{50}$ (ERK$</em>{1/2}$ activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without L-Phe</td>
<td>With L-Phe</td>
</tr>
<tr>
<td>WT</td>
<td>3.0 ± 0.2</td>
<td>2.0 ± 0.1$^*$</td>
</tr>
<tr>
<td>L173F</td>
<td>1.2 ± 0.1$^#$</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>P221L</td>
<td>1.1 ± 0.1$^#$</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>L173P</td>
<td>10.7 ± 1.0$^#$</td>
<td>9.0 ± 0.9</td>
</tr>
<tr>
<td>P221Q</td>
<td>6.1 ± 0.3$^#$</td>
<td>4.5 ± 0.4$^*$</td>
</tr>
</tbody>
</table>

HEK293 cells were transiently transfected with the WT CaSR or disease-associated CaSR mutants. Cells were then treated with various levels of [Ca$^{2+}$]$_o$. The EC$_{50}$s of the IP$_1$-[Ca$^{2+}$]$_o$ concentration responses and the ERK$_{1/2}$ phosphorylation activity-[Ca$^{2+}$]$_o$ concentration responses were obtained from curve fitting using the Hill equation as described in Methods. # indicates significance with respect to wild type CaSR, p < 0.05; * indicates significance with respect to the corresponding mutants in the absence of L-Phe, p < 0.05.

5.2.4 The ERK$_{1/2}$ responses of the WT and mutants CaSRs correlate with their [Ca$^{2+}$]$_i$ responses to [Ca$^{2+}$]$_o$.

In order to understand the effects of the “toggled” mutations on the function of the CaSR and its downstream signal pathways (Figure 5.1c) as well as to explore whether L-Phe can
regulate these mutants as suggested by the computer simulation results, we further analyzed [Ca^{2+}]_i oscillation patterns induced by [Ca^{2+}]_o in HEK293 cells transfected with those mutations.

Exposure of WT CaSR-transfected HEK293 cells to increasing concentrations of [Ca^{2+}]_o in the range of 0.0–2.0 mM for 10 minutes had little effect on the phosphorylation of p44/42 ERK. Greater increases of [Ca^{2+}]_o resulted in the accumulation of p44/42 ERK, which exhibited a maximum response at 8.0 mM [Ca^{2+}]_o. In the presence L-Phe, the ERK(1/2) activity was enhanced. As shown in Figure 5.6 and Table 5.2, the amount of phosphorylated ERK(1/2) was increased significantly at 2.0 mM [Ca^{2+}]_o compared with that observed at the same level of [Ca^{2+}]_o in the absence of L-Phe. The ERK(1/2) activity in the presence of L-Phe reached a maximum at 4.0 mM [Ca^{2+}]_o. For the activating mutation, L173F, the phosphorylated ERK(1/2) signal could be detected at 2.0 mM [Ca^{2+}]_o which was in line with its enhanced intracellular calcium response compared to WT CaSR. However, although L-Phe didn’t significantly shift the [Ca^{2+}]_o triggered [Ca^{2+}]_i response curve, it enhanced the phosphorylation of ERK(1/2) even at physiological [Ca^{2+}]_o concentrations in the mutant L173F. The maximum ERK(1/2) activity in cells transfected with the L173F mutant was achieved at 3.0 mM [Ca^{2+}]_o with 5.0 mM L-Phe. The mutation P221L enhanced ERK(1/2) activity in the transfected cells, which approached a maximum at 3.0 mM [Ca^{2+}]_o. The addition of L-Phe failed to activate the ERK(1/2) signaling at lower levels of [Ca^{2+}]_o, but the maximal p-ERK(1/2) activity was enhanced ~15% in the presence of L-Phe. On the other hand, the ERK(1/2) responses of the loss-of-function mutants, L173P and P221Q, correlated with the patterns observed for [Ca^{2+}]_i oscillations, as the phosphorylation of ERK(1/2) started at 10.0 mM and 3.0 mM [Ca^{2+}]_o, respectively, similar to the initiation of [Ca^{2+}]_i oscillations. The addition of 5 mM L-Phe shifted the [Ca^{2+}]_o-stimulated p-ERK(1/2) concentration response curves of mutant P221Q to the left but had little influence on the rescue of the mutant
Figure 5.6 L-Phe potentiates $[\text{Ca}^{2+}]_o$-activated ERK signaling in CaSR-transfected HEK293 cells.

HEK-293 cells transfected with WT CaSR or its mutants were incubated in serum-free high glucose MEM medium containing 0.2% BSA overnight. Cells were washed with HBSS and then incubated in the presence of various Ca$^{2+}$ concentrations ($0.0$--~$25.0$ mM) in the absence or presence of 5 mM L-phenylalanine for 10 min at 37 °C. The incubations were stopped by exposure to the lysis buffer and processed for SDS/PAGE and Western blotting as described in the Methods. The western blot results were further quantified using Image J. All $[\text{Ca}^{2+}]_o$-concentration response curves were normalized to the maximum response in each individual experiment. The Hill equation was employed to fit the data. Solid dots: with L-Phe; Empty dots: $[\text{Ca}^{2+}]_o$ only.
5.2.5 CaSR mutants exhibit different sensitivities to L-Phe

To determine whether the four mutations impacted the apparent affinity of the receptor for L-Phe, concentration responses for L-Phe at physiological calcium levels of 1.5 mM [Ca$^{2+}$]$_o$ and 2.5 mM [Ca$^{2+}$]$_o$ (intermediate levels of [Ca$^{2+}$]$_o$ at which the two gain-of-function mutants are not desensitized based on their responses to [Ca$^{2+}$]$_o$) were compared among cells transfected with different CaSR mutants. Figure 5.7 shows that at 1.5 mM [Ca$^{2+}$]$_o$ mutant L173F has a higher sensitivity (EC$_{50}$ = 2.9 ± 0.1) than WT and the other mutants. On the contrary, the loss-of-function mutant L173P shows a lower sensitivity (EC$_{50}$ = 5.5 ± 0.3 mM) to L-Phe compared to the other mutants. P221L and P221Q do not show any differences from WT at this level of [Ca$^{2+}$]$_o$. When analyzing the sensitivity to L-Phe at their respective EC$_{50}$s for [Ca$^{2+}$]$_o$, P221Q exhibited a reduced EC$_{50}$ of the [L-Phe] response (2.5 ± 0.2 mM, p<0.05) while L173P remained a similar sensitivity to L-Phe (4.9 ± 0.5 mM, p>0.05) (Figure 5.7, Table 5.3).

In order to explore how the four mutants have such different sensitivities to L-Phe, the solvent accessibility at the hinge region has been analyzed. As shown in Figure 5.8, mutation L173F, P221L and P221Q barely altered the surface potential at the predicted site of entry to the L-Phe binding site, which is formed by residues S170, S169, and T145 (221,223); however, the mutation from leucine to proline at position 173 contributes significantly to the clustering of positive charges in the cleft between lobe 1 and lobe 2. On the other hand, the mutation also decreased the original distance between residue L173 and one of the critical calcium binding site ligand residues, Y218 from 10.8 Å to 8.7 Å (in the holo form) and from 14.2 Å to 12.2 Å (in the apo form), which might make it more difficult for a hydrophobic amino acid to enter into its
binding site.

Figure 5.7 Sensitivity of various CaSR mutants to L-Phe in HEK293 cells.

(a). HEK-293 cells transfected with CaSR or its mutants were loaded with Fura-2 AM for 15 min. The intracellular Ca$^{2+}$ level was assessed by monitoring emission at 510 nm with excitation alternately at 340 or 380 nm using fluorescence microscopy as above. Each experiment started with 1.5 mM mM Ca$^{2+}$ followed by stepwise increases in the level of L-Phe (up to 12.0 mM) while [Ca$^{2+}$]$_o$ maintained at 1.5 mM. (b). The sensitivity of P221Q to L-Phe at 5.0 mM [Ca$^{2+}$]$_o$. The [Ca$^{2+}$]$_i$ signal was measured using Frua-2 AM as mentioned above. The experiment was carried out in the presence of 5.0 mM [Ca$^{2+}$]$_o$ while increasing the L-Phe concentration. (c). The sensitivity of L173P to L-Phe was monitored in the presence of 15.0 mM [Ca$^{2+}$]$_o$. 
Table 5.3 Summary of EC$_{50}$s of concentration-response curves for L-Phe at different [Ca$^{2+}$]$_{o}$.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>EC$<em>{50}$ (at 1.5 mM [Ca$^{2+}$]$</em>{o}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>L173F</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>P221L</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>L173P</td>
<td>5.5 ± 0.3# (4.9 ± 0.5)</td>
</tr>
<tr>
<td>P221Q</td>
<td>4.5 ± 0.4 (2.5 ± 0.2*)</td>
</tr>
</tbody>
</table>

The [Ca$^{2+}$]$_{i}$ responses of HEK-293 cells transfected with CaSR or its mutants upon stepwise increases of L-Phe in the presence of 1.5 mM [Ca$^{2+}$]$_{o}$ were recorded. For L173P and P221Q, the L-Phe-induced [Ca$^{2+}$]$_{i}$ change were also measured in the presence of high [Ca$^{2+}$]$_{o}$ corresponding to their EC$_{50}$ values for [Ca$^{2+}$]$_{o}$, specifically 15.0 mM [Ca$^{2+}$]$_{o}$ for mutant L173P and 5.0 mM for P221Q. The results are shown in brackets. The EC$_{50}$s were calculated from the concentration-response curves fitted using the Hill equation. # indicates significance with respect to wild type CaSR, p < 0.05; * indicates significance with respect to the corresponding mutants in the presence of 1.5 mM [Ca$^{2+}$]$_{o}$, p < 0.05.
Figure 5.8 Surface potential of the hinge region in the extracellular domain of CaSR.

The zoomed in view of the cleft between lobe1 and lobe2 of WT CaSR or its mutants was depicted using pymol. Upper panel: using the apo form of the CaSR models; Lower panel: using the holo form of the CaSR models. Residues involving the “toggle” disease mutations are labeled in black. Negative potentials are shown in red, and positive potentials are shown in blue.

5.2.6 The protein correlation profiling of the disease-related mutations as assessed by molecular dynamic simulations.

Since it has been reported that 18 out of 34 newly found naturally-occurring missense mutations on the ECD are within 10 Å of one or more of the predicted Ca$^{2+}$-binding sites (208), and a few disease-associated human mutations severely affect the functional cooperativity of CaSR (161), an investigation on the mechanisms of the cooperativity alterations induced by
mutations as well as allosteric modulators is highly desirable. Molecular dynamic simulation provides an approach complementary to in vitro experiments for understanding biomolecular structure, dynamics, and function (220). To obtain additional insights into the impacts of these various mutations on the structure and function of the CaSR, we carried out molecular dynamics simulations, as shown in Figure 5.9. The correlation map in Figure 5.9 illustrates the protein motions predicted using such simulations. The X- and Y-axes correspond to residue numbers of the CaSR ECD minus 24 (in order to align with the structure of mGluR1). In the WT model, site 1 is shown to have strong correlated motions with sites 2, 3 and 4, which are shown in blue for negative correlations (motions between each pair of residues taking place in the opposite direction) and red for positive correlations (movements in the same direction) (Figure 5.9). The correlation maps of the gain-of-function mutations, L173F and P221L, exhibited dramatic increases in the regions with either blue or red color indicating an enhancement of correlated motions between the respective residues in these two mutants (Figure 5.9). A closer analysis of the correlated motions of L173F and P221L revealed that the negative correlations between Site 1 and Site 3 and between Site 1 and Site 2 observed in the WT correlation map became positive correlations in the gain-of-function mutants indicating that the mutations might have profound impacts on the dynamic properties of the CaSR-ECD. Moreover, greater correlated motions were observed among residues 344-454, among residues 24-124, and between these two groups of residues. Especially for P221L, strong negative correlations between lobe 1 and lobe 2 are observed as indicated by the abundant blue area between residues 200-300 and residues 24-170. On the other hand, the loss-of-function mutations, L173P and P221Q, exhibit weaker correlated motions compared to the WT CaSR (Figure 5.9).
Since L-Phe is considered as one of the positive allosteric modulators to the CaSR, we investigated whether L-Phe can change the dynamic features of these mutants. The introduction of L-Phe and calcium to the simulation system dramatically changed the correlated motions of WT (data not shown) and mutant L173F, P221L and P221Q, but had less effect on the correlation map of L173P (Figure 5.9). These results suggested that the allosteric modulator may have biased influences on changing the correlation motions among these mutants.

Figure 5.9 The correlation map of the modeled WT and mutant CaSR ECD structure.

The correlation map is depicted based on molecular dynamics (MD) simulations for 50 ns. The X axis and Y axis are residue numbers of CaSR ECD sequences minus twenty four.
The strongest negative correlation is given the value -1 while the strongest positive correlation is defined as +1. Residues that have the strongest negative correlated motions are shown in blue, while those involved in positive correlated motions are shown in red. Green stands for no apparent correlated motion between the two residues.

Principal component analysis (PCA) was also applied to the four mutations and the WT CaSR as well based on the trajectories of molecular dynamics simulations in order to predict the effect that L-Phe could have on the CaSRs at the atomic level. PCA separates out the protein motions into principal modes ranked according to their relative contributions (199). Projection of the trajectories of the CaSR mutants in both the apo form (upper panel) and the holo form (lower panel) onto the first three modes that accounted for the majority of the total fluctuations is shown in Figure 5.10. In the Ca\(^{2+}\)-free and L-Phe-free forms, the conformations of the CaSR mutants sampled are similar to those of the WT CaSR. However, in the L-Phe and Ca\(^{2+}\)-loaded forms, the conformations of the CaSR mutant L173P is essentially distinguishable from other mutants and WT CaSR (Figure 5.10). The results suggest that Ca\(^{2+}\) and L-Phe might shift the population of conformational ensembles of mutant L173P in a different way compared with WT or the other three mutants.
Figure 5.10 Principal component analysis (PCA) of the wild type and mutant CaSR ECDs.

The trajectories of the molecular dynamics simulations were analyzed using principal component analysis (PCA), which separates out the motions of different CaSR ECD mutants into principal modes ranked according to their relative contributions. The first three principal modes were included in the present study to analyze two different states of the protein: upper: apo form; lower: holo form. Different colors stand for each individual CaSR or its mutants: WT (black), L173F (Green), L173P (Red), P221L (Magenta), P221Q (Blue).
5.3 Discussion

The loss-of-function mutations impaired the functional cooperativity of the CaSR

We have reported 71 CaSR mutations identified in 220 patients with previously undiagnosed hyper- or hypocalcemic disorders. More than 50% of the mutations are located within 10 angstroms of our predicted Ca$^{2+}$-binding sites (208). The hinge region is considered to be crucial in terms of the CaSR’s sensing of its agonists (e.g., polyvalent cations and amino acids) (68,212). Notably, 12 mutations can be mapped in the vicinity of the major Ca$^{2+}$-binding site 1, which is in the hinge region between lobes 1 and 2, linking other four potential calcium binding sites. Thus, mutations near Ca$^{2+}$-binding site 1 possibly influence the whole receptor’s functional cooperativity (68,212).

By analyzing the changes in [Ca$^{2+}$]$_i$ in individual HEK293 cell elicited by increasing [Ca$^{2+}$]$_o$, the impact of the ‘toggle’ mutations on the function of CaSR has been further evaluated. The in vitro study showed that the L173F and P221L mutations enhance the sensitivity of the CaSR to [Ca$^{2+}$]$_o$ compared to the WT receptor, but barely change the cooperativity of the receptor, while the L173P and P221Q mutations render a less cooperative receptor with impaired capability to sense [Ca$^{2+}$]$_o$.

Disease related mutations change the [Ca$^{2+}$]$_o$ elicited [Ca$^{2+}$]$_i$ oscillation pattern and intracellular signaling.

Oscillations have been postulated to be the result of complex responses from multiple signaling pathways. Since the [Ca$^{2+}$]$_o$-triggered [Ca$^{2+}$]$_i$ oscillation pattern is believed to be modulated by the activity of phosphoinositide pathway (262) as well as a negative feedback loop involving the inhibitory effect of protein kinase C (PKC) on IP$_3$ production (263), the
downstream intracellular signaling responses (such as the production of IP$_1$ and the activity of ERK$_{1/2}$, which will be discussed later) can be reflected in the oscillation patterns associated with the different disease-related mutations. Although most research focusing on the relationship between intracellular calcium mobilization and CaSR activation has utilized CaSR expressed heterologously in HEK293 cells, CaSR-induced [Ca$^{2+}$]$_i$ oscillations have been found in cells with endogenously expressed CaSR, for instance in parathyroid cells (264), bovine anterior pituitary cells (265), opossum kidney (OK) cells (106) and medullary thyroid carcinoma cells (266). Oscillations in [Ca$^{2+}$]$_i$ have been reported not only to modulate the rate of parathyroid hormone (PTH) secretion, but also to regulate gene expression (24,119,126,130). The pattern of [Ca$^{2+}$]$_i$ oscillations is thus one of the most important signatures reflecting the state of CaSR activity. As [Ca$^{2+}$]$_o$ is increased, the [Ca$^{2+}$]$_i$ oscillations reach a plateau in CaSR-transfected cells as the desensitization process occurs and/or there is depletion of [Ca$^{2+}$]$_i$ stores. Although the loss-of-function mutation L173P still oscillated at 30.0 mM [Ca$^{2+}$]$_o$, considering the elevated and broad range of its sensitivity to [Ca$^{2+}$]$_o$ it is possible that a plateau would be reached at even higher levels of [Ca$^{2+}$]$_o$.

The studies of the accumulation IP$_1$ (a metabolite of IP$_3$) and the ERK pathways in HEK293 cells transfected with the various CaSR mutants conveyed two messages: Firstly, the mutations caused changes in the patterns of [Ca$^{2+}$]$_i$ oscillations that at least partially involve activation of the G$_{q/11}$ pathway. The changes in the accumulation of IP$_1$ upon stimulation with various concentrations of [Ca$^{2+}$]$_o$ correlated well with the patterns of [Ca$^{2+}$]$_i$ oscillations. Secondly, stimulation of the ERK$_{1/2}$ activity in CaSR transfected HEK293 cells has been reported to involve PKC-mediated as well as a PTX-sensitive, tyrosine kinase-dependent pathways (260). Although it is believed that the carboxyl terminus of the protein is involved in the activation of
MAPK signaling by interacting with the scaffold protein filamin A (80, 81), the data showed that alterations in the extracellular domain can also affect the ERK cascade which is in agreement with previous studies (267). Since all the mutations presented similar levels of expression on the membrane (Figure 5.1b), these downstream signaling changes may at least partially be contributed by the disruption of cooperativity among different calcium binding sites introduced by different mutations. On the other hand, the gain-of-function mutations may increase the stability the receptor as suggested by other studies (268).

**L-Phe induced heterotropic cooperativity rescued CaSR activity**

We next investigated the effects of the positive allosteric modulator L-Phe on these four mutations. The interaction between CaSR and L-Phe has been reported to modulate the Gα12/13-RhoA pathway (121); however, L-Phe could also potentially enhance [Ca\(^{2+}\)]\(_o\)-induced alterations in intracellular signaling pathways through Gαq/11 pathways as reflected by the IP\(_1\) accumulation assay. The alterations in the oscillation patterns after the introduction of the allosteric activator could be the result of the activation of signaling downstream of Gα12/13 or a combined effect with the positive heterotropic cooperativity between the multiple Ca\(^{2+}\) binding sites induced by L-Phe.

The present study also confirms that the ERK signaling pathway in CaSR-transfected HEK293 cells can be modulated not only by [Ca\(^{2+}\)]\(_o\) but also by the positive allosteric modulator, L-Phe (100). It should be noted that the ability of L-Phe to enhance the intracellular response varied among different mutations. L-Phe enhanced the maximum response of the ERK signaling to a greater extent than it altered the apparent affinity in the two gain-of-function mutants, although it showed barely any influence on the [Ca\(^{2+}\)]\(_o\)-triggered [Ca\(^{2+}\)]\(_i\) and IP1 responses for
these mutants, which may explain, in part, the dramatic changes in correlated motions from the computational simulation results using molecular dynamics. As a signaling messenger further downstream than either IP$_1$ or [$\text{Ca}^{2+}$], the phosphorylation of ERK$_{1/2}$ may not merely reflect activation of the $G_{q/11}$ pathway, but could be contributed to by other G-proteins (e.g., $G_i$). In agreement with the in silico results, L-Phe exhibited less effect on the regulation of the ERK1/2 pathway in mutant L173P. The predicted L-Phe-binding site has been reported to be located near the hinge region between lobe 1 and lobe 2 and to involve residues S169A/S170A/S171A (221). L173 is located within 5 Å of this region. The Leu to Pro change could potentially prevent generating additional steric effects on the closure of lobe 1 and lobe 2 so that addition of exogenous L-Phe is has relatively little impact on the function of the CaSR’s response to changes in [$\text{Ca}^{2+}$]. Meanwhile, the surface electrostatic charge analysis indicated that the mutation could cause charge redistribution, leaving the $\text{Ca}^{2+}$ binding pocket slightly more positive, on one hand, and reduce the interaction between calcium and its binding site 1. Thus, the hetero-cooperativity between the allosteric modulator and calcium binding sites could be disturbed, resulting in the lack of a left-shifted concentration-response curve in response to [L-Phe].

**Computational studies revealed different behavior of the four mutants though simulation.**

Based on the known structure of the agonist-bound and apo forms of mGluR$_1$, which is in the same family C of the GPCRs as the CaSR, an analogous hinge motion of the CaSR is potentially involved in forming the activated form of the latter upon interaction with ligands binding to this region. The correlation maps generated from molecular dynamic simulations for the model structures of the ECDs of the WT and mutant CaSRs provide a complementary approach that is useful to compare the influence of mutations on the interaction networks within
the CaSR ECD. In the current CaSR ECD model structure, it seems that the gain-of-function mutations tend to have stronger correlations between the residues than the WT receptor. Meanwhile, the loss-of-function mutations showed opposite effects on the correlation maps, suggesting a disrupted cooperativity among different calcium binding sites. The simulation results revealed limited effects of L-Phe on changing the correlation motions in the mutant L173P. Meanwhile it dramatically changed other mutants as well as WT indicating that L-Phe may not alter the cooperativity in the same way as the others which have subsequently been confirmed by experiment data.

The results from PCA could also be an explanation for the biased L-Phe effects. L-Phe could not effectively shift the inactive population of conformational ensembles of L173P to an active ensemble of conformations as it did to other mutants and WT as well. However, whether the in silico simulations provide a novel method to predict mutational effects on CaSR will ultimately require the kind of structural detail available for the mGluRs whose structures have been solved. Certainly, the model structure does not take into consideration the possible interactions between the ECD and the extracellular loops and/or transmembrane domain as well as the intracellular domains. Thus, caution is merited in applying the calculated in silico results to those observed with the CaSR in intact cell or in vivo situations. Nevertheless, the predicted structural motions may help to identify the mutations or ligands that have increased or diminished coupling among specific structural elements within the CaSR ECD. While this type of analysis is limited for the moment to the environment of the orthosteric site, this strategy could facilitate drug design for modulating the functions of disease-related mutations in the CaSR as well as other members of the family C GPCRs
In conclusion, through the analysis of the $[\text{Ca}^{2+}]_i$ oscillation and the $[\text{Ca}^{2+}]_o$ and L-Phe triggered downstream signaling changes, we found in the present study that the functional cooperativity was disrupted in the identified FHH associated CaSR mutations L173P and P221Q but not in the ADHH related mutations L173F and P221L. The addition of L-Phe rescued the function of P221Q via the introduction of heterotropic cooperativity. The distinctive correlated motions between the gain-of-function mutations and the loss-of-function mutations and potential revealed by the molecular dynamic simulations provide insights into the mechanism for the disease associated receptor functional alterations.
CHAPTER 6. CHARACTERIZE THE BIOPHYSICAL PROPERTIES OF THE EXTRACELLULAR DOMAIN OF CALCIUM SENSING RECEPTOR

6.1 Introduction

The calcium sensing receptor (CaSR) is a seven transmembrane protein which belongs to family C of the G protein-coupled receptor super family (269). The CaSR is named after its major function—regulating extracellular calcium homeostasis in the body—and its predominant expression in parathyroid and kidney cells allows this receptor to modulate parathyroid hormone (PTH) secretion as well as renal calcium reabsorption (38,41,270,271). The CaSR is also widely expressed in other tissues including the gastrointestinal system(165), the central nervous system(272), several kinds of cancer tissues etc. where it may play other crucial roles. Besides responding to the primary physiological agonist ‘calcium’, the CaSR can also respond to various other stimuli, such as other cations (273), amino acids(118,272), polyamines(242), polypeptides(274) etc.

The CaSR is comprised of the three major structural features of GPCR family C: an extracellular domain (ECD) which includes residue 20 to 612 (38); a seven-transmembrane domain (613-862) (275) and an intracellular tail covers 216 amino acids(276) which bridge the extracellular stimuli into intracellular signal pathways. Based on the amino acid homology of the CaSR’s ECD to metabotropic glutamate receptor (mGluR), a modeled structure has been proposed for the purpose of exploring the biophysical properties of the protein(68). The generated ECD model has a bilobed Venus-fly-trap (VFT) structure (68,70), and is proposed to contain five Ca$^{2+}$-binding pockets (68). Among those calcium binding pockets, it has been demonstrated that binding site 1, comprising residues Ser 147, Ser 170, Asp190, Tyr218 and Glu297, is critical for binding
Eleven highly conserved putative N-linked glycosylation sites, Asn-Xaa-Ser/Thr were identified in the ECD which are considered to be critical for cell surface expression (88). However, due to the lack of biochemical and structural information about the ECD, how extracellular stimuli induce conformational changes of the CaSR leading to further intracellular downstream responses still remains to be defined. The expression of the CaSR ECD has been reported by Goldsmith et. al. (277) and Ryan et. al. (278) using CaSR ECD stably transfected HEK293 cell line and insect cells respectively. Both of the expressed proteins can form dimers and are recognized by specific anti-CaSR antibodies. A few biophysical properties of the insect cell expressed CaSR-ECD have also been explored in Ryan’s study including the secondary and tertiary structure as well as metal-binding properties. Nevertheless, little is known about the influence of glycosylation on this protein and the differences of the expressed proteins with distinct glycosylation modifications.

UDP-GlcNAc:α-D-mannoside-β1,2-N-acetylglucosaminyltransferase I (GnTI) EC 2.4.1.101 is a type II integral membrane protein, localized to medial-Golgi cisternae, and catalyzing the first step in the conversion of high mannose N-glycans into complex and hybrid structures (279,280). Although the complex N-glycans are essential for the viability of the developing embryo, as mice lacking a functional GnTI gene die before birth (281,282), the complex N-glycans are not crucial for viability of cells cultured in vitro as many mutants have been isolated which lack of GnTI activity (283,284). One such mutant is the leuco-phytohemagglutinin (L-PHA) resistant Lec1 HEK293F cell (285). Due to the deficiency at GnTI, Lec1 cells are unable to synthesize complex and hybrid N-glycans and the oligosaccharides will be accumulated in the form of
Man5-GlcNAc2-Asn-structures (286). Thus, protein expressed using Lec1 HEK293F cells could have a uniform high mannose glycan modification at the surface.

In the present study, the ECD of CaSR was secreted from human CaSR (hCaSR)-ECD transiently transfected HEK293F or Lec1 HEK293F cells. The protein was further purified through affinity columns. The biophysical properties of the native CaSR ECDs were characterized using fluorescence titration spectroscopy, circular dichroism technique as well as NMR spectra. Our studies indicate that glycosylation could dramatically alter the protein’s thermal stability; meanwhile it also affected the calcium induced conformational changes as well as its metal binding capability.

6.2 Results

6.2.1 The CaSR ECD forms dimer in culture medium

HEK293F cells or the Lec1 mutant HEK293F cells were transiently transfected with engineered hCaSR ECD cDNA (Figure 6.1). The signal peptide at the N-terminal led to the secretion of the CaSR ECD into the culture medium. Those His-tagged CaSR ECDs were efficiently separated from other proteins with a HisPrep column using an imidazole gradient (Figure 6.2). The fractions containing CaSR ECD were collected and detected by SDS-PAGE under reducing conditions (Figure 6.2). Immunoblot was further applied to verify the samples using the specific anti-CaSR antibody. As shown in Figure 6.2 (c~e) WT CaSR ECD exhibited a smeared band in the range from 100 kDa ~ 130 kDa suggesting various degrees of glycosylation while the Lec1 mutant hCaSR has one single band with a molecular weight of approximately 95 kDa. The non-reducing samples of WT CaSR ECD and Lec1 CaSR ECD have molecular weights at around 240
kDa and 200 kDa, respectively, corresponding to the calculated dimerization form of the receptor’s extracellular domain.

Figure 6.1 Schematic representation of extracellular domain of the CaSR.
The first 25 amino acids are signal peptide from vector. The arrow indicates the beginning of CaSR ECD sequences. The introduced stop codon mutation is immediately after residue 612, resulting in the secretion of the receptor into the cell culture medium. Putative glycosylation sites are highlighted using branches. Underlined and labeled regions represent peptide sequences used to generate peptide-specific monoclonal antibody ADD. The loop inserted between D371 and T372 is the engineered Flag-tag.

Figure 6.2 CaSR ECD protein fractions isolated from cell culture medium using FPLC

(a). HEK293F cells transfected with WT CaSR ECD were continuously cultured for 6 days. Then cell culture medium was collected and underwent protein purification procedure
using a nickel affinity column. Representative tracing of A280 measured in milli AU for protein eluted by imidazole. Inset: Coomassie blue staining of the representative fractions from the affinity column separation. The lanes marked “M” are protein molecular weight standards. (b). The same chromatography of protein purification using secreted proteins from Lec1 cells transfected with the CaSR ECD. (c). WT CaSR ECD proteins (50 μg) from indicated fractions were incubated at 100 °C in sample dissociation buffer containing β-mercaptoethanol for 5 mins before loading on the SDS gels. (d). the same treatment as (a) but with Lec1 CaSR ECD proteins (e). Left two lanes were loaded with WT/Lec1 CaSR ECD incubated at 4°C for 5 mins in sample dissociation buffer without β-mercaptoethanol; the right two lanes were loaded with WT/Lec1 CaSR ECD and incubated at 100°C for 5 mins with reducing reagent.

6.2.2 The secreted CaSR ECD is stable and glycosylated

The stability of the purified extracellular domain was further investigated using immunoblot. Samples from CaSR ECD aliquots were kept at room temperature continuously for seven days. Both the WT CaSR ECD and the Lec1 CaSR ECD was quite stable as no degradation bands could be detected by Western blot (Figure 6.3). In order to verify whether the secreted WT CaSR ECD is glycosylated or not, Ricinus communis agglutinin I (RcaI) agarose beads, which preferentially binds to oligosaccharides, were incubated with the purified protein. A high concentration of galactose (200 mM) would compete with the glycoproteins for interaction with the lectin agarose beads. Figure 6.4a shows that the WT CaSR ECD was able to interact with the RcaI agarose beads which indicate that the protein was glycosylated. N-Glycosidase F, known as PNGase F, can cleave between the innermost acetylglucosamine (GlcNAC) and asparagine
residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins (287). Treatment with heat and detergent denatured WT CaSR ECD and exposed enzyme cleavage sites. As shown in Figure 6.4b, with the presence of N-glycosidase, the molecular weight of WT CaSR ECD decreased from ~100 kDa to ~80 kDa. Meanwhile, Endo F1 cleaves asparagine-linked or free high mannose and hybrid structures but not complex oligosaccharides, leaving a truncated sugar molecule with one N-acetylglucosamine residue on the asparagines(288). The Lec1 CaSR ECD showed a lower band on the gel at molecular weight around 87 kDa after treatment with Endo F1 (Figure 6.4c), suggesting that the Lec1 CaSR ECD has homogenous high mannose form.

Figure 6.3 The stability of CaSR ECD at room temperature.

The purified ECD protein aliquots were incubated in 10 mM Tris buffer (pH 7.2) at room temperature for the indicated number of days. Samples were then added with the sample dissociation buffer with or without β-mercaptoethanol before loading in the gel. R: without β-mercaptoethanol. (a) WT CaSR ECD; (b) Lec1 CaSR ECD.
Figure 6.4 The secreted ECDs were glycosylated.

(a.) WT CaSR ECD protein (1.0 mg) was incubated with RCA-1 lectin agarose beads overnight at 4°C followed by washing with PBS. Lane 1: Eluted fractions using PBS without addition of galactose. Lane 2: Eluted fractions using PBS containing 200 mM galactose. Lane 3: 5 µl of the Rca-1 lectin agarose beads after incubating with protein were loaded into the gel as a positive control. The elution fractions were further analyzed by immunoblot using anti-flag antibody. (b.) Denatured purified WT CaSR ECD (20 µg) was treated with PNGase F for 2 hours at 37°C. Protein aliquots treated with or without PNGase F were loaded in the gel and further analyzed by Western blot using an anti-flag antibody. (c.) The purified Lec1-hCaSR-ECD protein was mixed with Endo F1 at mass ratio 1:3 in 10 mM Tris buffer, pH 7.4 for overnight shaking at 4 °C. Samples were detected using anti-flag antibody by immunoblot under reducing condition.

6.2.3 Calcium and glycosylation caused little secondary structure change of the CaSR ECD.

In order to analyze the secondary structure of the purified CaSR ECDs, circular dichroism measurements were performed. Figure 6.5 shows typical far-UV CD spectra of the proteins.
Analysis of all the spectra indicated that the purified WT and Lec1 CaSR ECD shared similar secondary structure with α helical content of 52%~58% and β-sheet content of 17% ~25%. The addition of calcium had caused minimal changes in the spectra of both WT CaSR ECD and Lec1 CaSR ECD (Table 5.1).

The proteins were subjected to stepwise increases in temperatures to assess thermal stability. Molar ellipticity was monitored from 4~ 95 °C at 222 nm, which is considered to be a sensitive position for proteins have >50% α helical structure. As shown in Figure 6.5 (a&b), the WT CaSR ECD demonstrated extraordinary thermal stability as the signal at the given wavelength did not change. The high mannose form of the CaSR ECD, the Lec1 CaSR ECD, showed a rapid disruption of secondary structure at 92°C in the absence of Ca$^{2+}$. In the presence of Ca$^{2+}$, the melting points of the Lec1 CaSR ECD were decreased dramatically to 80°C possibly due to protein precipitation. However, the calcium induced alterations in melting temperature were not observed in the WT CaSR ECD. The heating process resulted in irreversible structural changes in the Lec1 CaSR ECD as the high temperatures caused marked precipitation of the protein. To determine the enthalpy of the unfolding process for the CaSR ECD, the Van’t Hoff equation could be employed for the analysis (289), however, in the present study, the precipitation could due to calcium binding induced instability of the protein.
Figure 6.5 Circular Dichroism measurement of purified ECD in the presence or absence of calcium.

(a) Far-UV CD spectrum of WT CaSR ECD in 10 mM Tris, pH 7.2 with or without 2.0 mM Ca\(^{2+}\), at 25 °C. (b) Far-UV CD spectrum of Lec1 CaSR ECD in 10 mM Tris, pH 7.2 with or without 2.0 mM Ca\(^{2+}\), at 25 °C. (c) Temperature dependence of the molar ellipticity at 222 nm for WT CaSR ECD in 10 mM Tris, pH 7.2. (d) Temperature dependence of the molar
ellipticity at 222 nm for Lec1 CaSR ECD in 10 mM Tris, pH 7.2. The addition of calcium may also cause the precipitation of Lec1 protein at high temperature, resulting in a decrease of the melting temperature.
Table 6.1 Secondary structure of CaSR ECDs at 25°C.

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<tr>
<td></td>
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<td>Ca²⁺(+)</td>
<td>Ca²⁺(-)</td>
<td>Ca²⁺(+)</td>
</tr>
<tr>
<td>WT CaSR ECD</td>
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<td>55% ± 6%</td>
<td>17% ± 1%</td>
<td>21% ± 4%</td>
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<tr>
<td>Lec1 CaSR ECD</td>
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<td>60% ±4%</td>
<td>25% ± 3%</td>
<td>18% ± 1%</td>
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</tbody>
</table>

The secondary structure contents were estimated according to SELCON and CONTIN algorithms. Numbers in the table show the average of secondary structure content of both algorithms.
6.2.4 Fluorescence spectra indicate folded structure of the CaSR ECD.

There are twelve tryptophan (Trp) residues in the ECD, thus, by analyzing the intrinsic tryptophan emission spectra from 300 to 400 nm at the excitation wavelength of 282 nm, the Trp environment can be monitored. The emission spectrum of free Trp has a peak at around 350 nm. The emission peak undergoes a blue-shift if the tryptophan is embedded in a hydrophobic environment. All the Trp emission spectra of various types of ECD showed a peak at around 334 nm, indicating that the proteins retained their native structures (Figure 6.6).

Meanwhile, ANS, a fluorescence probe extensively used for the detection and analysis of protein conformational changes, was applied to the study of the two proteins (290). This dye has low fluorescence yield when exposed to polar environments, but a blue shift of the emission spectrum and an increase of the fluorescence intensity would occur once its fluorophore is located in less polar media (291). Compared to the free ANS dye, which has an emission peak at around 500 nm, the ANS fluorescence spectra of both the CaSR ECDs underwent a more than 30 nm blue shift and further exhibited a 10-fold increase in fluorescence intensity (Figure 6.7). The fluorescence spectra changes indicate the hydrophobic portion at the protein surface can bind with ANS and calcium can induce a conformational change of CaSR ECDs. These results correlate well with the Trp spectrum, demonstrating that the purified ECDs, both from WT HEK293F cells and Lec1 mutant system, were possibly in their native forms.
Figure 6.6 Tryptophan fluorescence titration of CaSR ECDs.

(a) Trp fluorescence spectra of WT CaSR ECD in the presence of 0 (---) or 5 mM Ca$^{2+}$ (—).

(b) Trp fluorescence spectra of Lec1 CaSR ECD in the presence of 0 (---) or 5 mM Ca$^{2+}$ (—).

(c) Calcium titration curve of WT CaSR ECD. (d.) Calcium titration curve of Lec1 CaSR ECD.
Figure 6.7 Ca\textsuperscript{2+}-induced changes in ANS fluorescence in CaSR ECDs.

ANS fluorescence spectra of CaSR ECD in the presence (—) or the absence of (—•) 5 mM Ca\textsuperscript{2+}. The spectrum of ANS alone is shown in gray. The fluorescence intensity at 465 nm was recorded and plotted against calcium concentration. The data were further fitted using Hill equation. (a) WT CaSR ECD; (b) Lec1 CaSR ECD.
6.2.5 \( \text{Ca}^{2+} \)-induced conformational changes in CaSR ECDs.

As shown in Figure 6.6 & 6.7, the addition of \( \text{Ca}^{2+} \) led to changes in both Trp fluorescence and ANS spectrum. Binding to \( \text{Ca}^{2+} \) resulted in 2–3 nm blue shift of the emission maxima of the Trp fluorescence spectra of both the WT CaSR ECD and the Lec1 CaSR ECD. Furthermore, a decrease of 20% in Trp fluorescence intensity in WT CaSR ECD was observed upon the addition of saturating amounts of \( \text{Ca}^{2+} \), while a 14% decrease in signal was shown in the case of Lec1 CaSR ECD. Compared to the calcium induced signal changes of the Trp spectra, the ANS intensity alterations upon \( \text{Ca}^{2+} \) titrations rendered different patterns for the two proteins. Calcium produced an exposure of more hydrophobic regions in the complex oligosaccharides form (WT CaSR ECD) as there was a 25% increase in the fluorescence signal. Similar intensity enhancements, though to lesser extents (~10%), were detected in Lec1 CaSR ECD.

The calcium induced conformational changes reflected in the Trp spectra were further assessed by monitoring the calcium titration processes. As expected, the \( K_d \)s for mammalian system expressed CaSR ECDs were in mM range for WT CaSR ECD 3.5 ± 0.3 mM and for Lec1 CaSR ECD 3.2 ± 0.3 mM, respectively. The Hill numbers obtained from the Trp fluorescence spectra were 2.6 ± 0.5 for WT CaSR ECD and 2.2 ± 0.2 for Lec1 CaSR ECD, indicating that the two proteins may share similar multiple binding processes (Table 5.2). The calcium induced ANS spectral alterations were also fitted by the Hill equation. The WT CaSR ECD had a \( K_d \) of 3.8 ± 0.2 mM while the Lec1 CaSR ECD had a \( K_d \) value at 3.2 ± 0.1 mM. Their corresponding Hill numbers calculated from the \( \text{Ca}^{2+} \)-ANS titration curves were 2.6 ± 0.4 and 2.3 ± 0.5 respectively.
Table 6.2 Summary of dissociation constants and Hill numbers for CaSR ECDs.

<table>
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<th>Mutants</th>
<th>Trp Spectrum</th>
<th>ANS Spectrum</th>
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<tr>
<td></td>
<td>EC50</td>
<td>Hill number</td>
</tr>
<tr>
<td>WT CaSR ECD</td>
<td>3.5 ± 0.3</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>Lec1 CaSR ECD</td>
<td>3.2 ± 0.3</td>
<td>2.2 ± 0.2</td>
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6.2.6 Tb³⁺ binding process confirmed the weak binding affinity of CaSR ECD

Considering the potential presence of background Ca²⁺ in the buffer, which might affect the accurate measurement of binding affinity, the metal binding capability of ECDs were further analyzed using Tb³⁺ luminescence resonance energy transfer (Tb³⁺-LRET). There are 12 Trp residues in the CaSR ECDs. According to the model structure of the CaSR ECD, more than 5 of the Trp residues are within 15 Å of those potential calcium binding pockets, thus enabling aromatic residue-sensitized Tb³⁺-LRET. Due to their similarities in ionic radii and coordination chemistry, Tb³⁺ has been ubiquitously used to probe Ca²⁺ binding sites as a trivalent Ca²⁺ analogue. There are several advantages of using Tb³⁺. Firstly, the spectroscopic properties of energy transfer between Tb³⁺ and the aromatic residues near the binding pockets can facilitate the measurements of Kd as well as revealing structural information. Secondly, the higher affinity of Tb³⁺ than Ca²⁺ also helps to probe the weak Kd values. Last, but not least, the background contamination of Tb³⁺ in the titration system is lower than that of Ca²⁺. The Tb³⁺ binding processes of the two different proteins turned out to be one binding step instead of a biphasic process as observed in the subdomain studies (172). The Tb³⁺ binding affinity for WT CaSR ECD was about 36.6 ± 0.4 μM with a Hill number at 1.2 ± 0.1 suggesting a 1:1 binding mode.
(Figure 6.8). In terms of Lec1 CaSR ECD, the $K_d$ for Tb$^{3+}$ was $31.8 \pm 0.3$, which is comparable with the WT CaSR ECD. On the other hand, the Ca$^{2+}$-Tb$^{3+}$ competition assay suggested apparent dissociation constants for the ECDs, from which the binding constants for calcium can be deduced (Figure 6.9). The Ca$^{2+}$ binding dissociation constant calculated from Ca$^{2+}$-Tb$^{3+}$ competition assay, using equation; $K_{d,Ca} = K_{app} \frac{K_{d,Tb}}{K_{d,Tb} + [Tb]}$, for the WT CaSR ECD and the Lec1 CaSR ECD were $3.7 \pm 0.2$ mM and $2.4 \pm 0.2$, respectively. This is comparable to the $K_d$ calculated from Ca$^{2+}$ induced changes in native Trp signals as well as the ANS spectra.

Figure 6.8 Tb$^{3+}$ titration curve of CaSR ECDs.
Tb\textsuperscript{3+} titration curve of purified CaSR ECDs. The buffer for Tb\textsuperscript{3+} titration consisted of 20 mM PIPES, 135 mM NaCl, and 10 mM KCl (pH 6.8). The arrow indicates the increase in fluorescence. The titration curve was fitted using the Hill equation. (a.) WT CaSR ECD; (b.) Lec1 CaSR ECD.

Figure 6.9 Tb\textsuperscript{3+} competition assay of CaSR ECDs.

Tb\textsuperscript{3+}-Ca\textsuperscript{2+} competition assay of purified CaSR ECDs. The buffer for Tb\textsuperscript{3+} titration consisted of 20 mM PIPES, 135 mM NaCl, and 10 mM KCl (pH 6.8). The arrow indicates the decrease of the fluorescence with Ca\textsuperscript{2+} addition. (a) 2.0 μM of WT CaSR ECD; (b) 2.0 μM of Lec1 CaSR ECD.
Table 6.3 Summary of the Tb<sup>3+</sup> FRET assay.

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<tr>
<th>Mutants</th>
<th>Tb&lt;sup&gt;3+&lt;/sup&gt; Titration (µM)</th>
<th>Tb&lt;sup&gt;3+&lt;/sup&gt; Competition (µM)</th>
<th>K&lt;sub&gt;d,Ca&lt;/sub&gt; (mM)</th>
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<tr>
<td></td>
<td>EC50</td>
<td>Hill number</td>
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<tr>
<td>WT CaSR ECD</td>
<td>36.6 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>16.1 ± 0.2</td>
</tr>
<tr>
<td>Lec1 CaSR ECD</td>
<td>31.8 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>10.0 ± 0.2</td>
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6.2.7 NMR spectrum revealed calcium induced tertiary structure changes in CaSR ECDs

Disruption of chemical shift can be used to monitor the interaction between metals and the protein due to the sensitivity of the chemical shifts of protein amides to even diminutive conformational changes occurring at the interacting interface. As depicted by Tb<sup>3+</sup> binding process, as well as the ANS fluorescence, a multiple binding process can be observed in both the one-dimensional (1D) (Figure 6.10) and two-dimensional (2D) NMR (Fig. 6.11) when calcium was titrated into both <sup>15</sup>N L-Phe labeled WT CaSR ECD and <sup>15</sup>N L-Phe labeled Lec1 mutant CaSR ECD. The chemical shift changes for each protein are shown in Figure 6.11. During the titration, a progressive disappearance of the amide signals at 8.05 ppm of the WT CaSR ECD was accompanied by the concomitant emergence of a new set of peaks at 7.85 ppm as well as a chemical shift between amide signal at 7.95ppm and 8.21ppm which move towards each other upon titration of Ca<sup>2+</sup> (Figure 6.11 a,b.&c), indicating complex binding states.

The chemical shifts of the peaks were further plotted into calcium response curves as shown in Figure 6.11. The calcium induced conformational changes revealed a 1:1 ligand-protein complex formation process, Figure 6.11 left panel. In agreement with data from fluorimetric systems, the
binding constant was in the millimolar range (1.1 mM). Meanwhile, the analysis on the peaks in Figure 6.11c suggested a slow exchange process in which the exchange rate of the bound and unbound conformation could not catch up with the amide frequency. The Ca$^{2+}$-concentration response curve indicated a multiple ligand binding process with a Kd at 2.5 mM and a Hill number at 2.5.

The Lec1 mutant CaSR ECD with its high mannose form demonstrated a less dispersed 2D spectrum after the addition of calcium. A few peaks appeared with the addition of 3 mM calcium with characteristics as slow chemical exchanges indicate a strong binding process (Figure 6.12).

![Figure 6.10 Ca$^{2+}$ titration of CaSR ECD monitored by 1D $^1$H NMR.](image)

(a) Ca$^{2+}$-induced, gradual chemical shift changes in WT CaSR ECD were observed at resonances in the main chain amide proton region, such as 7.35 and 8.42 ppm . (b) Ca$^{2+}$-induced, gradual chemical shift changes in Lec1 CaSR ECD were observed at resonances in the main chain amide proton region, such as 7.40 and 8.40 ppm
Figure 6.11 \(^{1}H,^{15}N\)-HSQC spectra of WT CaSR ECD.

Left panel: Ca\(^{2+}\)-induced chemical shift changes of WT CaSR ECD. Red: WT CaSR ECD without addition of Ca\(^{2+}\); Orange: 1 mM Ca\(^{2+}\); Yellow: 3 mM Ca\(^{2+}\); Green: 5 mM Ca\(^{2+}\); Cyan: 10 mM Ca\(^{2+}\). Right panel: Ca\(^{2+}\) chemical shift curves of peak b and peak c in WT CaSR ECD fitted by the Hill equation.
The binding of L-Phe to the CaSR ECD can be modulated by Ca$^{2+}$

The protein yield of $^{15}$N-labeled Lec1 mutant CaSR ECD was much lower compared with $^{15}$N-labeled WT CaSR ECD. Therefore, the application of the heteronuclear single quantum coherence (HSQC) NMR to monitor the protein-ligand interaction with the purified $^{15}$N-labeled Lec1 mutant CaSR ECD will be fraught with pitfalls. Recently, saturation transfer difference (STD) NMR has become a popular NMR technique in terms of analyzing protein-ligand interactions due to the fact that it detects the signals from the ligands and only small amount of nonlabeled proteins or macromolecules are required. As shown in Figure 6.13, the NMR signal change from the L-Phe STD spectrum indicated the conformational change of this CaSR positive allosteric regulator during the interaction with CaSR ECD (Figure 6.13). This phenomenon provides direct evidence to support the earlier hypothesis that L-Phe can bind to the extracellular
domain of CaSR with a $K_d$ at 4.7–6.5 mM (Figure 6.13a&b). Intriguingly, the addition of 2.5 mM calcium decreased the signal from L-Phe STD spectrum, suggesting the interaction between Ca$^{2+}$ and the receptor may influence the binding between L-Phe and the CaSR ECD (Figure 6.13 c&d).

Figure 6.13 Monitoring the ligand-protein interaction via STD NMR.
(a) STD spectrum of L-Phe titration to the CaSR ECD in the absence of Ca$^{2+}$. The increased L-Phe concentration is indicated in the figure. (b) STD spectrum of L-Phe titration to the CaSR ECD in the presence of Ca$^{2+}$. (c) The overlaid 1.0 mM L-Phe STD spectra in the presence of Ca$^{2+}$ (cyan line) and in the absence of Ca$^{2+}$ (red line). (d) The intensity of the three major peaks shown in panel c was integrated respectively and further plotted against L-Phe concentration. The plots were further fitted by a 1:1 binding
equation. The blue line represents the left peak, red for the middle one and black for the right one.

6.3 Discussion

It has been suggested that the ECD of the mGluRs and CaSR share similar Venus Fly Trap structures based on several conserved amino acid regions and predicted secondary structures (216). However, there is limited structural and biochemical information available for CaSR due to its large molecular weight (~70 kDa as monomer) and heterogeneous glycosylation. In the present study, we were able to purify CaSR ECDs from both normal mammalian expression systems and the complex N-glycans deficient expression system. In the two systems, the target protein was expressed by mammalian cells and was secreted into the cell culture media due to a signal peptide at the beginning of the ECD sequence. It is known that CaSR functions as a dimer on the surface of the cell membrane. Importantly, the secreted ECD proteins from both the complex glycan and the homogenous high mannose form also present in the culture medium as a dimer form.

Glycosylation is one of the most important posttranslational modifications occurring in protein biosynthesis and secretary pathways. The secreted WT CaSR ECD was well glycosylated as it can bind with lectin agarose beads and the treatment with PNGase reduced its molecular weight. The Lec1 hCaSR ECD can be further cleaved by Endo F1 but it could not interact with the lectin beads. The thermodynamic stability calculations indicate that the stability of a protein may be enhanced when a glycan is covalently bound to the protein surface, which can be measured by an increase in melting temperature (292). A stabilization by glycosylation has been reported for other membrane proteins such as AQP2 mutants (293) and a shaker potassium channel (294).
How the glycosylation could affect the biophysical properties of CaSR or other family C GPCR proteins has yet to be analyzed. In the present study, the influence of the complexity of polysaccharide on the thermal stability of CaSR-ECD is remarkable. The ECD protein with heterogeneous saccharides was able to maintain its secondary structure at high temperature (>90°C). On the other hand, the CaSR ECD with homogenous glycan was comparatively less stable. These phenomena would be importantly contributed to the differences of protein tertiary structures altered by the post-translational modification since their secondary structures were relatively similar.

Calcium, as a predominant agonist for CaSR, is believed to induce conformational changes of the receptor to convey the extracellular signal to intracellular messengers. However, the lack of evidence to prove such a change makes it worthwhile to analyze alteration of the receptor’s biophysical characteristics before and after the Ca^{2+} binding. One of the biophysical changes on CaSR, upon the interaction with Ca^{2+}, is the thermal stability of its ECD. The addition of calcium altered the structural stability of CaSR-ECD, resulting in loss of the secondary structure at much lower temperatures. Another possible interpretation is the positive charges of the metal accelerate the aggregation of the protein at higher temperatures. Different changes in enthalpy corresponding to different states, measures a change in the strength of the interactions between molecules (295). The enthalpy change of Lec1 CaSR ECD, measured in the present study, is comparable to the results from previous subdomain studies in our lab (172), confirming the weak binding of Ca^{2+} to the CaSR ECDs. These lines of evidence are also in line with results from tryptophan spectra, as well as the ANS assay, regarding the metal-binding capability.
The results in the present study suggested that the full length of WT CaSR ECD has Ca\(^{2+}\) binding affinities (3.5~3.7 mM) comparable with the EC50s calculated from CaSR functional assays (2.7~3.1 mM). The high mannose form of the CaSR ECD showed similar Ca\(^{2+}\) binding capability as the complex N-glycans form. This indicates that the additional post-translational modification did not impose large structural changes on the protein. There are twelve tryptophan residues in the ECD, among them four are on the molecular surface between lobes 1 and 2. The decrease of tryptophan intensity might be explained by a closed form of the receptor resulting from calcium binding which could lead to a more imbedded position of those tryptophan residues. On the other hand, the signal from the ANS spectrum decreased upon titration with Ca\(^{2+}\) suggesting the ligand-induced exposure of more hydrophobic regions of the protein to interact with the ANS probe. The Ca\(^{2+}\) induced ANS signal increase of the WT CaSR ECD was larger than with the Lec1 CaSR ECD which indicated that the glycans on the WT CaSR ECD might contribute to the hydrophobicity changes in the protein triggered by Ca\(^{2+}\).

Functional studies of the CaSR in cells, as well as our previous metal binding studies, using grafting approaches have demonstrated that the CaSR has multiple Ca\(^{2+}\)-binding sites (87). The Hill coefficients of both the WT CaSR ECD and its Lec1 mutant, calculated from calcium induced spectrum changes in the current study (2.2~4.0), were also in agreement with the multiple Ca\(^{2+}\) binding modes. In our lab’s previous studies, when titrated with Tb\(^{3+}\), CaSR ECD subdomain 1, which includes predicted Ca\(^{2+}\) binding site 1, site 2 and site 3, showed biphasic binding processes while subdomain 2 (including Ca\(^{2+}\) binding site 2 &3) and subdomain 3 (including Ca\(^{2+}\) binding site 4 &5) had monophasic responses to Tb\(^{3+}\). However, in the present studies, the fitted Tb\(^{3+}\)-response curve for the WT and Lec1 CaSR ECD did not show an
observable cooperative binding curve. It could possibly be due to the fact that in the grafting approaches, the subdomains were not constrained by the additional structural conformations as it would be when they are in the intact ECD structure. Concomitantly, the CaSR ECDs had much stronger binding affinities for Tb$^{3+}$ than for Ca$^{2+}$ with $K_d$s at $\mu$M range. Thus the differences between potentially weak metal-binding sites may not be detected. Moreover, the intensive FRET signal between Tb$^{3+}$ and more than one Trp residues might produce an overall strong signal hiding behind the possible cooperativity among these metal binding sites. There are more than 30 Phe on the ECD of CaSR, however, limited peak numbers were detected using a 900 MHz magnet, suggesting similar local environment for multiple Phe residues. Data from NMR spectra indicated that the local environment around several Phe residues had been changed during the calcium titration process in various ways, supporting that CaSR ECDs may interact with calcium cooperatively.

Calcium-induced conformational changes in the intact CaSR ECD were observed in both fluorescence and NMR spectra. Such conformational changes are fundamental initial signals for activation of the CaSR and transduction of these signals to the cytoplasm, calling on numerous downstream molecules that are involved in critical signaling cascades and resulting in the regulation of numerous processes, such as the control of parathyroid hormone (PTH) secretion. The ligand triggered tertiary structure alterations in the extracellular domain have been observed in other GPCR family C members. Kunishima et al. have reported ligand-bound forms and unliganded forms of mGluR1, showing alterations in the bi-lobed protomer architecture associated with the receptor’s active and resting status through the modulation of the dimeric interface by a packed $\alpha$-helical geometry (175). Ligands stabilize the mGluR1 in the active form
by maintaining the closed bi-lobed receptor structure as the dominant form in the receptor’s
dynamic equilibrium between open and closed state. Although the changes from the Trp
spectrum and the ANS probes may, in a way, support a wrapped form of the CaSR, mutagenesis
work on the CaSR ECD will still be necessary in order to assign the peaks of the NMR spectra.
Only by gathering the detailed information about the authentic structural movements of the
CaSR, will we understand whether correcting the deviations from homeostatic balance is via
modulating the “open” and “close” form of the CaSR.

Overall, the present study provides further insights into the structure-function relationships for
the CaSR and should permit further studies to define which are the key residues involving the
interaction with the ligands for this receptor, including cations, nutrient amino acids, as well as
antibiotics. This information will be crucial to understanding how CaSR responds to variety of
stimuli and pave the way for future research works on this and other members in GPCR family C.
CHAPTER 7. COMPARISON OF BIOPHYSICAL CHARACTERISTICS OF BACTERIA AND MAMMALIAN EXPRESSED CASR ECD

7.1 Introduction

Prokaryotic expression systems have been widely used in scientific research. It is considered as one of the most efficient and economic ways to produce proteins. Among various prokaryotic expression systems, including *Escherichia coli* (*E.coli*), *Lactococcus lactis* and other bacteria, (*e.g. Bacillus* species) *E.coli* is the most proficiently explored organisms and has been prevalently applied for heterologous expression of proteins (molecular biology and genomics pg.191).

The advantages of the bacterial expression system are multifaceted. Firstly, bacterial cells have simple physiology compared with eukaryotic cells so they are relatively easy to culture. Secondly, their short generation time makes them grow and multiply much quicker than eukaryotic cells, which is a characteristic favorable to large scale expression of recombinant proteins. Moreover, it is inexpensive to culture bacterial cells. Last, but not least, the post-translational modification in bacteria cells is different with eukaryotic cells, such as glycosylation, the most common post-translational modifications.(296). Thus, the bacterial expression is widely applied in protein isotopic labeling methods and the produced homogenous samples allow structural related studies using NMR.

A number of GPCR proteins have been expressed using bacterial systems. Link et. al. have expressed two proteins, cannabinoid receptor (CB1) and bradykinin receptor 2 (BR2), in *E.Coli* system using the shake flasks culture method (297). Four GPCRs, namely chemokine receptors (hCRs) CCR5, CCR3, CXCR4 and CX3CR1, have been discovered to be involved in HIV-1 infection, asthma and cancer metastasis. These proteins have recently been purified using a
bacterial system for biochemical, biophysical as well as structural studies (298). In 2013, the functional N-terminal domain of the T1R3 taste receptor was expressed using an *E. coli* system by Maitrepierre et. al (299). Similar to CaSR, T1R3 taste receptor also belongs to GPCR family C, the successful purification of functional T1R3 taste receptor suggested feasibility to express CaSR ECD in *E. coli*.

Along with the advantage in utilizing *E.coli*, there are also several disadvantages intrinsic to the prokaryotic system. First, the expressed proteins may not fold properly and thus lose its biological activity. Misfolding of disulfide bonds are one of the frequent errors observed in manipulation with prokaryotic expression. Second, the synthesized protein, especially exogenous protein, can be toxic to bacteria. Decreasing the bacteria cell number and/or degradation of the expressed protein can be the following consequences. Additionally, the lack of post-translational modification can work as double-edged sword. Protein expressed from a bacteria system is not attached with sugar residues, which may affect its *in vitro* functional studies.

In this chapter, the ECD of CaSR was expressed using a bacteria system (bCaSR) and was further purified through affinity columns. The biophysical properties of the native CaSR ECDs were characterized using the fluorescence titration spectroscopy, circular dichroism technique as well as the NMR spectrum.

**7.2 Results**

**7.2.1 Expression of triple labeled and non-labeled CaSR ECD in bacteria system.**

The non-labeled human CaSR ECD was expressed overnight at 30°C using the Rosetta-gami (DE3) pLys bacteria strain. Since the Rosetta-gami (DE3)pLys strain does not grow or express
well in minimum growth medium, the triple labeled protein was expressed in the Tuner strain. Those His-tagged CaSR ECDs were efficiently separated from other proteins with a HisPrep column using an imidazole gradient. The fractions containing CaSR ECD were collected and revealed by SDS-PAGE under reducing conditions (Figure 7.1a &b). Immunoblot was further applied to verify the samples using specific anti-CaSR antibody. As shown in Figure 7.1 c&d, the bacterial expressed CaSR ECD (bCaSR ECD) showed a clear band at around 72 kDa, which is close to its predicted molecular weight (70 kDa). Moreover, the bCaSR ECD can not interact with Ricinus communis agglutinin I agarose beads, which preferentially binds to oligosaccharides, suggesting that the bCaSR ECD has minimal glycosylation modification compared with its mammalian expressed counterpart (Figure 7.1 e). Unlike the mammalian expressed CaSR ECD, the bCaSR ECD forms high-order polymers instead of dimer under a non-reducing environment. It is also worthwhile to notice that the bCaSR ECD was less stable when compared with ECD expressed from mammalian cells, as there were multiple degradation bands below 72 kDa.
Figure 7.1 Bacterial expressed CaSR ECD isolated by FPLC is non-glycosylated

a: WT bCaSR ECD was expressed in Rosetta-gami (DE3) pLys at 30°C. The cell pellet was collected and the soluble protein was extracted by a cell disruptor. After centrifugation, the supernatant fraction of the cell lysate then underwent protein purification procedure using a nickel affinity column. Representative tracing of A280 measured in milli AU for protein eluted by imidazole. Inset: Coomassie blue staining of the representative fractions from the affinity column separation. b: The same chromatography of protein purification using triple labeled $^{15}$N$^{13}$C$^2$D WT bCaSR ECD expressed in Tuner at 30°C. c: WT bCaSR ECD protein (50 µg) from indicated fractions were incubated at 100 °C in sample dissociation buffer containing β-mercaptoethanol 5 mins before loading on the SDS gels. The band of bCaSR ECD was detected using anti-flag antibody. d: the same treatment as (a) but with triple labeled bCaSR ECD proteins e: WT bCaSR ECD protein (0.5 mg) was incubated
with RCA-1 lectin agarose beads overnight at 4°C followed by a washing step with PBS. 
Lane 1: Elution fraction from FPLC as input protein. Lane 2: Elution fraction using PBS 
containing 200 mM galactose. Lane 3: 5 μl of the RCA-1 lectin agarose beads after 
incubating with protein were load into the gel. The elution fractions were further analyzed 
by immunoblot using anti-flag antibody.

7.2.2 Fluorescence spectra indicate folded structure of the bCaSR ECD

There are twelve tryptophan (Trp) residues on the bCaSR ECD, thus, by analyzing the intrinsic 
tryptophan emission spectra from 300 to 400 nm at the excitation wavelength of 282 nm, the Trp 
environment can be monitored. The emission spectrum of free Trp has a peak at around 350 nm. 
The emission peak undergoes blue-shift if the tryptophan is embedded in the hydrophobic 
environment. As shown in Figure 7.2, bCaSR ECD showed a peak at around 334 nm indicating 
that the proteins are retained in their native structure (solid line).

Meanwhile, ANS, a fluorescence probe extensively used for the detection and analysis of protein 
conformational changes, was applied to the study of bacterial expressed CaSR ECD in the same 
way as in the application to the mammalian expressed proteins mentioned in Chapter 6. This dye 
has low fluorescence yield when exposed to polar environments, but a blue shift of the emission 
spectrum and an increase of the fluorescence intensity would occur once its fluorophore relocates 
into a less polar media (291). Compared to the free ANS dye, which has an emission peak at 
around 500 nm, the ANS fluorescence spectra of the bCaSR ECDs underwent a more than 30 nm 
blue shift and further exhibited a 10 fold increase in fluorescence intensity (Figure 7.3). The
results from the ANS spectra thus correlates with the Trp spectrum in terms of demonstrating the purified ECD from the bacterial system was at least partially folded.

Figure 7.2 Tryptophan fluorescence titration of CaSR ECDs.

Trp fluorescence spectra of WT CaSR ECD in the presence of 0 (---) and 139 μM Ca²⁺ (—).
Figure 7.3 $\text{Ca}^{2+}$-induced changes in ANS fluorescence in bCaSR ECDs.

ANS fluorescence spectra of CaSR ECD in the presence (—) or the absence of (—–) 1 mM $\text{Ca}^{2+}$. The spectrum of ANS alone is shown in gray.

7.2.3 Calcium induced little secondary structure change of the bCaSR ECD.

In order to analyze the secondary structure of the bacterial expressed protein, the far-UV circular dichroism measurements were performed as shown in Figure 7.4. Analysis of all the spectra indicated that the purified bCaSR ECD shared similar secondary structure with CaSR ECD expressed from a mammalian expression system, possessing $\alpha$ helical content of 47%~58% and $\beta$-sheet content of 17%~27%. The addition of 200 μM calcium did not change the spectrum of bCaSR ECD significantly (Figure 7.4).
The proteins were subjected to step-wise increasing temperatures to measure the thermal stability. The molar ellipticity signal was monitored from 4–95 °C at 222 nm, which is considered to be a sensitive position for proteins that have >50% of the α helical structure. As shown in Figure 7.4, the melting point (T_m) of bCaSR ECD is around 80 °C in the absence of calcium. Interestingly, in the presence of Ca^{2+}, the melting point of bCaSR ECD was dramatically dropped to 55°C and 80°C respectively. The heat denaturation is irreversible as the high temperatures lead to the formation of precipitation of the bCaSR ECD. In the presence of 200 μM Ca^{2+}, the tendency of protein precipitation at lower temperatures was seen.

Figure 7.4 Circular Dichroism measurement of purified ECD in the presence or absence of calcium.

Left panel: Far-UV CD spectrum of bCaSR ECD in 10 mM Tris, pH 7.2 with or without 200 μM Ca^{2+}, at 25 °C. Right panel: Temperature dependence of the molar ellipticity at 222 nm for bCaSR ECD in 10 mM Tris, pH 7.2. In the presence of 200 μM Ca^{2+}, bCaSR ECD tended to form precipitants.
7.2.4 Metals induced conformational changes in bCaSR ECD

As shown in Figure 7.2 & 7.3, the addition of Ca\(^{2+}\) led to changes in both the Trp fluorescence and the ANS spectrum. Binding to Ca\(^{2+}\) resulted in 2~3 nm blue shift of the emission maximum on the Trp fluorescence spectrum of the bCaSR ECD. Furthermore, similar to mammalian expressed CaSR ECDs, a decrease of 17% in Trp fluorescence intensity in bCaSR ECD was observed upon the addition of saturating amounts of Ca\(^{2+}\). Compared to the calcium induced signal changes on the Trp spectra, the ANS intensity alterations upon Ca\(^{2+}\) titrations rendered different patterns between the proteins generated from the two expression systems. For bCaSR ECD, calcium caused less hydrophobic areas interacting with ANS, resulting in a 34% decrease of the fluorescence peak value; on the contrary, it induced exposure of more hydrophobic regions in the complex oligosaccharides form (WT hCaSR ECD) as there was a 25% increase in the fluorescence signal. Similar intensity enhancements, though to lesser extents (~10%), were detected in Lec1 hCaSR ECD (Figure 6.7).

The calcium induced conformational changes reflected in the Trp spectra were further assessed by monitoring the calcium titration processes. The calcium titrating bCaSR ECD step could be fitted by the Hill equation with a dissociate constant (Kd) of 0.8 ± 0.2 mM and a Hill coefficient of 1.1 ± 0.2, indicating a similar 1:1 binding process as bCaSR ECD (Figure 7.5). Meanwhile, the calcium induced ANS spectra alterations were also fitted by the Hill equation. The K_d of bCaSR ECD measured via ANS spectra was 0.9 ± 0.2 mM with a Hill coefficient of 1.1± 0.2.
Figure 7.5 Ca\textsuperscript{2+}-induced changes in native tryptophan and ANS fluorescence

(a.) Left panel: Trp fluorescence spectra of WT bCaSR ECD in the presence of 0.0 (---) and 2.0 mM Ca\textsuperscript{2+} (—). Right panel: Calcium titration curve of WT bCaSR ECD. The fluorescence intensity was normalized to the initial read-out when the calcium concentration was 0.0 mM and further plotted against calcium concentration. The plot was fitted using the Hill equation. (b.) Left panel: ANS fluorescence spectra of CaSR ECD in the presence (—) or the absence of (---) 1 mM Ca\textsuperscript{2+}. The spectrum of ANS alone is shown in
Considering the potential background $\text{Ca}^{2+}$ in the buffer, which might affect the accurate measurement of binding affinity, the metal binding capability of bCaSR ECD was further analyzed using Tb$^{3+}$ luminescence resonance energy transfer (Tb$^{3+}$-LRET). There are 13 residues on the bCaSR ECD and according to the modeled structure of the CaSR ECD, some of the Trp residues are within 10 Å of those potential calcium binding pockets, thus enabling the aromatic residue-sensitized Tb$^{3+}$-LRET. Due to their similarities in ionic radii and coordination chemistry, Tb$^{3+}$ has been ubiquitously used to probe $\text{Ca}^{2+}$ binding sites as a trivalent $\text{Ca}^{2+}$ analogue. The advantages of using Tb$^{3+}$ have been elucidated earlier in Chapter 6. The Tb$^{3+}$ binding processes of the bCaSR ECD turned out to be one binding step instead of a biphasic process as observed in the subdomain studies (Huang 2009). The Tb$^{3+}$ binding affinity for bCaSR ECD was about $5.1 \pm 0.3$ μM with a Hill coefficient of $1.2 \pm 0.2$ (Figure 7.6). The $\text{Ca}^{2+}$-Tb$^{3+}$ competition assay gave the apparent dissociation constant for the ECD. For bCaSR ECD, the $\text{Ca}^{2+}$ binding dissociation constant calculated from $\text{Ca}^{2+}$-Tb$^{3+}$ competition assay using equation 2 was $38.4 \pm 2.2$ μM (Figure 7.7).
Figure 7.6 Tb$^{3+}$ titration curve of hCaSR ECDs.

Tb$^{3+}$ titration curve of purified CaSR ECDs. The buffer for Tb$^{3+}$ titration consisted of 20 mM PIPES, 10 mM KCl (pH 6.8). The arrow indicates the increase of the fluorescence with increasing [Tb$^{3+}$]. The titration curve was fitted using Hill equation.

Figure 7.7 Tb$^{3+}$ competition assay of CaSR ECDs.
Tb$^{3+}$-Ca$^{2+}$ competition assay of purified bCaSR ECD. The buffer for Tb$^{3+}$ titration consisted of 20 mM PIPES, 135 mM NaCl, and 10 mM KCl (pH 6.8). The arrow indicates the decrease of the fluorescence with increasing [Tb$^{3+}$].

7.2.5 NMR spectrum revealed calcium induced tertiary structure changes in bCaSR ECD.

The fluctuations in the chemical shift can be used to monitor the interaction between metals and the protein due to the sensitivity of chemical shifts of protein amides to even subtle conformational changes occurring at the interacting interface. Figure 7.8 depicts the one-dimensional (1D) NMR of the N$^{15}$ labeled bCaSR-ECD expressed from the Tuner strain. The proton signal from the aromatic group and side chain HN (chemical shift from 8 ppm~6ppm) indicated that the bacterial protein was at least partially folded. However, the large size of the protein (72 kDa) may also influence the resolution as well as the dispersion of spectrum.
Figure 7.8 1D $^1$H spectrum of $^{15}$N labeled bCaSR ECD.

The bCaSR ECD was expressed in the Tuner Strain at 25°C. The protein sample was prepared in 10 mM Tris buffer with pH at 7.4. The NMR spectrum was collected from an 800 MHz magnet.

The chemical shifts observed in the 2D spectrum of bCaSR-ECD showed a more complicated pattern compared with the mammalian expressed ECDs. The calcium induced chemical shift changes suggested multiple binding processes instead of 1:1 binding as demonstrated in fluorescence spectroscopy. As shown in Figure 7.8, the concentration bCaSR ECD is constantly maintained at 100 µM while the calcium concentration is increased stepwise. When the ratio of protein concentration versus [Ca$^{2+}$] equals 1:4, the
spectrum appeared similar as the ratio was 10:1. The spectrum of 1:0 is similar to the one with 1:1 (Figure 7.8).

Figure 7.9 (1H,15N)-HSQC spectra of bCaSR ECD with Ca\(^{2+}\) titration.

15N labeled CaSR ECD was expressed in the Tuner strain at 25°C. The sample was initially prepared in 10 mM Tris buffer with a pH at 7.2. Calcium was introduced to the system stepwise before each scan of the HSQC spectra using a 900 MHz NMR instrument.
7.2.6 Optimization for the expression condition of CaSR ECD

Due to the fact that the bacterial expressed CaSR ECD tends to degrade within a short time, efforts have been made to optimize the expression conditions using different cell strains, temperature, as well as expression time. By collaborating with Dr. Jian Hu from Michigan State University, we designed mutations on several cysteine residues and changed the rare codon in the human CaSR sequence. The optimized CaSR ECD sequence was then synthesized from the Genscript Company. Figure 7.10 shows the sequence alignment of the CaSR among different species.
Figure 7.10 Sequence alignment of CaSR ECD among different species.
Predicted secondary structures of CaSR are highlighted in colors. Red: Helix structure; Blue: Strand. Conserved Cysteines are highlighted in yellow; Green labeled cysteines are cysteines unique in the CaSR ECD.

Cysteines are generally considered to be important in the disulfide bond formation and protein folding. In order to facilitate the correct protein folding, we mutated non-conservative cysteines and cysteines that are involved in intermolecular dimerization to alanines (Figure 7.11). Six cysteines involved in three pairs of intramolecular disulfide bonds are kept in the sequence.

Query METABOTROPIC GLUTAMATE RECEPTOR SUBTYPE 1 (PDB: 1EWV)

Sbjct Human CaSR

Non-conserved Cys

May be involved in dimerization, but disordered in 1EWV

Mutated into Alanine in our constructs
Figure 7.11 sequence alignment of mGluR1 and the CaSR ECD domain. Intramolecular disulfide bonds are labeled with red arrows. Three cysteines that will be mutated to alanines are circled in red.

7.2.7 Problems associated with the CaSR ECD expression using bacterial system.

One of the problems associated with the CaSR ECD expression using *E. Coli* is protein degradation. As shown in Figure 7.12, the SDS page on the left indicated a dark band at around 70 kDa which is corresponding to the calculated CaSR ECD molecular weight. However, there are smeared bands at lower molecular weights. Western blot on the right side, shows that the smeared bands are at least partially due to the protein degradation. It is also possible that the degradation may start during protein expression.

Figure 7.12 Protein degradation problem observed during protein expression and purification

Left side: Coomassie blue staining of purified CaSR ECD collected from FPLC fractions. Right side: western blot of the same fractions. Protein was blotted using anti-flag antibody at 1:3000 dilution.
In order to solve the issue, several conditions have been screened for increasing protein stability. As shown in Figure 7.13, proteins aliquoted into six conditions started to degrading from Day 1. There were no significant differences among these reagents. However, the results on Day 3 suggested dramatic variation among the six conditions. Calcium free buffer may prevent the protein from severe degradation. Meanwhile, the decrease of the pH condition to 4.0 could also help in slowing down the degradation process. Other conditions, including addition of protein inhibitor (PMSF), mixing with 5% glycerol, as well as 20 μM of La³⁺, help to increase the stability of the protein to a certain extent, but not as promising compared to EDTA and an acidic environment.
Figure 7.13 Stability test of bacteria expressed CaSR ECD

Aliquots of CaSR ECD samples were prepared in different buffer conditions and incubated at room temperature or -20°C for up to 5 days. Samples are further loaded onto the SDS page gels.
Although some reagents might be helpful for preventing protein degradation, it would be better if the CaSR ECD can be expressed with less degradation bands. Several methods are considered to be feasible for dealing with this problem. First, deleting the cysteine rich domain as well as the signal peptide at the beginning of CaSR ECD sequence might increase the yield and decrease the misfolding of disulfide bonds during protein expression. Second, decreasing the expression temperature to 20 or 16 °C can be helpful for the protein folding correctly. Third, we can switch to different bacterial strains (e.g. SHuffle). SHuffle have DsbC prokaryotic disulfide bond isomerasers, which help disulfide bond formation and functions in bacterial cells.

7.2.8 Comparing the bacterial expressed protein with mammalian expressed CaSR ECDs

As can be seen from those aforementioned results, bCaSR ECD and hCaSR ECDs have few similarities in their biophysical studies. First, all of them showed a blue shift from their intrinsic Trp spectrum indicating the protein was at least partially in its native form. Second, all of their secondary structures are dominated by α-helix (Table 7.1). Third, protein from either of the two systems exhibited conformational changes upon their interaction with Ca$^{2+}$ as well as Tb$^{3+}$.

Table 7.1 Secondary structure comparison between bCaSR ECD and hCaSR ECD.

<table>
<thead>
<tr>
<th></th>
<th>α-Helix</th>
<th>β-Strands</th>
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<td>With Ca$^{2+}$</td>
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<td>With Ca$^{2+}$</td>
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<td>bCaSR</td>
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<td>48% ± 1%</td>
<td>27% ± 5%</td>
<td>25% ± 2%</td>
</tr>
<tr>
<td>mCaSR-WT</td>
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<td>55% ± 6%</td>
<td>17% ± 1%</td>
<td>21% ± 4%</td>
</tr>
<tr>
<td>mCaSR-Lec1</td>
<td>52% ± 5%</td>
<td>60% ± 4%</td>
<td>25% ± 3%</td>
<td>18% ± 1%</td>
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</table>
Despite the fact that the CaSR ECDs expressed from bacterial and mammalian systems share several similarities, the properties of CaSR ECDs expressed from the two systems have quite a few differences. The most apparent difference is the size of the CaSR ECDs. The bCaSR ECD showed a clear band at around 72 kDa; on the other hand, WT hCaSR ECD exhibited a smeared band in the range from 100 kDa~130 kDa while the Lec1 mutant hCaSR has one single band with a molecular weight of approximately 95 kDa. Under non-reducing conditions, the WT hCaSR ECD and Lec1 hCaSR ECD have molecular weight at around 240 kDa and 200 kDa respectively, corresponding to the calculated dimerization form of the receptor’s extracellular domain. However, the bCaSR ECD forms oligomers (Figure 7.14).

The second difference is the glycosylation pattern and it is one of the major factors that affected the thermal stability of the proteins. The melting point ($T_m$) of bCaSR ECD is around 80 °C in the absence of calcium. Interestingly, the WT hCaSR ECD demonstrated extraordinary thermal stability as the signal at the given wavelength did not change. The high mannose form of the hCaSR ECD, the Lec1 hCaSR ECD, showed a rapid disruption of the secondary structure at 92°C (Figure 7.15).

Moreover, the calcium induced conformational changes of the CaSR ECDs expressed from the two different systems are dissimilar. For bCaSR ECD, calcium caused less hydrophobic areas interacting with ANS, resulting in a 34% decrease of the fluorescence peak value; on the contrary, it induced exposure of more hydrophobic regions in the complex oligosaccharide form (WT hCaSR ECD) as there was a 25% increase in the fluorescence signal. Similar intensity enhancements, though to lesser extents (~10%), were detected in Lec1 hCaSR ECD.
Figure 7.14 Bacterial expressed CaSR ECD under reducing and non-reducing condition (Data adopted from Ling Wei). Immunoblotting using anti-CaSR antibody ADD shows that bCaSR ECD formed oligomers under non-reducing condition (Left lanes).
Figure 7.15 The thermal stability of CaSR ECDs from different expression systems.  

Temperature dependence of the molar ellipticity at 222 nm for CaSR ECDs in 10 mM Tris, pH 7.2 in the absence of calcium is shown in the figure. Square: bCaSR ECD; Open circle: Lec1 hCaSR ECD; Closed circle: WT hCaSR ECD.

Fourth, the binding affinities calculated from the fitting curves for titration experiments with bCaSR ECD and hCaSR ECDs are different. The observed Kds of hCaSR ECDs for Ca\(^{2+}\) were in the range of mM, however, the Kd of bCaSR ECD for Ca\(^{2+}\) was in \(\mu\)M range. It is also worthwhile to notice that during the titration of calcium into the buffer containing 2 \(\mu\)M bCaSR ECD, protein precipitation was observed. This phenomenon may be due to the fact that bCaSR ECD has less solubility compared with the hCaSR ECDs, which have glycosylation to increase their solubility.
7.3 Discussion

It has been suggested that the ECD of the mGluRs and CaSR share similar Venus Fly Trap structure based on several conserved amino acids regions and predicted secondary structures (Mei Bai 2004). However, there is limited structural and biochemical information available for CaSR due to its large molecular weight (~70 kDa as monomer) and heterogeneous glycosylation. Since the amino acid sequence of a protein determines its three-dimensional structure, it is worthwhile to utilize the bacterial system to express the CaSR ECD. In the present study, the target protein was expressed in the cytosol of bacteria and we were able to purify the bCaSR ECD via affinity columns. It is known that CaSR function as a dimer on the surface of cell membrane, the mammalian secreted ECD proteins, both the complex glycan form and the homogenous high mannose form, also present in the culture medium as a dimer form (Chapter 5). However, the bacterial expressed ECD forms an oligomer in the non-reducing environment.

Glycosylation is one of the most important posttranslational modifications occurring in protein biosynthesis and secretory pathways. As expected, distinctive from the mammalian expression system, the bCaSR ECD did not have glycosylation modifications. The thermodynamic stabilization calculations indicate that the stability of a protein may be enhanced when a glycan is covalently bound to the protein surface, which can be measured as an increase in melting temperature (292). The ECD protein with heterogeneous saccharides was able to maintain its secondary structure at high temperature (> 90°C) (Chapter 5). On the other hand, bCaSR ECD, which has no glycosylation modification, was comparatively less stable than its mammalian expressed counterparts. These phenomena may be attributed to the differences of protein tertiary
structures altered by the post-translational modification since their secondary structures were relatively similar.

Calcium, as a predominant agonist for CaSR, is believed to induce conformational changes of the receptor to convey the extracellular signal to intracellular messengers. However, the lack of evidence to prove such change makes it worthwhile to analyze the alteration of the receptor’s biophysical characteristics before and after the Ca\(^{2+}\) binding. One of the biophysical changes on CaSR, upon the interaction with Ca\(^{2+}\), is the thermal stability of its ECD. The addition of calcium altered the structural stability of CaSR-ECD, resulting in losing the secondary structure at a much lower temperature. Another possible interpretation is the positive charges of the metal accelerate the aggregation of the protein at higher temperatures.

The enthalpy change usually measures a change in the strength of the interactions between molecules. However, the decreased Tm value of bCaSR ECD as well as Lec1 CaSR ECD, in the presence of calcium, indicates that proteins could tend to aggregate with the addition of calcium.

Data from spectroscopy and NMR spectrum indicated that calcium induce conformational changes in CaSR ECD. There are fourteen tryptophan residues on the ECD, among them four are on the malleolar surface between lobe 1 and 2. The decrease of tryptophan intensity might be explained by a close form of receptor resulting from calcium binding which could lead to a more imbedded position of those tryptophan residues. However, the Trp spectrum relies on the environment surrounding the Trp residues. Since those Trp residues are not only near the predicted calcium binding site, but also spread out on the entire ECD, sensitivity to monitor the
real binding process can be affected. Interestingly, the decreased ANS signal resulting from calcium titration into the bCaSR ECD, revealed the difference between the bacterial expressed protein and the mammalian expressed protein.

In our lab’s previous studies, when titrated with Tb$^{3+}$, CaSR ECD subdomain 1, which includes predicted Ca$^{2+}$ binding site 1, site 2 and site 3, showed biphasic binding processes while subdomain 2 (including Ca$^{2+}$ binding site 2 & 3) and subdomain 3 (including Ca$^{2+}$ binding site 4 & 5) had monophasic responses to Tb$^{3+}$. However, in the present studies, bCaSR ECD presented a sigmoidal curve, suggesting multiple metal binding sites in the ECD may work cooperatively in order to accommodate the extracellular calcium concentration changes.

Overall, the present study provides further insights into the structure related CaSR functions and should permit further studies to define which are the key residues involving the interaction with the ligands for this receptor, including cations, nutrient amino acids, as well as antibiotics. This information will be crucial to understanding how CaSR responds to a variety of stimuli and will pave the way for future research of other members in GPCR family C.
CHAPTER 8. CALMODULIN, AN ASSISTANT FOR THE RECEPTOR INSERTION ON CELL MEMBRANE.

8.1 Introduction

The calcium sensing receptor (CaSR), which is predominately expressed in the parathyroid glands and kidneys, is a major player in sensing fluctuations of the extracellular calcium concentration $[\text{Ca}^{2+}]_o$ and in maintaining calcium homeostasis by regulating parathyroid hormone (PTH) secretion as well as renal $\text{Ca}^{2+}$ reabsorption (38). Since 1995 when CaSR was found universally in the gastrointestinal tract (41,166) and antral G cells (166), the exploration of its physiological function has been extended.

In the digestive system, proteins from dietary intake can be ultimately transformed to free amino acids which are essential molecules as they are the metabolic precursors of crucial substances which could be ligands for receptors, for instance L-histidine for histamine, L-tryptophan for 5-hydroxytryptamine (serotonin), and L-tyrosine for dopamine, catecholamines and thyroid hormone (165). Fluctuation of the plasma amino acid level can regulate the rate of hormone synthesis and secretion as well as calcium metabolism through the control of cellular signal transduction pathways (165).

It has been reported that aromatic amino acids can enhance the sensitivity of CaSR to extracellular calcium, which could be one potential explanation for how dietary protein modulates calcium homeostasis in normal individuals as well as patients with chronic renal failure (165,167). However, the mechanisms by which these two nutrients, calcium and L-Phe,
cooperatively regulate the function of the receptor are barely understood, other than the fact that they activate the CaSR through G_{q11} and G_{12/13} respectively\(^{(121)}\).

Recently, a novel concept which is termed “agonist-driven insertional signaling” (ADIS) has been brought to the field of CaSR signaling by Dr.Breitwieser’s group\(^{(144)}\). ADIS is considered to be contributing to the high degree of the receptor’s cooperativity as well as lack of desensitization\(^{(144)}\). The subsequent insertion of additional CaSR upon the activation of plasma membrane CaSRs is the fundamental feature of ADIS model. Since ADIS integrates the cell signaling with protein trafficking, it would be interesting to analyze whether ADIS contributes to the L-Phe modulated CaSR activity.

One of the scaffold proteins that have been reported to interact with the CaSR is filamin \(^{(80,81)}\). Filamin is ubiquitously expressed as a homodimer which is composed of monomers with 2647 amino acids forming 24 immune globulin-like repeats, two hinge regions, a dimerization domain at the C terminus, and an actin-binding domain at the N terminus \(^{(300)}\). Filamin crosslinks actin to cell surface receptors, for instance, dopamine D2 receptor (a G protein-coupled receptor), the Fc\_\gamma receptor, platelet glycoprotein Ib\_\alpha, \(\beta_1\) and \(\beta_2\) integrins, tissue factor, MAP kinases, the tumor necrosis factor receptor-associated factor-2 (TRAF2), Rho GTPases, and Smad proteins \(^{(301-304)}\). Filamin is also involved in protein trafficking and cycling of proteins by interacting with caveolin \(^{(305,306)}\). Filamin binds to the c-tail of the CaSR as demonstrated by yeast two hybrid assays, and there are two potential interaction regions, one 972-1031, and the other 907-997.
CaM is a ubiquitously expressed protein in the cytosolic environment. It has been reported that the interaction between the CaSR and CaM is essential for maintaining $[\text{Ca}^{2+}]_i$ oscillations triggered by $[\text{Ca}^{2+}]_o$(148). The previous study by our lab using a calmodulin binding domain (CaMBD) peptide indicated that CaM could interacted with the potential CaMBD at the c-tail of the CaSR in 1-8-14 class binding mode and it induced significant changes in the secondary and tertiary structure of this region (148). Since it has been reported that CaM can modulate the surface expression of other GPCR proteins (307), it is worthwhile to analyze how CaM, as one of many proteins that interacting with CaSR (see Chapter 1 for details), can regulate the trafficking as well as the function of this particular receptor. Calmodulin can stabilize the CaSR by interacting with the c-terminal of the receptor, specifically in the region between amino acids 881-894 (148). Moreover, the presence of calmodulin (CaM) inhibitors can diminish the extracellular calcium $[\text{Ca}^{2+}]_o$ as well as the L-Phe induced intracellular calcium concentration $[\text{Ca}^{2+}]_i$ change(120). However, how CaM can regulate the the agonist triggered receptor function as well as the following downstream signaling pathways is unclear.

In the present study, we report our observation that in the presence of L-Phe, the ADIS was enhanced in HEK293 cells either expressing wild type CaSR or disease related loss-of-function mutant CaSR, which possibly contributes to the L-Phe potentiated receptor activity. The deletion of potential CaM and filamin interaction regions changed the $[\text{Ca}^{2+}]_i$ oscillation patterns and abolished the L-Phe promoted ADIS. Our findings may provide a novel angle to investigate how calcium and L-Phe cooperatively work together to promote the function of the CaSR.
8.2 Results

8.2.1 L-Phe can promote the function of CaSR by working cooperatively with Ca$^{2+}$.

In the presence of L-Phe, the [Ca$^{2+}$]$_i$ oscillation pattern was changed as the [Ca$^{2+}$]$_i$ started oscillating at lower [Ca$^{2+}$]$_o$, and a [Ca$^{2+}$]$_i$ plateau required less [Ca$^{2+}$]$_o$ to be reached to (Figure 8.1a). Next, we utilized immunostaining assay to analyze the Ca$^{2+}$ triggered ADIS in CaSR transfected HEK293 cells with or without the addition of L-Phe. The transfected CaSR has a flag tag in the extracellular domain. Cells were first labeled with a rabbit monoclonal FLAG antibody to block all plasma membrane FLAG epitopes, followed by stimulation with different calcium concentration. Cells were then fixed with formaldehyde and incubated with a mouse monoclonal FLAG antibody to identify newly exocytosed receptor. After permeabilization with triton, cells were stained with goat anti-rabbit Alexa488 and goat anti-mouse Alexa568 to differentiate the original surface CaSR and newly translocated CaSR. The immunostaining assay revealed that the agonist driven receptor insertion phenomena induced by [Ca$^{2+}$]$_o$ emerged at 3.0 mM [Ca$^{2+}$]$_o$ with the addition of L-Phe instead of 4.0 mM [Ca$^{2+}$]$_o$ without L-Phe (Figure 8.1b). Quantitative analysis of the surface receptor intensity using Image J was able to generate an ADIS-[Ca$^{2+}$]$_o$ response curve which correlated with their [Ca$^{2+}$]$_i$ change (Figure 8.1c). Meanwhile, the results of the biotin-cleavable western blot assay showed that the CaSR internalization in the presence of both L-Phe and Ca$^{2+}$ compared to Ca$^{2+}$ only was similar (Figure 8.1d).
Figure 8.1 L-Phe and $[\text{Ca}^{2+}]_o$ influenced the activity and trafficking of CaSR.

(a.) Representative oscillation pattern from a single HEK-293 cell transfected with WT CaSR. The $[\text{Ca}^{2+}]_i$ was measured using Fura-2AM as described in methods. Each experiment was carried out in 0.1% DMSO as W7 solvent control. Cells were first submerged in Ca$^{2+}$-free Ringer’s buffer followed by treatment with various $[\text{Ca}^{2+}]_o$ buffers until $[\text{Ca}^{2+}]_i$ reached a plateau (up to 8 mM $[\text{Ca}^{2+}]_o$) in the presence or absence of 5 mM L-Phe.

(b.) HEK293 cells transiently expressing FLAG-CaSR were labeled with polyclonal anti-FLAG antibody (pFLAG) for 1 hour (4°C), then incubated in various $[\text{Ca}^{2+}]_o$ buffer for 10 min (37°C). Cells were labeled with monoclonal anti-FLAG antibody (mFLAG) for
1 hour (room temperature) after fixing with formaldehyde. Cells were permeabilized and labeled with goat anti-rabbit Alexa488- and goat anti-mouse Alexa555-conjugated secondary antibodies. Incubation in high \( [\text{Ca}^{2+}]_o \) buffer increased binding of monoclonal anti-FLAG antibody (mFLAG) to CaSR, demonstrating ADIS. Images shown are representative of \( N = 25 \) cells. (c.) Quantitative analysis of ADIS: The fluorescent intensity from Alexa555 was measured by Image J which was further divided by the fluorescent intensity from Alexa488. The values were calculated for all cells at each indicated \( [\text{Ca}^{2+}]_o \) with (grid) or without (blank) L-Phe. (d.) Effects of L-Phe on internalization of the WT CaSR. CaSR transfected HEK293 cells were labeled with disulfide-cleavable biotin in calcium-free buffer for 30 min, and then stimulated with various \( [\text{Ca}^{2+}]_o \) for 10 mins. The internalized CaSR was immunoprecipitated with the anti-FLAG antibody and detected by streptavidin peroxidase. Top panel: Non-cleaved biotin labeled CaSR stands for internalized receptor. Lower panel: total CaSR.

8.2.2 L-Phe enhanced the ADIS in inactive CaSR mutants.

We next examined whether this L-Phe enhanced ADIS existed in the disease related mutations. The CaSR L173P and P221Q are two loss-of-function mutants recently reported by Hannan et. al. The immunostaining results showed that the translocation of the mutated receptors from cytosolic reservoir to cell membrane can also be facilitated by L-Phe (Figure 8.2). Although the L-Phe enhanced the ADIS in mutant CaSRs, it did not increase the surface receptor internalization as shown in Figure 8.3.
Figure 8.2 L-Phe enhanced ADIS in disease related mutants.

Cells transfected with L173P and P221Q were exposed to various \([Ca^{2+}]_o\) for the measurement of ADIS as described in Figure 6.1(b). Quantitative analysis of ADIS was shown in the scatter plot. The fluorescent intensity from Alexa555 (I_{555}) and Alexa488 (I_{488}) was measured by Image J. The ratio of I_{555}/I_{488} was further plotted against \([Ca^{2+}]_o\) and normalized to the intensity ratio when \([Ca^{2+}]_o\) was at 1.0 mM. Open circle: without L-Phe; Closed circle: in the presence of L-Phe.

Figure 8.3 Receptor internalization in disease related mutants.

CaSR mutants transfected HEK293 cells were labeled with disulfide-cleavable biotin in calcium-free buffer for 30 min, and then stimulated with various \([Ca^{2+}]_o\) for 10 mins. The
internalized CaSR was immunoprecipitated with the anti-FLAG antibody and detected by streptavidin peroxidase.

8.2.3 CaM inhibitor W7 affected the trafficking of the CaSRs.

Previously studies have reported that CaM plays a pivotal role in regulating the traffic of the CaSRs (148) and it may also be involved in modulating the L-Phe triggered [Ca$^{2+}$]$_i$ oscillation(120). We further investigated whether CaM could also be involved in regulation of the ADIS effects. We first incubated the cells with CaM inhibitor W7 followed by monitoring the [Ca$^{2+}$]$_o$ induced [Ca$^{2+}$]$_i$ changes using Fura-2 AM in the presence or absence of L-Phe. As shown in Figure 8.4, the [Ca$^{2+}$]$_i$ oscillation has gone through dramatic pattern alterations. Most cells exhibited transient peaks instead of continuous sinusoidal changes (Figure 8.4a). The confocal images suggested the ADIS induced by Ca$^{2+}$ was still observable (Figure 8.4b); on the contrary, the L-Phe enhanced ADIS was abolished. Meanwhile, the ERK activity which once could be promoted by L-Phe was also reduced by W7 (Figure 8.4c). The [Ca$^{2+}$]$_o$ induced internalization of the receptor had no significant change with the pretreatment of W7 either in the presence or the absence of L-Phe (Figure 8.4d).
Figure 8.4 W7 reduced the Ca^{2+} and L-Phe induced ADIS.
(a.) Cells were pretreated with 50 μM W7 or DMSO for 30 mins. Figures show representative oscillation pattern from a single pretreated WT CaSR transfected HEK-293 cell with or without L-Phe. Right panel: Statistic analysis of total responded cells. Empty bar: DMSO only; Black: with 50 μM W7; Red diagonal: with 5 mM L-Phe and DMSO; Red solid: with 5 mM L-Phe, DMSO and 50 μM W7. (b.) HEK293 cells transiently expressing FLAG-CaSR were first treated with either W7 or DMSO for 30 mins at 37°C. Cells were labeled with polyclonal anti-FLAG antibody (pFLAG) for 1 hour (4°C), then incubated in various [Ca2+]o buffer with 5 mM L-Phe for 10 min (37°C). Cells were labeled with monoclonal anti-FLAG antibody (mFLAG) for 1 hour (room temperature) after fixing with formaldehyde. Cells were permeabilized and labeled with goat anti-rabbit Alexa488- and goat anti-mouse Alexa555-conjugated secondary antibodies. Right panel: Quantitative analysis of ADIS as described in Fig. 1c. (c.) W7 inhibited [Ca2+]o activated ERK phosphorylation in CaSR-transfected HEK293 cells. HEK-293 cells transfected with WT CaSR or its mutants were incubated in serum-free high glucose MEM medium containing 0.2% BSA overnight. Cells were pretreated with DMSO alone or W7 in DMSO for 30 mins, cells were then incubated in various Ca2+ concentrations (0.0~8.0mM) for 10 min at 37 °C. The incubations were stopped by exposure to the lysate buffer and processed for SDS/PAGE and Western blotting as described in the Methods. The western blot results were further quantified using Image J. Empty bar: without W7; Black bar: with W7. (d.) Effects of W7 on Ca2+ and L-Phe triggered internalization of the WT CaSR. CaSR transfected HEK293 cells were incubated with DMSO or W7 for 30 mins. Cells were then labeled with disulfide-cleavable biotin in calcium-free buffer for 30 min, followed by
stimulation with various $[\text{Ca}^{2+}]_o$ for 10 mins. The internalized CaSR was immunoprecipitated with the anti-FLAG antibody and detected by streptavidin peroxidase.

8.2.4 Mutations on the CaM interaction region changed the L-Phe enhanced intracellular calcium response pattern.

Since CaM is a ubiquitous molecule that participates in hundreds of intracellular signaling pathways, the addition of W7 could affect other signal transductions besides the potential disruption of the interaction between CaSR and L-Phe. In order to analyze the specific role of CaM in the trafficking of the CaSR, we designed experiments with several CaSR C-tail mutants (CaSR F881E/V894E, F881E/T888E, F881E/T888E/V894E) which their binding with CaM are believed to be interrupted (148). The $[\text{Ca}^{2+}]_i$ changes upon the $[\text{Ca}^{2+}]_o$ stimulation were altered in those mutants as shown in Figure 8.5. Majority of the cells transfected with C-tail mutants F881E/V894E, F881E/T888E, F881E/T888E/V894E showed transient peaks as their responses to the increase of $[\text{Ca}^{2+}]_o$. The addition of L-Phe could not change the $[\text{Ca}^{2+}]_i$ response as the way it changed the oscillation patterns in WT CaSR, i.e. starting oscillation at lower $[\text{Ca}^{2+}]_o$, increasing the $[\text{Ca}^{2+}]_i$ oscillation frequencies; instead, the $[\text{Ca}^{2+}]_i$ responded in a special way in the presence of L-Phe. Noticeably, the width of each transient peak became wider in the mutant F881E/V894E, mutant F881E/T888E and mutant F881E/T888E/V894E as well. Moreover, the peak descending rates were also decreased compare to WT CaSR.

Because the CaM CaSR interaction region also includes T888, which is a potential phosphorylation site of CaSR, in order to rule out the possibility that mutations at the phosphorylation site would complicate the conclusion, single mutation to the residue T888 was
employed for an in depth analysis. As indicated in (Figure 8.5), the T888V mutation, which abolished the phosphorylation site, forced the receptor to start oscillating at lower $[Ca^{2+}]_o$ compared with WT CaSR. However, with L-Phe, the oscillation patterns were changed likely as in WT CaSR. Thus, the L-Phe caused peak transformation is more related to CaM other than phosphorylation.
Figure 8.5 The $[\text{Ca}^{2+}]_i$ oscillation in cells expressing CaSR with mutations in the CaM binding region.
Representative oscillation patterns from a single cell. HEK-293 cells transfected with CaSR or one of its mutants were loaded with Fura-2 AM for 15 min. The \([\text{Ca}^{2+}]_i\) was assessed by monitoring emission at 510 nm with excitation alternately at 340 or 380 nm as described in methods. Each experiment carried out with or without 5 mM L-Phe began in \([\text{Ca}^{2+}]_o\)-free Ringer’s buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, and 1.0 mM MgCl\(_2\), pH 7.4) followed by stepwise increases in \([\text{Ca}^{2+}]_o\) until \([\text{Ca}^{2+}]_i\) reached a plateau (up to 10 mM \([\text{Ca}^{2+}]_o\)).

8.2.5 Filamin A is involved in the CaM regulated CaSR trafficking.

The next question is how CaM could modulate the traffic of the receptor. The C-tail of the CaSR has been reported to interact with the cytoskeleton protein, filamin. Truncation of these potential binding domains on CaSR led to the weakened interaction with filamin as shown in (Figure 8.6). Interestingly, the disruption of the CaSR-filamin interaction also influenced the communication between CaM and CaSR. Notice that the molecular weight of CaM shown in the western blot results indicated its existence as a dimer form. It is worthwhile to look into the \([\text{Ca}^{2+}]_i\) oscillation pattern in those mutants as well as the ADIS effects.
Figure 8.6 Filamin A and CaM interacts with CaSR.

HEK293 cells expressing FLAG-CaSR or its Filamin truncation mutants (Δ860/Δ906-998/Δ907-999/Δ860971) or CaM truncation mutants (Δ880-894) or CaM interaction mutation (3E3) were immunoprecipitated with anti-flag antibody. The input receptor (CaSR), Filamin A and CaM were immunoblotted with their specific antibodies respectively.

8.3 Discussion

In the present study, we characterized how the interaction between CaSR and CaM could transduce the extracellular amino acids triggered signaling into the intracellular environment and modulate the trafficking of the receptor. We have demonstrated that the disruption of the CaM binding site on the C-tail of CaSR altered the L-Phe and [Ca^{2+}]_o cooperatively induced [Ca^{2+}]_i oscillation patterns in the HEK293 cells transfected with the CaSR. The cross-talk between the two proteins and filamin A appear to be critical in the L-Phe enhanced agonist-driven receptor insertion signaling.

The addition of L-Phe can potentiate the responses of HEK293 cells transfected with the CaSR to the changes of extracellular calcium concentration, characterizing by starting as well as terminating [Ca^{2+}]_i oscillation at lower [Ca^{2+}]_o and an increase in the oscillation frequency at the EC50 for [Ca^{2+}]_o. The data reported herein suggested that L-Phe also enhanced the [Ca^{2+}]_o driven receptor insertion while stimulating more surface receptor underwent internalization. The CaSR specific allosteric modulator NPS R-568 has been reported to have the similar ADIS(144). Since
the L-Phe induced [Ca$^{2+}$]$_i$ change via the CaSR requires a minimum of 1.5 mM [Ca$^{2+}$]$_o$, the potentiated ADIS effect was barely observable when the [Ca$^{2+}$]$_o$ was in the physiological range. This phenomenon was also detected in the disease related mutants and was well correlated with their respective [Ca$^{2+}$]$_i$ oscillation patterns, suggesting that both active and inactive receptors could be modulated in the similar way as WT CaSR. On the other hand, the L-Phe enhanced ADIS was diminished in the mutant L173P, of which the rescue ability of the L-Phe was impaired possibly due to the sterically hindering by the Leu to Pro mutation, indicating the appropriate interaction between the CaSR and the L-Phe should be crucial in transducing the extracellular signaling into the cytosol. Considering the fact that the [Ca$^{2+}$]$_i$ alterations responded much faster than the ADIS, the oscillation patterns could be a necessary messenger for the regulation the trafficking of the CaSR.

In the present study, we confirmed that not only the application of CaM inhibitor W7 but also the mutations on the CaMBD, which interrupted the interaction with CaM could change the [Ca$^{2+}$]$_i$ oscillation patterns upon the step wise increase of [Ca$^{2+}$]$_o$. The reduced ADIS in these CaSR mutants suggested a linkage between the two events. The addition of L-Phe failed to rescue the [Ca$^{2+}$]$_i$ oscillation as it would to WT CaSR indicating CaM probably involved in the L-Phe regulation which is in a way correlated with the findings from Rey et. al. that CaM inhibitors impaired the L-Phe induced [Ca$^{2+}$]$_i$ changes(120). Meanwhile, the L-Phe enhanced ADIS was also abolished suggesting CaM participates both in the [Ca$^{2+}$]$_o$ stimulated ADIS as well as in the L-Phe signaling.
Based on the results, a model of the L-Phe enhanced Ca\(^{2+}\) induced ADIS was proposed as presented in the schematic figure (Figure 8.7). CaM binds to the CaSR to stabilize the receptor during its interaction with agonists. In the presence of Ca\(^{2+}\) the ADIS is predominately regulated by 14-3-3 protein (144). The addition of W7 did not completely abolish the transferring of novel receptor to the cell membrane. The L-Phe cooperatively regulated the CaSR with Ca\(^{2+}\), meanwhile it activated Gi/o signal pathway other than Gq/α. CaM is a crucial element in the L-Phe regulated [Ca\(^{2+}\)]\(_i\) change. The disruption of its interaction with CaSR not only alter the [Ca\(^{2+}\)]\(_o\) induced [Ca\(^{2+}\)]\(_i\) oscillation pattern but the [L-Phe] promoted [Ca\(^{2+}\)]\(_i\) responses. CaM may participate in the L-Phe enhanced ADIS in a dimer form.

Figure 8.7 Schematic presentation of the L-Phe enhanced ADIS under the modulation of CaM
Phosphorylated CaM forms a dimer and binds to the C-tail of CaSR. The stimulation with \([\text{Ca}^{2+}]_o\) and L-Phe facilitates the phosphorylation of ERK. The later may accelerate the opening of cytosolic CaSR reservoir and potentiate the exocytosis.

In the present study, we found that in the presence of L-Phe, the ADIS was enhanced in HEK293 cells transfected with either wild type CaSR or disease related loss-of-function mutant CaSR. Further analysis with the calmodulin (CaM) inhibitor suggested CaM could be involved in regulating the L-Phe enhanced ADIS. Studies on the CaSR with mutations at calmodulin binding domain which interrupted CaM-CaSR interaction and the CaSR with Filamin A binding domain truncation showed that not only the L-Phe induced intracellular calcium\([\text{Ca}^{2+}]_i\) oscillation was altered, but the L-Phe promoted receptor insertion phenomena was abolished. Thus, L-Phe possibly regulates the receptor through CaM and Filamin A modulation.

Several experiments still need to be done in order to finalize the story. The interaction between Filamin A and the c-tail of CaSR need to be confirmed either using pull down assay or immunofluorescence study. Whether Filamin A is truly involved in the CaM modulated CaSR trafficking needs to be further investigated. The relationship among CaM stabilized surface receptor and receptor internalization as well as the ADIS might be much clearer with bungotoxin linked SEP-CaSR. Our approach will shed light on understanding the mechanisms underlying the agonist regulated receptor trafficking for other GPCR family members and will provide new strategies for drug designing targeting at various GPCR proteins.
CHAPTER 9. PROBING THE CORRELATION BETWEEN THE $[\text{Ca}^{2+}]_{\text{ER}}$ AND THE TRAFFICKING OF CASR

9.1 Introduction

The endoplasmic reticulum (ER) is considered as a major storage reservoir and a crucial regulator of intracellular $\text{Ca}^{2+}$ as it stores more than 90% of the $\text{Ca}^{2+}$ in the cells, although its volume is less than one tenth of the total cell (308-310). ER is also the most important suborganelle for global protein maturation, as approximately one third of all open reading frames code for proteins that enter the ER in human. A polypeptide starts with translocation across or into the ER membrane before its secretory pathway. It will not be transported via the Golgi apparatus to the cell surface unless it is folded and modified correctly in the ER. The ER lumen has a unique environment for the proper folding of protein, including complex pathways, tons of folding factors like Hsp90, Cyp, FKBP, PDI, UGGT etc., quality control, recycling (e.g. ER-to-Golgi intermediate compartment (ERGIC)), Especially for membrane proteins, which usually contain glycosylation sites, the ER provides a good compartment for proper modification such as glycosylation and disulfide bond formation to stabilize their conformation before they are secreted out and exposed to extracellular milieu (311). In this case, protein folding and secretion can be related with the ER calcium concentration. An accurate way for quantifying the ER $\text{Ca}^{2+}$ concentration is thus in great need.

Certain mutations on CaSR can lead to reduced receptor surface expression on the cell membrane. The treatment with the CaSR specific positive allosteric modulators can increase its surface expression while the negative allosteric modulators have the opposite effects. Since ER is one of the major subcellular organelles involving the synthesis, modification and transportation
of proteins, it would be worthwhile to test how those allosteric modulators can affect ER, mainly focusing on the ER calcium concentration.

How these allosteric modulators can change the receptor expression level is still obscure. One of the hypotheses is that the drug treatment can influence the ER calcium concentration change thereby affecting the foldings of the receptors and the following trafficking pathways.

In order to analyze the ER calcium concentration, an ER targeted calcium sensor should be an essential tool

Currently, there are two major generations of ER Ca\(^{2+}\) sensors. The first one is small molecular based Ca\(^{2+}\) dye, Mag-fura-2 which can majorly measure Ca\(^{2+}\) concentration in calcium stores (e.g. mitochondria or ER) due to its small size and low affinity to Ca\(^{2+}\). The other type is based on fluorescent proteins consisting of a Ca\(^{2+}\) regulated protein such as calmodulin or troponin C(181,312,313). The advantage of those protein based Ca\(^{2+}\) indicators is that they can target subcellular organelles with high spatial and temporal resolution (9). However, they still have some limitations during the application in excitable cells (18). Our group has engineered a new type of Ca\(^{2+}\) sensors, named CatchER(calciium sensor for detecting high concentration in the ER), based on the green fluorescence protein. CatchER has an ER retention sequence KDEL and its EGFP based property allows it to have intensity change upon calcium binding. CtachER meets the requirements for a better Ca\(^{2+}\) indicator such as accurate targeting to certain cellular compartments, the ability to quantitatively measure the ER Ca\(^{2+}\) concentration and, fast release kinetics, and minimized quenching problems (185).
Although CatchER has been successfully applied for detecting the Ca\textsuperscript{2+} release in mouse muscle sarcoplasmic reticulum (SR), the lower temperature requirement for the expression of CatchER in HEK293 cells limited its further application, e.g. co-expression with other proteins in cells. Our lab further improved this calcium sensor to ensure its chromophore formation at 37 °C and named it CatchERT.

In the present study, we aim to answer how ER Ca\textsuperscript{2+} signaling regulates CaSR functional cooperativity via modulation of receptor surface expression. Either CatchER or CatchERT were co-transfected with CaSR and the ER calcium concentration changes were monitored using these designed calcium sensors after treating with various drugs. We found that the positive allosteric modulator NPS R-568 could increased the calcium concentration in ER, while NPS 2134 had minimum effect on the ER.

9.2 Results

9.2.1 The CaSR allosteric regulators can influence the receptor expression level.

It has been reported that the not only the addition of proteasome inhibitor e.g. GM132 can increase the amount of the CaSR expressed at the cell surface, but also overnight incubation with positive allosteric CaSR modulator NPS R-568 can increase the expression of the receptor (268). Here we confirmed that the 0.1%DMSO as solvent only treatment did not change the expression level of WT CaSR while the negative regulator NPS-2143 down regulated the total amount of CaSR and the positive modulator NPS-R568 had the opposite effect.
Figure 9.1 Total CaSR expression in HEK293 cells with various treatments.

After transfection with CaSR for 48 hours, HEK293 cells were treated with 10 μM CaSR positive allosteric modulator NPS R-568 in 0.1% DMSO or 10 μM negative allosteric modulator NPS 2143 in 0.1% DMSO or 0.1%DMSO (as solvent control) for 16 hours. Cells were collected and lysed in RIPA buffer. The total CaSR was detected by anti-Flag antibody. GAPDH was used as internal control.
9.2.2 Monitoring the $[\text{Ca}^{2+}]_{\text{ER}}$ using calcium sensor CatchER.

In the present study, we coexpressed Catcher and the CaSR in HEK293 cells in order to detect the extracellular calcium induced intracellular calcium concentration change as well as the corresponding calcium concentration alteration occurring in sub cellular organelles. Because CatchER has to be expressed at 30°C while the CaSR exhibited best expression at 37°C, we used two different protocols to optimize the condition for co-expression of the two proteins simultaneously. Figure 9.2 shows the first protocol in which cells were initially cultured at 37°C for 12 hours after transfection followed by 12 hours incubation at 30°C. Cells were continued in culture for another 4 hours before proceeding to the imaging experiments. The green line represents the fluorescent intensity from CatchER while the red line represents the signal from Rhod-2, which is a calcium sensing dye to measure the cytosolic calcium concentration, from the same single cell. Cells responded to upon the addition of extracellular calcium indicating the CaSR had been expressed. However the dynamic range of CatchER is relatively small compared with Rhod-2. Figure 9.3 indicated the intracellular responses using the second protocol. Apparently the CaSR did not properly fold and express on the cell surface as suggested by impaired the intracellular calcium responses. After comparing the two methods, the expression with two temperatures was chosen for future measurements.
Figure 9.2 Monitoring the $[\text{Ca}^{2+}]_{\text{ER}}$ using CatchER (first method).

After co-transfection with CaSR and CatchER, cells were first incubated at 37 °C overnight then the temperature was reduced to 30°C for continuing expression another 12 hours. Cells were further moved to a 37°C incubator for another 4 hours prior to experiments. The calcium concentration is indicated above the lined bar. Ionomycin is used as ionopore to transport the extracellular calcium to the intracellular enviroment. Red: cytosolic calcium changes measured using Rhod-2. Green: signals from CatchER.

Figure 9.3 Monitoring the $[\text{Ca}^{2+}]_{\text{ER}}$ using CatchER (second method).

After co-transfection with CaSR and CatchER, cell were first incubated at 30 °C for 2 days then the temperature was increased to 37°C for continuing 1 day expression. Red: cytosolic calcium changes measured using Rhod-2. Green: signals from CatchER.
9.2.3 Measuring the drug induced $[\text{Ca}^{2+}]_{\text{ER}}$ in CaSR transfected HEK293 cells using CatchER.

We next analyzed the intracellular responses upon the treatment with different CaSR modulators. HEK293 cells co-transfected with CatchER and CaSR were treated with the CaSR positive modulator NPS R-568, the negative modulator NPS 2143 or DMSO as solvent control overnight at 37°C. As shown in Figure 9.4, 3.0 mM of $[\text{Ca}^{2+}]_o$ can trigger the increase of cytosolic calcium concentration. Some of the cells started to show $[\text{Ca}^{2+}]_i$ oscillation (Figure 9.4B) while some of the cells responded as transient peak of cytosolic $[\text{Ca}^{2+}]$ (Figure 9.4A). The incubation with 0.1% DMSO barely had any influence on the extracellular induced intracellular calcium change. Incubation with NPS 2143 diminished the cytosolic calcium oscillation frequency as well as the peak amplitude. On the other hand, NPS R568 had the opposite effect. The calcium alterations in the ER were less pulsatile compared with its cytosolic changes. Only a sudden decrease of the $[\text{Ca}^{2+}]_{\text{ER}}$ was able to be detected when cells were exposed to 3.0 mM $[\text{Ca}^{2+}]_o$ and the $[\text{Ca}^{2+}]_{\text{ER}}$ started to increase when the $[\text{Ca}^{2+}]_o$ mM dropped to 1.0 mM. 1.5 $\mu$M of ionomycin was introduced as a positive control. The addition of ionomycin induced a $[\text{Ca}^{2+}]_i$ climbing and a similar $[\text{Ca}^{2+}]_{\text{ER}}$ descending was able to be detected. Although the fluorescence quenching phenomenon is quite notable, the overall $[\text{Ca}^{2+}]_{\text{ER}}$ responses were able to be recorded.
Figure 9.4 [Ca\textsuperscript{2+}]\textsubscript{ER} measurement in HEK293 cells treated with various CaSR allosteric modulators.

Cells were seeded on coverslips and transfected with CatchER T and CaSR simultaneously. Cells were continuously cultured for another 48 hours at 37°C and further treated with
various drugs for 16 hours. After loading with Rhod-2, the coverslip was mounted onto the
microscope working stage. The chamber was first perfused with 3 mM Ca\(^{2+}\), followed by
calcium free washing buffer. Then cells were then treated with 1.5 \(\mu\)M ionomycin. The
cytosolic calcium concentration was measured using Rhod-2 (Red), while the \([\text{Ca}^{2+}]_\text{ER}\) was
analyzed using CatchER-T(Green). A. WT CaSR expressed cells as controls. B. Cells were
treated with DMSO. C. Cells were incubated with NPS-2143, which is a negative allosteric
modulator for CaSR. D. Cells were treated with NPS R-568, which positively regulated
CaSR.

Next, we measured the \([\text{Ca}^{2+}]\) in subcellular organelles using Mag-Fura-2 and CatchER. The
former compartmentalizes in the ER, mitochondria as well as cytoplasm while the latter targets
to the ER only. The calibration using Mag-fura-2 suggested an increase in the subcellular
organelles \([\text{Ca}^{2+}]\) after incubation with the CaSR positive modulators NPS R-568. Further
analysis using CatchER showed an increase in the \([\text{Ca}^{2+}]_\text{ER}\) upon the treatment with NPS R-568.
Other treatments did not have significant influences on the \([\text{Ca}^{2+}]_\text{ER}\).
Figure 9.5 Calibration of ER calcium concentration using Mag-fura 2.

The $[\text{Ca}^{2+}]_{\text{ER}}$ was calculated using $[\text{Ca}^{2+}]_i = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$ (314) and the results from different drug treatments were further normalized to WT. The calcium binding constant for Mag-Fura-2 is around 25 $\mu$M.

![Graph showing relative $[\text{Ca}^{2+}]_{\text{ER}}$ with WT, 0.1% DMSO, 2143, and 568. * p<0.05]

Figure 9.6 Calibration of ER calcium concentration using CatchER.

The $[\text{Ca}^{2+}]_{\text{ER}}$ was calculated using $[\text{Ca}^{2+}]_{\text{ER}} = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$ The $K_d$ of CatchER was 0.18 mM and the results from different drug treatments were further normalized to WT.

9.2.4 Insertion of Furin tag in CaSR functional mutants.

White et.al has categorized CaSR loss-of-function mutants into four types: Class Ia mutants have little expression on the plasma membrane regardless of the presence of proteasomal inhibitor MG132. They went through the mature glycosylation process and traverse from ER to the Golgi;
Class Ib mutants are also not significantly localized to the plasma membrane, but they are targeted for degradation from the ER or ER-Golgi intermediate compartment (ERGIC). Class Ila can be expressed on the cell membrane without MG132 and their function can be rescued by CaSR positive allosteric regulator NPS R-568. Class IIb CaSR mutants can locate on the plasma membrane but their function is barely rescued by NPS R-568. Figure 9.7 shows the schematic diagram of the four types of mutants. The pro-protein convertase furin cleavage sequence “ARRRRKGRLDV” was cloned right behind flag-tag in our pcDNA-CaSR (Figure 9.8). Furin is a trans-Golgi network (TGN)-resident type I membrane protease with a catalytic site facing the Golgi lumen (Furin at the cutting edge: from protein traffic to embryogenesis and disease.).
Figure 9.7 Schematic diagram of the trafficking pathways of the disease related mutants (315).

A.

B.

Figure 9.8 Schematic diagram of Furi-CaSR constructions.

A. Furi sequence inserted into pcDNA CaSR. B. Furi sequence inserted into pEGFP CaSR.
9.2.5 Analysis of the constructed Furin-CaSR and its mutants

A few CaSR loss-of-function mutants from each subclass have been selected for the present studies. As shown in Figure 9.10, the furin tagged WT CaSR, either constructed in the pcDNA plasmid or in the pEGFP plasmid, traversed from ER to Golgi would be cleaved by furin resulting in an additional band at 50 kDa in reduced environment on a denatured SDS-page gel. P39A and G778D are two inactive mutants that can not traffic to the Golgi, therefore they maintained in an intact form. In the absence of β-mercaptoethanol, WT Furin-CaSR was still able to keep all the fragments together even though it interacted with pro-protein convertase. Further analysis of other mutants confirmed this phenomenon (Fig. 8.11) as majority of the mutants had an intense western blot band with fragment molecular weight at 50 kDa except for L174R which also belongs to class Ib as P39A and G778D.

The pull down experiment using anti-flag antibody revealed similar results compared with the experiment directly using cell lysates in terms of the band at 50 kDa (Fig. 8.12). Multiple bands are observed under the reducing environment due to the fact that the secondary antibody not only recognizes the primary anti-CaSR ADD antibody but also the anti-flag antibody which was used to pull down the receptor.
Figure 9.9 Schematic illustration of location of CaSR loss-of-function mutations.

Mutations involved in the present studies are highlighted with frames. CaSR contains 1078 amino acids. The amino-terminal, ECD is comprised of a VFTD, divided into lobes I (horizontal bars) and II (vertical bars), and a CysRD (diagonal bars). The TMD contains seven helices (gray boxes). Extra- and intracellular loops and carboxyl terminus are white. Yellow: Class Ia, Green: Class IIa, Red: Class Ib, Blue: Class IIb.
Figure 9.10 Western blot of Furin-CaSR constructions with or without reducing reagent (R). (-G) represent plasmids constructed in pEGFP vectors.

Forty-eight hours after transfected with different mutants, cells were lysated and underwent western blot. R: with the addition of reducing reagent (β-mercaptoethanol).

Yellow: Class Ia, Green: Class IIa, Red: Class Ib, Blue: Class IIb. (The clone of R227Q was not correct).

Figure 9.11 Western blot of disease related mutations in pcDNA-Furin-CaSR constructs.

Figure 9.12 Pull down assay analysis of disease related mutations in pcDNA-Furin-CaSR constructs.
Forty-eight hours after transfection with different mutants, cell lysate was co-immunoprecipitated with anti-flag antibody overnight at 4°C. Proteins were further eluted from beads with sample buffer and underwent western blot. **R:** with the addition of reducing reagent (β-mercaptoethanol). **Yellow:** Class Ia, **Green:** Class IIa, **Red:** Class Ib, **Blue:** Class IIb.

### 9.3 Discussion

How the calcium concentration in the endoplasmic reticulum (ER) change during cytosolic calcium oscillation is a question that has puzzled scientists for a long time. The absence of a proper ER calcium indicator is one of the major barriers. The development of CatchER enables us to monitor the ER calcium concentration change in living cells due to its engineered ER targeting peptide and the relative weak binding affinity (0.19 ± 0.02 mM) (185). The fact that we only observed a decrease in the [Ca^{2+}]_{ER} during the [Ca^{2+}]_{i} oscillation should not be attributed to the failure of dissociation of calcium from CatchER since the k_{off} rate of CatchER is 700 s^{-1}. To our knowledge, it is the fastest off-rate of all reported Ca^{2+} sensors. The [Ca^{2+}]_{ER} is the consequence of the entrance and exit of calcium from the organelle while the cytosolic calcium concentration reflects the dynamics calcium change as a result of various membrane pumps and calcium inlets. The potential increases of [Ca^{2+}]_{ER} contributed by the presumed SERCA pump and other factors may not be dramatic enough to be captured by the ER calcium indicator during [Ca^{2+}]_{i} oscillation.

The calibration of the [Ca^{2+}]_{ER} suggested the positive allosteric modulator NPS R-568 can increase [Ca^{2+}]_{ER} which is expected as a potential explanation that the addition of the NPS R-568 can facilitate the expression of the CaSR. Similar findings have been reported by Ong et al on the
study of glucocerebrosidase (316). Their group found out that increasing the ER calcium concentration can regulate the post-translational protein folding pathway which can facilitate the chaperone system to fold misfolding-prone enzymes and increase folded protein population.

In the present study, we first documented how the dynamics of $[\text{Ca}^{2+}]_{\text{ER}}$ changed upon the extracellular induced intracellular calcium oscillation. Second, we analyzed the influence of CaSR allosteric regulators on the intracellular calcium store. Third, this approach can be applied to other receptors located on the cell membrane.
CHAPTER 10.  MAJOR CONCLUSIONS AND SIGNIFICANCE

Overall, these series of studies provide a mechanistic view to understand how CaSR integrates the Ca\textsuperscript{2+} as well as amino acids-mediated signaling and depict the molecular basis for diseases associated CaSR mutations resulting in alterations in Ca\textsuperscript{2+} homeostasis. We have met different challenges, nevertheless, we are able to achieved the goal via addressing the following five major questions.

1. How CaSR functional cooperativity is regulation by extracellular binding of Ca\textsuperscript{2+} and amino acids?

2. How the disease associated CaSR mutants can affect the functional cooperativity mediated by Ca\textsuperscript{2+} and amino acids?

3. How can we determine the binding of Ca\textsuperscript{2+} and L-Phe to the CaSR ECDs and detect the potential conformational change induced by the interaction?

4. How the multifunctional intermediate messenger protein---CaM regulates the CaSR trafficking and signaling?

5. How ER Ca\textsuperscript{2+} signaling regulates CaSR functional cooperativity via modulation of receptor surface expression?

A. In Chapter 3, we focused on the regulation of CaSR functional cooperativity by extracellular binding of Ca\textsuperscript{2+} and amino acids.

Although previous lab members predicted five potential Ca\textsuperscript{2+} binding sites using computational algorithm as well as grafting approaches, how these Ca\textsuperscript{2+} binding sites are correlate with each other in tuning the function of CaSR is still a mystery. Calcium binding sites are usually invisible even in determined structures of the ECD such as mGluR1. Combined with
the challenge, the lack of direct binding methods for determining cooperativity in its responses to Ca\textsuperscript{2+} and amino acids and the high off rates of these binding ligands associated with their weak binding affinities discourage the structural and functional study of the CaSR.

The major findings in the chapter are summarized in the following figure (see Fig. 3.10 for details).

The studies in Chapter 3 are important and novel for several reasons:

First, we have documented for the first time the predicted Ca\textsuperscript{2+}-binding site 1 within the hinge region of the CaSR’s ECD plays a central role of molecular connectivity in tuning the positive cooperativity caused by changes in [Ca\textsuperscript{2+}]\textsubscript{o}. Second, We also identified a potential L-Phe binding pocket in the hinge region of CaSR ECD which plays a key role in regulating the
heterotropic cooperativity of CaSR. Third, we were able to demonstrate of the interplay among extracellular Ca\(^{2+}\), amino acids and the CaSR via molecular connectivity that modulates the positive homotropic and heterotropic cooperativity of CaSR-mediated intracellular Ca\(^{2+}\) signaling. The positive cooperative co-activation of the CaSR by Ca\(^{2+}\) and L-Phe and the importance of the positive homotropic and heterotropic cooperativity, respectively, exhibited by the two agonists may be further extended to other members of the family C GPCRs to facilitate an understanding of the molecular basis for related human disorders and the development of new therapeutic strategies.

B. In Chapter 4 & 5, we analyzed how the disease associated CaSR mutants can affect the functional cooperativity mediated by Ca\(^{2+}\) and amino acids.

Several naturally occurring mutations in the CaSR are located in the extracellular domain of CaSR. However, due to the lack of structure information about this receptor, it is not clear how calcium modulates those CaSR mutants as well as the molecular basis of those clinical disorders associated with this receptor. By collaborating with Prof. Thakker’s group at University of Oxford, we have demonstrated the importance the predicted Ca\(^{2+}\)-binding sites play in regulating the function of the receptor. Among the thirty-four VFTD missense mutations we have identified, 18 mutations were located within 10 Angstroms of one or more of the Ca\(^{2+}\) binding sites, particularly at the calcium binding site 1, which is the principal site of Ca\(^{2+}\) binding. Moreover, our studies strongly suggested that the Ca\(^{2+}\) binding site 1, which is encoded in the VFTD, is the key for connecting the other calcium binding sites and regulating the receptor activity. Two residues 173 and 221 located at the entrance to the VFTD cleft binding site, were associated with
both receptor activation (L173F, P221L) and inactivation (L173P, P221Q), thereby highlighting the importance of the molecular connectivity linked by the $\text{Ca}^{2+}$ binding site 1. We also found that the heterotropic cooperativity introduced by L-Phe can be limited by gain-of-function mutants. Both \textit{in silico} and \textit{in vivo} results indicated that the residue Leu$^{173}$, which is close to residues that are part of the L-Phe-binding pocket, exhibited impaired heterotropic cooperativity in the presence of L-Phe.

The following schematic figure summarizes our work in Chapter 4 and 5.
These naturally occurring mutations, therefore, may be useful for further probing the functions of Ca\(^{2+}\)-binding site 1 and the adjacent L-Phe binding site in producing the positive cooperative interactions that are critical for the capacity of the CaSR to sense both mineral ions and amino acids. Last but not least, our findings may provide key insights into molecular mechanisms shared with other members of the class C GPCRs, including metabotropic glutamate receptors (mGluRs), GABA\(_B\) receptors, pheromone receptors, and taste receptors, that are regulated by Ca\(^{2+}\)\(_o\).

C. In Chapter 6 and 7, we have probed the Ca\(^{2+}\) and L-Phe binding affinity, cooperativity, and related conformational change of both mammalian and bacteria expressed ECD by various biophysical methods.

The challenges associated with probing the key question, how CaSR senses the alterations of calcium concentration, are multifaceted. First, the full length of CaSR is huge (260 kDa), even the extracellular domain of the CaSR is comparably larger (~70 kDa based on sequence only) than most of the proteins that can be expressed and purified using bacterial system (< 60 kDa). In order to get the protein within its native conformation, eukaryotic system may be the best choice. Second, the multiple glycosylation sites (11) in the ECD can cause difficulties in crystallization. Besides, those glycans also introduce structural heterogeneity into the entire system make the protein in unfavorable condition for structure studies using NMR. Third, despite extensive functional studies of CaSR by different groups, how calcium and L-Phe interact with the extracellular domain is still under debate due to the lack of structural information. Fourth, the direct visualization of interaction of Ca\(^{2+}\) and Phe to the receptor ECD has also been hampered by the high off rate associated with weak binding during X-ray crystallography. Last but not least,
direct monitoring of such interactions in solution and characterization the biophysical properties of the large size protein and collection of structurally related information using spectroscopic methods also requires a large amount of homogenous native protein.

In these two chapters, we demonstrated our progress in coping with the challenges stated in the last paragraph. By collaboration with Dr. Kelley W. Moremen, we expressed and purified the CaSR ECD, which was shown to play important role in intracellular calcium responses, using bacteria and mammalian expression systems. The native conformation of the bacterial expressed protein was compared to the mammalian expressed protein using by immunoblotting technique and metal binding FRET assay. The secondary structures of the purified proteins were verified using circular dichroism. Using various spectroscopic methods, we have shown that both protein variants bind Ca$^{2+}$ with a to $K_d$ of 3.0~5.0 mM, which were hardly affected by the level of glycosylation. We have demonstrated for the first the direct interaction between the CaSR and its ligands. The calcium binding induced local conformational changes of the protein can be visualized by NMR with both uniformly labeled and specific $^{15}$N Phe-labeled ECDs. By utilizing the saturation transfer difference (STD) NMR technique, we report for the first time the direct interaction between the CaSR ECD and its positive allosteric modulator L-Phe. We further demonstrate that L-Phe increases the binding affinity of the CaSR ECD for Ca$^{2+}$. This current study provides valuable molecular basis for studying the connectivity between different calcium binding sites. Our findings also present new insights into the mechanism of how Ca$^{2+}$ and amino acids regulate the CaSR and other proteins in the G protein coupled receptors (GPCR) superfamily and may pave the way for exploration of the structural properties of CaSR.
D. In Chapter 8, we have studied the molecular mechanism underlying receptor trafficking upon the interaction with various agonists.

Given the fact that the CaSR has low affinity for Ca$^{2+}$ or allosteric regulators such as amino acids, small peptides (in the range from μM to mM), structural and functional studies about this receptor are mainly through mutagenesis induced changes. And the regulation about the CaSR is largely from signaling readouts of intracellular calcium, lipids or mitogen-activated protein kinase (MAPK) pathways. The complex and hysteretic regulation of the downstream signaling pathways independent of the activated receptors, combined with receptor desensitization, contribute to the difficulties to describe the responses exclusively to the CaSR. Understanding the factors that regulate the life cycle of the receptor in the context of physiological condition is crucial for developing strategies to regulate CaSR activities.

In our previous study, we have demonstrated a CaM-binding domain (CaMBD) located within the C terminus of CaSR (residues 871 – 898) interacting with CaM in a Ca$^{2+}$-dependent, stoichiometric way. Our studies suggest a wrapping around 1 – 14-like mode of interaction that involves global conformational changes in both lobes of CaM with concomitant formation of a helical structure in the CaMBD. More importantly, the Ca$^{2+}$-dependent association between CaM and the C terminus of CaSR is critical for maintaining proper responsiveness of intracellular Ca$^{2+}$ responses to changes in extracellular Ca$^{2+}$ and regulating cell surface expression of the receptor.

In chapter 8, we particularly investigated how calmodulin regulates of the CaSR mediated cytosolic Ca$^{2+}$ signaling and the receptor cell surface expression. We found that in the presence of L-Phe, the agonist driven insertion signaling (ADIS) was enhanced in HEK293 cells transfected with either wild type CaSR or disease related loss-of-function mutant CaSR. Further analysis with the calmodulin (CaM) inhibitor suggested CaM could involve in regulating the L-
Phe enhanced ADIS. Studies on the CaSR with mutations at calmodulin binding domain which interrupted CaM-CaSR interaction and the CaSR with Filamin A binding domain truncation showed that not only the L-Phe induced intracellular calcium $[Ca^{2+}]$ oscillation was altered, but the L-Phe promoted receptor insertion phenomena was abolished. Thus, L-Phe possibly regulates the receptor through CaM and Filamin A modulation. Our working model is summarized in the following figure (see Fig. 8.7 for details).

The significance of the work relyis on the fact that CaM not only interact with the CaSR, but also its activity is prevalently involved in the function of other GPCR family members. Since GPCRs meet at least a subset of the criteria for the ADIS mechanism, such as a large intracellular pre-Golgi pool of receptors, chronic or extended exposure to agonist during receptor life cycle, relatively low steady-state plasma membrane amounts of receptor and a CaM binding domain at C-tail, our findings may be a relevant regulatory mechanism for other GPCRs, include mGluRs, adrenergic, dopamine and cannabinoid receptors etc.
E. In Chapter 9, we have investigated the regulation of CaSR functional cooperativity by modulation of surface expression via \([\text{Ca}^{2+}]_{\text{ER}}\) signaling.

\(\text{Ca}^{2+}\) signaling is one of the most ubiquitous cellular events in living organisms. It regulates tremendous amount of biological functions. The endoplasmic reticulum (ER) lumen, which occupies less than 10% of cell volume, stores >90% of intracellular \(\text{Ca}^{2+}\) and is crucial in controlling \(\text{Ca}^{2+}\) signaling. Directly monitoring fast ER \(\text{Ca}^{2+}\) dynamics in living cells is still a new field, and understanding how the ER \([\text{Ca}^{2+}]\) can regulate the trafficking of receptors is a challenging project.

In Chapter 9, we reported that our lab has used the ER calcium probe developed in our lab -- CatchER as well as the commercial available ratiometric dye Mag-Fura 2 to investigate how \([\text{Ca}^{2+}]_{\text{ER}}\) regulates the expression of the receptor. The working model is summarized in the following schematic diagram.
The significances are in several layers. First, we first documented how the dynamics of 
$[\text{Ca}^{2+}]_{\text{ER}}$ upon the extracellular induced intracellular calcium oscillation. Second, we analyzed the influence of CaSR allosteric regulators on the intracellular calcium store. Third, this approach can be applied to other receptors located on the cell membrane.
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APPENDICES

Appendix A: EMOC, a Novel Endoplasmic Reticulum Protein that Regulates Cell Growth and Ca\textsuperscript{2+} Store-Operated Entry

INTRODUCTION

The endoplasmic reticulum (ER) is a key organelle involved in controlling major cellular functions including protein synthesis and modification as well as intracellular Ca\textsuperscript{2+} homeostasis [1, 2]. In recent years, the role of ER in the regulation of intracellular signal transduction is beginning to become clear. A number of ER-associated proteins are known to modulate intracellular Ca\textsuperscript{2+} signaling, including, for example, calreticulin, glucose-regulated protein 78 (Grp78/Bip), 94(Grp94), and calsequestrin [reviewed in 3]. Other types of ER-associated proteins are ER-membrane calcium channels; for example, the ER Ca\textsuperscript{2+} uptake channels, formed by sarcoplasmic/ER Ca\textsuperscript{2+}-transporting ATPases (SERCAs), and the Ca\textsuperscript{2+}-releasing channels, constituted by the inositol-1,4,5-triphosphate receptor (InsP\textsubscript{3}R) or ryanodine receptors (RYRs) [3]. Many of these proteins are known to regulate several cellular processes including proliferation, adhesion, motility, metabolism, differentiation, gene expression and apoptosis [4, 5].

Store-operated calcium (SOC) channels play important roles in regulation of a series of distinct cellular processes including cell migration, gene expression, cell growth and apoptosis [reviewed in 6, 7]. Store-operated Ca\textsuperscript{2+} entry (SOCE) is evoked by depletion of Ca\textsuperscript{2+} from the ER. It has been noted that depletion of intracellular calcium store triggers the opening of plasma membrane Ca\textsuperscript{2+} channels that leads to Ca\textsuperscript{2+} influx [6-8]. Although the phenomenon of SOCE was noted many years ago, little is known about proteins that regulate SOCE process. Recent studies
have identified two proteins, STIM1 (stromal interaction molecular 1) and Orai1 (also named CRACM1) that are believed to work together to regulate SOCE. While STIM1 functions as the ER Ca\(^{2+}\) sensor [9-11], Orai1 is an essential pore-forming subunit of the plasma membrane Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) [11-14]. Several lines of evidence suggest that when the ER Ca\(^{2+}\) stores are replete, STIM1 predominantly localizes in the ER-membrane, however, in the event that ER Ca\(^{2+}\) stores are depleted, STIM1 forms oligomers and redistributes to ER-plasma membrane junctions as STIM1 clusters (aggregates) [9, 15, 16]. Evidence also suggests that STIM1 recruits Orai1 protein to the regions directly opposite of the STIM1 clusters and STIM1-Orai1 interactions activate Orai1 channels resulting in Ca\(^{2+}\) influx through the Orai1 channels [8, 15, 17-19]. While these findings significantly enhance our understanding of how SOCE is regulated, several questions still remain unanswered and warrant further investigations. Chief among those are how STIM1 and Orai1 are regulated and whether other cellular protein(s) is/are also involved in the regulation of SOCE process.

In the present study, we report the characterization of a novel ER membrane protein named EMOC (ER Membrane protein Overexpressed in Cancer). Our study shows that EMOC is an integral ER membrane protein that harbors two transmembrane domains. Our study also shows that EMOC forms oligomers and binds to Ca\(^{2+}\). Importantly, we have also provided evidence that EMOC is linked to controlling of cell growth and appears to play a role in regulation of SOCE. Thus, our studies indicate EMOC to be another cellular protein involved in the regulation of the SOCE process.

**EXPERIMENTAL PROCEDURES**
Measurements of intracellular calcium concentration: Measurement of intracellular Ca\textsuperscript{2+} was performed as described [39]. Briefly, Hela and 293T cells with scrambled RNAi or EMOC RNAi were seeded on coverslips and further cultured for 48 hrs. The cells on the coverslips were subsequently loaded using 4 μM Fura-2 AM in 2 mL physiological saline buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.55 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2} and pH 7.4) for 15 mins. Then cells were washed with ringer buffer and coverslips were mounted in a bathing chamber on the stage of a Leica DM6000 fluorescence microscope. The Fura-2 emission signals at 510 nm from cells excited at 340 or 380 nm were recorded in real time during the experiments as 500 nM thapsigargin was added and the concentration of extracellular Ca\textsuperscript{2+} was changed to 2 mM. The data representing relative intracellular Ca\textsuperscript{2+} change were plotted as 340/380 fluorescence ratios.

The Mn\textsuperscript{2+} Quench Assay: The Mn\textsuperscript{2+} quench assay was performed as described [9]. In brief, cells were seeded on 13.5 × 20 mm coverslips. Before experiments, 4 mM fura-2/AM was loaded into cells. After 20 mins, the remaining extracellular Fura-2/AM was washed out with Ringer buffer and the coverslips were transferred to a fluorescence cuvette. Before data acquisition, 2 mM Mn\textsuperscript{2+} in HBSS buffer was added to the cuvette. Thapsigargin was added 50 seconds later, and the experiment was continued for another 300 seconds. Quenching of Fura-2 fluorescence signal was measured at 510 nm with excitation at 360 nm.

RESULTS

EMOC plays a role in regulation of store-operated calcium influx: Because our results indicate that EMOC is an ER-membrane Ca\textsuperscript{2+}-binding protein, next, we examined the effect of EMOC on cellular Ca\textsuperscript{2+} homeostasis. To this end, we employed a lentiviral shRNA-mediated
knockdown (KD) approach to deplete EMOC and to determine the effect of EMOC on Ca\(^{2+}\) release from ER and on Ca\(^{2+}\) influx after intracellular Ca\(^{2+}\) store depletion. First, we performed EMOC knockdown and Figure 8A shows that EMOC expression was sufficiently depleted by the lentivirus-mediated EMOC shRNA. Next, we performed “Ca\(^{2+}\) add-back” experiments, in which the ER Ca\(^{2+}\) stores were first depleted by treating cells with thapsigargin (TG) in the absence of extracellular Ca\(^{2+}\), then 2 mM extracellular Ca\(^{2+}\) was added back to trigger intracellular Ca\(^{2+}\) depletion-induced store operated calcium (SOC) influx. Ca\(^{2+}\) release from the ER and extracellular Ca\(^{2+}\) influx were monitored in whole cell population by Furo-2-based assays. As shown in Figure 8B, there was no significant difference in TG-evoked Ca\(^{2+}\) transient (1\(^{st}\) peak) observed in the EMOC knockdown cells and in control scrambled RNAi cells. These results indicate that EMOC may not have a strong effect on ER Ca\(^{2+}\) store and release because when no extracellular Ca\(^{2+}\) is added, TG-evoked Ca\(^{2+}\) transient is mainly contributed by Ca\(^{2+}\) released from the ER. By contrast, when ER Ca\(^{2+}\) was depleted by TG and extracellular Ca\(^{2+}\) was added, the intracellular Ca\(^{2+}\) transient signal (2\(^{nd}\) peak) was significantly lower (by 40-60%) in the EMOC knocked down cells (Figure 8B). We also similarly performed the “Ca\(^{2+}\) add-back” experiments using another cell line 293T, and as shown in Figure 8C and D, EMOC depletion also led to a reduced Ca\(^{2+}\) transient signal in EMOC knockdown cells when extracellular Ca\(^{2+}\) was added after ER Ca\(^{2+}\) was depleted by TG. These results indicate that the absence of EMOC protein significantly alters Ca\(^{2+}\) transient. Because the suppression of Ca\(^{2+}\) signal in the EMOC knockdown cells seen in Figure 8 could be due to a mechanism involving a decreased Ca\(^{2+}\) influx from the store operated-channel or due to a mechanism that is involved in an accelerated plasma membrane Ca\(^{2+}\) extrusion, we performed the Mn\(^{2+}\) quench assays to further differentiate these two possible mechanisms. Like Ca\(^{2+}\), Mn\(^{2+}\) can enter cells via the SOC influx channels to form a
non-florescence complex with Fura-2. Thus, the Mn$^{2+}$ quench assay is widely used to investigate the regulation of SOC influx [9, 30]. We reasoned that, if EMOC were involved in the regulation of SOC influx, we should be able to observe a difference in intracellular Ca$^{2+}$ transient monitored by Fura-2 in cells depleted with the EMOC expression when extracellular Mn$^{2+}$ was present. As shown in Figure 9A&B, Hela EMOC knockdown cells (EMOC RNAi) exhibited significantly reduced Mn$^{2+}$ quench-rate evoked by TG (Figure 9A and B) compared with cells expressing the control RNAi (Scr. RNAi).

In addition, EMOC depletion also has similar effect in influencing SOC influx in 293T cells (Fig. 9C&D). Thus, these results indicate that EMOC depletion significantly reduces the rate of Mn$^{2+}$ entrance through the SOC channels and that EMOC plays a role in the regulation of SOC influx.

Interestingly, this behavior of EMOC in regulation of SOC influx appears to be similar to that of STIM1 protein, a key SOCE Ca$^{2+}$ sensor in the ER [9, 10, 31]. Furthermore, EMOC also has additional STIM1-like features including, for example, EMOC is an integral ER membrane protein that binds to Ca$^{2+}$ and forms oligomers. Whether EMOC functions in a manner similar to STIM1 in regulation of SOCE is an interesting and important issue that remains to be investigated.

In summary, the present studies have characterized a novel ER transmembrane protein, EMOC. EMOC protein binds to Ca$^{2+}$ and regulates Ca$^{2+}$ homeostasis. Our mechanistic studies also indicate that EMOC is involved in the regulation of the store-operated calcium influx. Thus, our data suggest EMOC is a new and critical factor, in addition to STIM1 and ORAI1, involved in regulating the SOCE process.
A  Hela

B  Hela

C  HEK293T

D  HEK293T
Figure 8. Depletion of EMOC expression significantly reduces thapsigargin (TG)- and extracellular Ca$^{2+}$-induced transient [$\text{Ca}^{2+}]_i$ (intracellular Ca$^{2+}$ concentration) increase. A&B, results obtained from Hela cells. C&D, results obtained from 293T cells. For these assays, TG (500 nM) was first added to deplete ER-stored Ca$^{2+}$ in the absence of extracellular Ca$^{2+}$. CaCl$_2$ (2 mM) was introduced after intracellular calcium concentration reached to a plateau to trigger calcium influx. The ratio of emission signals at 510 nm from cells excited at 340 or 380 nm were recorded in real time respectively for scrambled RNAi expressing cells (black line) or for EMOC RNAi expressing cells (red line). For Hela cells (A and B), results shown are the average Ca$^{2+}$ responses of 16 control (Scr. RNAi) and 18 EMOC-KD (EMOC RNAi) single cells; and for 293T cells, results shown are the average response of 46 control (Scr. RNAi) and 14 EMOC KD (EMOC RNAi) single cells. A and C are results of one representative experiment performed in Hela cells (A) or 293T cells (C). B and D, bar graphs represent averaged Ca$^{2+}$ influx peak ($\Delta F_{340/380}$ ratio) ± s.e.m. of three-independent experiments in Hela cells (B) or in 293T cells (D). Expression of EMOC mRNA in control and EMOC knockdown cells determined by RT-PCR assay is also shown (B and D).
Fig. 9. EMOC knockdown significantly suppresses the store-operated channel entry demonstrated by the Mn$^{2+}$ quench assay. The Mn$^{2+}$ quench assay was performed as described in the Materials and Methods. A&B, results obtained from Hela cells. C&D, results obtained from 293T. The relative Fura-2 fluorescence intensity was measured with 360 nm excitation as a function of time in the control (Scr. RNAi, black line) and EMOC knockdown cells (red line). Mn$^{2+}$ quench rate ($\Delta F/F$) measured in the scrambled RNAi and EMOC RNAi expressing cells in the absence (clear bars) or in the presence of thapsigargin (TG) (dark bars).
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Appendix B: Enhancing Near IR Luminescence of Thiolate Au nanoclusters by Thermo Treatments and Heterogeneous Subcellular Distributions

INTRODUCTION

Soluble gold nanoclusters can be categorized as very small gold nanoparticles. Unlike other nanomaterials normally described by size and shape, a definitive chemical composition of Au nanoclusters could be elucidated regarding the number of Au atoms forming an Au core and the number of chemically bonded molecules on core surface forming a monolayer of stabilizing ligands. Novel physicochemical properties distinct from larger Au nanoparticles have been discovered, which render the Au nanoclusters broad applications in fundamental research and biomedical and energy applications. Of particular relevance to this report is the intrinsic photoluminescence in near IR wavelength range from aqueous soluble Au nanoclusters with core diameter smaller than 2 nm stabilized by a monolayer of thiolate ligands. The small overall dimension makes them biocompatible, readily accessible to subcellular domains and ultimately body-clearable, while an emission maximum longer than 650 nm is highly advantageous for cell imaging due to drastically reduced background absorption, autofluorescence and other interferences. If the quantum efficiency (QE) of the near IR emission could be further enhanced and approach the QEs of organic dyes or semiconductor quantum dots, those aforementioned advantages of Au nanoclusters will make them superior candidates in studying the dynamic and heterogeneous cellular processes for biomedical applications over other counterparts. The luminescence properties of Au-thiolate nanoclusters have been studied from fundamental perspective and applied for bioimaging applications.1 An optical energy gap between the highest occupied and lowest unoccupied orbitals is required to observe appreciable photoluminescence,
albeit the emission energy does not match the band gap of the Au nanoclusters in most cases.2, 3 A large Stokes shift exists between the visible excitation and near IR emission. The observation suggests significant energy relaxation that is also supported by the long lifetime at nano to microseconds.4 An upper threshold in core size of 2.2 nm was determined by transient optical measurements from hexanethiolate stabilized Au nanoclusters.5, 6 Strong dependence on ligand polarity of the luminescence QE was initially reported via the comparisons through ligand exchanged and directly synthesized Au nanoclusters.7, 8 Possible charge interactions between the Au core and the ligands were further studied on well-defined monodispersed Au25 nanoclusters.9 Optimization of those parameters leads to enhanced QE of the near IR emission (i.e. emission max > 650-700 nm) at ca. 1% for aqueous soluble and ca. 8% for non-aqueous soluble Au nanoclusters. To make the near IR emitting Au nanoclusters competitive with organic dyes and nanoclusters that display intense visible emissions, further enhancement in QE would be favorable. Recently, a synthetic procedure involving heating induced aggregation was reported to generate Au nanoclusters displaying bright emission centered at ca. 600 nm.10 For the preformed Au nanoclusters, heating in the presence of excess thiols as ligands arguably reduced the core size, known as core etching process.11,12 An annealing procedure was developed to improve the electrochemical properties of the Au nanoclusters.13 It generally adopts milder conditions compared with etching, using limited amount of excess thiols (same as those ligands already on the Au nanoclusters) and lower temperatures. The nanomaterials quality is improved presumably by the elimination of those less thermodynamically stable species, or by the optimization of the thiolate-Au bonding,14-16 or both. Besides the introduction of multidentate dithiol ligands to tailor the core-ligand interfaces and particularly the luminescence,17-19 we have reported the observation of the individual Au nanoclusters and their
distribution inside fixed HeLa cells under confocal microscope via luminescence intensity and lifetime imaging.20 Au is FDA approved materials due to its non- or low toxicity. Various Au nanoclusters and Au complexes have been used in basic biomedical research and as diagnostic and therapeutic tools.21-23 Fundamental understanding regarding the fate of such small nanoclusters in cells is rather diffusive.24-29 One of the essential questions related to those contradictory reports is whether the nanoclusters could efficiently enter cell and nucleus, and what internalization pathway is responsible for the dynamic processes. Apparently, labeling nanomaterials with fluorescent probes adds additional variables and complicates the analysis. Ex-situ measurements by mass spectrometry, electron microscopy, or sedimentation etc. are less capable to capture living cell dynamics and heterogeneity, information essential for living subjects.22, 30 This report establishes a universal approach to enhance the near IR luminescence of Au nanoclusters with different sizes (less than 2 nm) and different protecting ligand molecules. Because the surface chemistry is known to be critical in the nano-bio interactions, a generalizable method to enhance the QE of Au nanoclusters with different surface functionalities could enable in-situ dynamic cellular studies previously inaccessible. Two types of monothiols, mercaptosuccinic acid (MSA) and tiopronin (Tio), were employed to synthesize different Au nanoclusters under a variety of conditions. Heating each batch of the purified Au nanoclusters in the presence of the corresponding thiols was found to drastically enhance the emission QE by ca. 5-10 folds. After the incubation with live human embryonic kidney (HEK) cells, confocal microscope analysis demonstrates the heterogeneous distribution of Au nanoclusters inside the fixed HEK cells. Furthermore, co-distribution with nucleus stain dye reveals their presence inside cell nucleus.
EXPERIMENTAL SECTION

Confocal Fluorescence Microscopy. Human embryonic kidney 293 (HEK) cells were incubated with Au-MSA-PEG at 37 °C in cell culture media over 24 hours. The cells were then washed in triplicate with PBS buffer followed by fixation with 4% ice cold 100% methanol in -20 °C for 5~10 minutes. Subsequently, cells were mounted on glass slides using Prolong Gold Antifade reagent with DAPI (P36391; Invitrogen) followed by triplicate washing with PBS prior to confocal imaging.

RESULTS

Confocal Fluorescence Imaging A key concern for the usage of nanomaterials as medicine or contrast enhancing agents for diagnostics and therapeutics is the prolonged body retention and subsequent potential toxicity. Effective renal clearance has been reported for nanoparticles with an overall dimension of 5 nm or smaller combined with proper surface chemistry design.35, 36

Because the kidneys play a major role in nanoparticles body retention and clearance, in this report, the distribution of the luminescent Au nanoclusters in kidney cells is investigated to offer fundamental insights at cell and subcellular levels for potential in-vitro and in vivo applications.

The enhanced QE of Au nanoclusters enables direct imaging using commercial confocal fluorescence microscope, albeit only a small portion of the emitted photons could be captured by the PMT detectors. Heterogeneous subcellular distribution inside individual HEK cells can be readily resolved in Figure 6, information inaccessible by ex-situ and ensemble measurements such as mass spectrometry, ICP OES. The membrane boundaries of individual HEK cells can be
seen under bright field. A fluorescent dye DAPI, widely used to stain DNA due to its high binding affinity to A-T, is co-distributed to highlight cell nucleus region. The blue and red channels record the shorter wavelength emission from DAPI and the near IR emission from the Au nanoclusters respectively. The co-distribution of the Au NCs inside the cells is further demonstrated in the two overlay panels. In comparison, Au NCs without heat treatments could not be observed in similar imaging studies at comparable dosage. Those treated Au NCs without PEGlation, though detectable directly under the PMT detectors, could not be observed inside the cells under comparable conditions.

To affirm that the Au NCs are inside cell nucleus, a series of z-stack images are presented in panel B (i.e. from the top toward the bottom of the cells). It is obvious that the emissions from both DAPI and Au nanoclusters become more intense toward the center of the cell. The features strongly suggest that the Au NCs are inside cell and cell nucleus instead of being adsorbed on membranes, which would otherwise display ring-like features along the membrane peripheries within focus, especially at middle z positions. Further, the Au NCs distribute heterogeneously both within individual cells and between different cells: some cells show very low intensity from Au NCs both in cytoplasm and in nucleus; some show high intensity in cytoplasm but low in nucleus; while other cells have a high intensity both in cytoplasm and nucleus. The more intense clustered red emission signals in cytoplasm suggest confinement or enriched Au NCs within unidentified organelles, presumably endosomes.

The kinetics and pathways of the cell internalization of nanomaterials are known to be heterogeneous and require more systematic studies. Internalization will differ at different phases of cell life cycle, which could explain the heterogeneous distributions observed.
Furthermore, the Au nanoclusters are not functionalized with specific biomarkers, and thus are expected to non preferentially distribute inside the cell if internalized. Due to the small dimension, passive diffusion is possible across the cell membrane and maybe nucleus membrane.26, 28, 37, 39 The kinetics of internalization and sub-cellular distribution of the Au NCs at different dosage and other conditions are under further investigation. Regardless, the consistent presence of Au nanoclusters at the center of multiple individual cells clearly demonstrated that these Au nanoclusters penetrated both the cytoplasm and nucleus of HEK cells that enable further cell dynamics and function studies.
Figure 6. Confocal images of fixed HEK293 cells. The cells were exposed to 50 μg/mL Au-MSA-PEG NCs (60% of original QE prior PEGlation) for 24 hours prior fixture. Panel A: clockwise from left: Au-MSA-PEG; Bright field; DAPI; Composite emissions from DAPI and Au-MSA-PEG; Composite image of bright field, DAPI and Au-MSA-PEG. The images
correspond to frame 10 in panel B. Panel B: Z-stack images of composite emissions from DAPI and Au-MSA-PEG. The images were taken in a series of 15-step 1-μm-step-size measurements, starting from above the cell (TOP) toward the bottom of the cell.

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Appendix C: CaSR May Play A Role In Osteoclastogenesis.

The bone turnover is a process that continuously exists throughout life. Osteoclasts which are a group of cells responsible for bone resorption and osteoblasts which take in charge of bone formation work collaboratively to ensure the old bones degradation and the new bones formation (317). The bone remodeling process is believed to be initiated by the recruitment of mononucleated osteoclast precursors from circulation (318). The osteoclast precursors then fused with these cells after them infiltrating the bone lining cell layer, thus forming multinucleated osteoclasts(319). The apoptosis of osteoclast ends the phase of resorption but begins the bone formation phase by recruiting osteoprogenitor cells to the resorption surface(320).

The skeleton is considered as a major reservoir of calcium and phosphorus. These bone derived products attract various cell types to the bone environment (321). For instance, Olszak et al. found that Ca\textsuperscript{2+} promote the migration of peripheral blood monocyte cells (PBMC)(322,323). Since Ca\textsuperscript{2+} can form great concentration gradient in the bone environment (up to 40 mM)(324,325), it is reasonable to hypothesize Ca\textsuperscript{2+} is one of the signals that involving the physiological and pathological process of bone modeling.

Arthritis is a form of joint disorder due to the inflammation on one or more joints. Osteoarthritis is the most common form of arthritis. Patients with arthritis could suffer from constant pain located to the joint affected, causing swelling, stiffness and limited movements. The basic treatment involves physical therapy and prescription of anti-inflammation drugs. However, only a few arthritis-related disorders can be completely cured, most of the rest are chronic conditions. It has been reported that the mean serum calcium level are lower in patients with rheumatoid
arthritis than health(326). Nevertheless how calcium concentration can affect the disease state is barely understood.

Calcium sensing receptor can sense the variation of calcium concentration and regulate the secretion of the parathyroid hormone (PTH) and calcitonin. By analyzing the receptor’s function in RAW264.7 cells, a well established osteoclast precursor model, recent studies have found that CaSR are intimately involved in regulating osteoclastic activities(57). Boudot et.al. reported in 2010 that CaSR could be a possible candidate for the initiation of the basic multicellular units (BMU), which is a temporary anatomical structures during the bone remodeling(321). However, little is known about how CaSR plays a role in the process of Arthritis.

In this study, we analyzed the CaSR expression level in RAW264.7 cells and carried out preliminary researches on the differentiation of the CaSR knock down RAW264.7 cells. A few tissue samples from mice with or without rheumatoid arthritis were tested with CaSR specific antibodies.

Preliminary Results
Figure. C-1 High [Ca^{2+}]_o and RANKL could promote the CaSR expression in RAW264.7 cells. Cells were incubated either in normal [Ca^{2+}]_o medium or 4.0 [Ca^{2+}]_o culture medium with or without the addition of RANKL. The mRNA was then extracted from those cells with various treatments. RT-PCR was performed to analyze the CaSR expression level. GAPDH was used as internal control.
Figure C-2 High $[\text{Ca}^{2+}]_{o}$ and RANKL stimulate the expression of CaSR. Cells were lysated in RIPA buffer after incubation in 1.5 mM $[\text{Ca}^{2+}]_{o}$ or 4.0 mM $[\text{Ca}^{2+}]_{o}$ in the presence or absence of RANKL. Subsequent to membrane transference, the membrane was incubated anti-CaSR specific antibody (ADD) or internal control antibody (GAPDH) overnight at 4°C. Upper panel: CaSR; Lower panel: GAPDH.
Figure C-3 Immunostaining of CaSR in RAW264.7 cells. A. Brightfield image of control group cells. B. Cells were fixed and further stained with anti-CaSR specific antibody (ADD). C. Bright field image of cells treated with 100 ng of RANKL for 6 days. D After treatment with RANKL for 6 days, cells were fixed and permeabilized with 0.2% tritonX100. Red: anti-CaSR antibody staining; Blue: DAPI staining.
Figure C-4. The proliferation of RAW264.7 cells in normal and high $[\text{Ca}^{2+}]_o$ medium. Cells were cultured for 5 continuous days in either 1.5 mM $[\text{Ca}^{2+}]_o$ medium or 4.0 mM $[\text{Ca}^{2+}]_o$ medium. The total cell numbers were determined using a hemocytometer.

Figure C-5. RT-PCR of CaSR knock down in RAW264.7 cells.
A

Untargeted

CaSR-KD

After 3 days
After 5 days

B

Percentage of OCL

Control  CaSR knock down

Raw Cell 100 ng Rankl Treatment
Figure C-6 CaSR knock down reduced the osteoclasts induced by RANKL. The CaSR knock down cells and the control group were subjected with 100ng/L of RANKL for 5 continuous days. A. Brightfield images of RANKL treated RAW264.7 cells. Red arrows: osteoclast like cells. B. Quantitative analysis of the number of osteoclasts after normalized to the control group.
Figure C-7 HE Staining of CaSR in physiological and pathological tissue samples. A. Paw tissues from normal mouse stained with anti-CaSR antibody (ADD) at 1:1000. B. Tissues from rheumatoid arthritis mouse model stained with the same antibody.
Appendix D: Sequence alignment of CaSR in various species

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Mus            NILHS 1079
Homo           NVVNS 1078
Bos            NMLRS 1085
Capra          NMLRS 1085
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Appendix E: Synergistic Effect

Simplified concept for synergy:

Drug A and Drug B are both agonists for Target T.

Assume: the effect of A only to T equals 1; the effect of B only to T equals 1

Then combination of the following conditions will be defined accordingly:

If A + B > 2; Synergism

If A + B = 2: Additive Effect

If A + B < 2; Antagonism

For a more detailed description, please see the manual of Calcusyn

In the present study,

A= Ca$^{2+}$

B= L-Phe (or other CaSR agonists)

T=CaSR

Our readout will be the average [Ca$^{2+}$].

Procedure:

First, measure the Calcium (Drug A) only induced receptor activity:

In the WT CaSR transfected HEK293 cells, Fura-2 AM will be loaded using the aforementioned protocol (normal loading). The intracellular calcium readout will be measured using Leica microscope.

Change the [Ca$^{2+}$]o stepwise from 0.0 mM to 10.0 mM for WT and upto 30.0 mM for inactive mutants, and record the ratio of F340/F380. In the current study, the 0.0 mM, 1.0 mM, 2.0 mM,
3.0 mM, 4.0 mM, 5.0 mM, 7.5 mM and 10.0 mM were chosen for measuring the Ca$^{2+}$ dosage-response.

**Second, measure the L-Phe (Drug B) only induced receptor activity:**

Change the [L-Phe]$_o$ stepwise from 0.0 mM to 10.0 mM, and record the ratio of F340/F380. In the current study, the 0.0 mM, 1.0 mM, 2.0 mM, 3.0 mM, 4.0 mM, 5.0 mM, 6.0 mM, 7.0 mM, 8.0 mM 9.0 mM and 10.0 mM were chosen for measuring the L-Phe dosage-response.

For the CaSR project, the activation of L-Phe requires the presence of minimum 1.0 mM Ca$^{2+}$, otherwise, there will be no change for the intracellular calcium readout.

The basal Ca$^{2+}$ can be varied among different CaSR mutants. For instance, for analyzing WT CaSR, [Ca$^{2+}$]$_o$ was fixed at 2.0 mM while changing the L-Phe concentration; on the other hand, 7.5 mM of [Ca$^{2+}$]$_o$ was chosen for analyzing the E297I CaSR during the stepwise increase of L-Phe. The ratio of F340/F380 was recorded for further analysis. The choice of fixed [Ca$^{2+}$]$_o$ is depended on the [Ca$^{2+}$]$_o$ of the mutant CaSR to start oscillating.

**Third, measure the combination effect (Drug A & B) induced receptor activity:**

The L-Phe concentration was fixed at either 5.0 mM or 10.0 mM, [Ca$^{2+}$]$_o$ was increased stepwise from 0.0 mM to 10.0 mM for WT and from 0.0 mM to 30.0 mM for inactive mutants. The average intracellular calcium change at each level of [Ca$^{2+}$]$_o$ was recorded accordingly.

**Using CalcuSyn for analyzing the synergistic effect:**

Choose “Drug wizard” for adding effects for individual drugs; choose “Drug manually” for input combined effects.

Because the software requires that the input drug effects shall be < 1, so the average intracellular calcium readout is subtracted from the basal fluorescence ratio value. For example, if the average
intracellular calcium level is 1.3 under 4 mM [Ca\textsuperscript{2+}]_o, and the initial readout for calcium level is 1.03, the number for inputting into the software as the drug effects shall be 1.3-1.03 = 0.27.

Results:

Figure E-1 Representative [Ca\textsuperscript{2+}]_i oscillation pattern from single-cell study. WT: calcium concentration increased stepwise. Control: typical [Ca\textsuperscript{2+}]_i response of non-CaSR transfected HEK293 cells upon the stimulation with extracellular calcium. Similar phenomena will be seen with increase of L-Phe in the absence of calcium.
Figure E-2 Representative $[Ca^{2+}]_i$ oscillation pattern from single-cell study. WT: L-Phe concentration increased stepwise in the presence of 2.0 mM Ca$^{2+}$. 

WT with L-Phe
Figure E-3 Representative $[\text{Ca}^{2+}]_i$ oscillation pattern from single-cell study. WT: calcium concentration increased stepwise in the presence of 5.0 mM L-Phe.

Similar experiments have been done for the CaSR mutants.

Figure E-4 Representative $[\text{Ca}^{2+}]_i$ oscillation pattern from single-cell study. E297I: L-Phe concentration increased stepwise in the presence of 7.5 mM Ca$^{2+}$. 
Figure E-5 Representative $[\text{Ca}^{2+}]_i$ oscillation pattern from single-cell study. E297I: Ca$^{2+}$ concentration increased stepwise in the presence of 5.0 mM Ca$^{2+}$. 
Figure E-6 Intracellular calcium response curve upon titration with calcium or L-Phe. Plots are fitted using Hill equations. A. WT CaSR transfected cellular response to the extracellular calcium concentration change in the presence of 5.0 mM of L-Phe. B. WT CaSR transfected cellular response to the extracellular L-Phe concentration change in the presence of 2.0 mM of calcium. C. E297I CaSR transfected cellular response to the extracellular calcium concentration change in the presence of 5.0 mM of L-Phe. D. E297I CaSR transfected cellular response to the extracellular L-Phe concentration change in the presence of 7.5 mM of calcium.
Table E-1. Analysis of possible synergy using CalcuSyn in the sensing of calcium and L-Phe to WT CaSR and mutant E297I. After transfection with WT CaSR for 48 hours, $[\text{Ca}^{2+}]_i$ was recorded upon incremental increases in $[\text{Ca}^{2+}]_o$ in the presence of 1 mM L-Phe or upon incremental increases in L-Phe in the presence of 2 mM $[\text{Ca}^{2+}]_o$. A similar experiment carried out in cells transfected with mutant E297I in the presence of 1 mM, 5 mM or 10 mM L-Phe. A CI number $>1.0$ indicates lack of a synergistic effect of the two ligands in WT CaSR and mutant E297I.