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Structure and function of the human calcium-sensing receptor: insights from natural and engineered mutations and allosteric modulators

Jianxin Hu [a], Allen M. Spiegel [b]

[a] *Molecular Signalling Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA*
[b] *Office of the Dean, Albert Einstein College of Medicine, Bronx, New York, NY, USA*

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Abstract

The human extracellular Ca^{2+}-sensing receptor (CaR), a member of the G protein-coupled receptor family 3, plays a key role in the regulation of extracellular calcium homeostasis. It is one of just a few G protein-coupled receptors with a large number of naturally occurring mutations identified in patients. In contrast to the small sizes of its agonists, this large dimeric receptor consists of domains with topologically distinctive orthosteric and allosteric sites. Information derived from studies of naturally occurring mutations, engineered mutations, allosteric modulators and crystal structures of the agonist-binding domain of homologous type 1 metabotropic glutamate receptor and G protein-coupled rhodopsin offers new insights into the structure and function of the CaR.

Keywords: G protein-coupled receptor • hypercalcaemia • hypocalcaemia • receptor mutations • allosteric modulators

Introduction

The extracellular Ca^{2+}-sensing receptor (CaR) plays a central role in the regulation of extracellular calcium homeostasis. It is expressed abundantly in parathyroid, thyroid C cells and kidney. Activation of the CaR by increased extracellular Ca^{2+} inhibits parathyroid hormone (PTH) secretion, stimulates

*Correspondence to: Jianxin HU
Molecular Signalling Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA
Tel: 1-301-594-5150
Fax: 1-301-480-3447
E-mail: jianxinh@niddk.nih.gov

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calcitonin secretion, and promotes urinary Ca^{2+} excretion, and thereby maintains the extracellular Ca^{2+} at the normal level [1–3]. The importance of the CaR in extracellular Ca^{2+} homeostasis is underscored by the identification of inactivating mutations in the CaR gene as the cause of familial hypocalciuric hypercalcaemia (FHH) and neonatal severe primary hyperparathyroidism (NSPHT), and the identification of activating mutations as the cause of autosomal dominant hypocalcaemia (ADH) [4]. Identification of CaR mutations in patients has been facilitated by the correlation between genotype and phenotype. As a result, the CaR is one of the very few G protein-coupled receptor (GPCR) with a large number of naturally occurring mutations identified [5].

The Ca^{2+}-binding sites in the CaR have not yet been unequivocally defined. Solution of the three-dimensional (3D) structure of the extracellular agonist-binding domain of structurally related rat type 1 metabotropic glutamate receptor (mGluR1) [6] offers important insights into the structure of the orthosteric site (agonist-binding site) and agonist-promoted conformational changes in this domain, both of which are likely relevant to the CaR.

Exciting progress has been made in recent years in screening for exogenous allosteric modulators selectively targeting the CaR by binding to allosteric sites in the seven-transmembrane domain (7TM) [7, 8]. They are potential drugs for treatment of various calcium metabolism disorders and one of them, Sensipar (cinacalcet) [9], is already in the market. Homology modelling of the CaR 7TM domain based on the single available 3D structure of a GPCR (bovine rhodopsin) [10] in inactive state, despite very limited sequence similarity, has shown to be useful in the study of binding sites for those allosteric modulators.

In this review, we summarize the major features of the structure and function of the CaR, focusing in particular on what has been learned from studying naturally occurring mutations, engineered mutations and allosteric modulators with assistance of modelling based on the crystal structures of rat mGluR1 agonist-binding domain and bovine rhodopsin.

The CaR is a unique family 3 GPCR

The human *CaSR* gene is located on chromosome 3q13.3–21 and spans over 50 kb of genomic DNA. It has a coding region of 3234 bp, which is contained within six exons [11]. The CaR (hCaR) is expressed as a 1078 amino-acid polypeptide [12] and the mature protein starts from tyrosine 20 after cleavage of a hydrophobic signal peptide [13] (Fig. 1). It contains 11 potential N-linked glycosylation sites in its extracellular N-terminal (Fig. 2) [14]. Immunoblotting of the CaR expressed in transfected HEK-293 cells identifies two major bands under reducing conditions, corresponding to an incompletely processed, high mannose intracellular form the receptor at ~130 kD and a fully glycosylated, cell surface-expressed form at ~150 kD. Glycosylation does not appear to be crucial for CaR function, but rather for proper protein folding and trafficking [14, 15]. Immunoblotting of the CaR under reducing and non-reducing conditions revealed that the CaR is expressed as an intermolecular disulphide linked homodimer.

The CaR is a GPCR which activates multiple G proteins including $G_{q/11}$ and Gi, and thereby activates different signal transduction pathways, depending on the cell type. Activation of the CaR by increased extracellular Ca^{2+} leads to inhibition of PTH secretion by a mechanism which remains poorly understood. Mutant mice that were parathyroid-specific G_q and G_{11} α-subunits double knockouts showed all the features of germline knockout of the CaR [16] including severe hypercalcaemia, hyperparathyroidism, skeletal abnormalities, retarded growth and early post-natal death, except hypocalcuria [17], underlining the importance of $G_{q/11}$ signalling pathway in the regulation of PTH secretion by the CaR. Most recently, a unique CaR autoantibody found in a patient with hypercalcaemia and elevated PTH-enhanced Gq-coupling but impeded G_i-coupling of the CaR in *in vitro* experiments, suggesting possible involvement of Gi signalling pathway as well in the regulation of PTH secretion [18], but since these studies were performed with transfected cells rather than parathyroid cells, the physiologic relevance of the G_i pathway for inhibition of PTH secretion remains unproven. In the parathyroid, the CaR also regulates PTH gene expression and parathyroid cellular proliferation. In calcitonin-secreting thyroid C cells, the CaR mediates the release of calcitonin that is stimulated by high calcium levels. In the kidney, the CaR is expressed at the plasma membrane of many tubular cell types. A large body of evidence supports the view that the CaR is directly involved in tight control of the renal tubular handling of calcium to match renal calcium excretion to the net amount of calcium entering the extracellular fluid. The

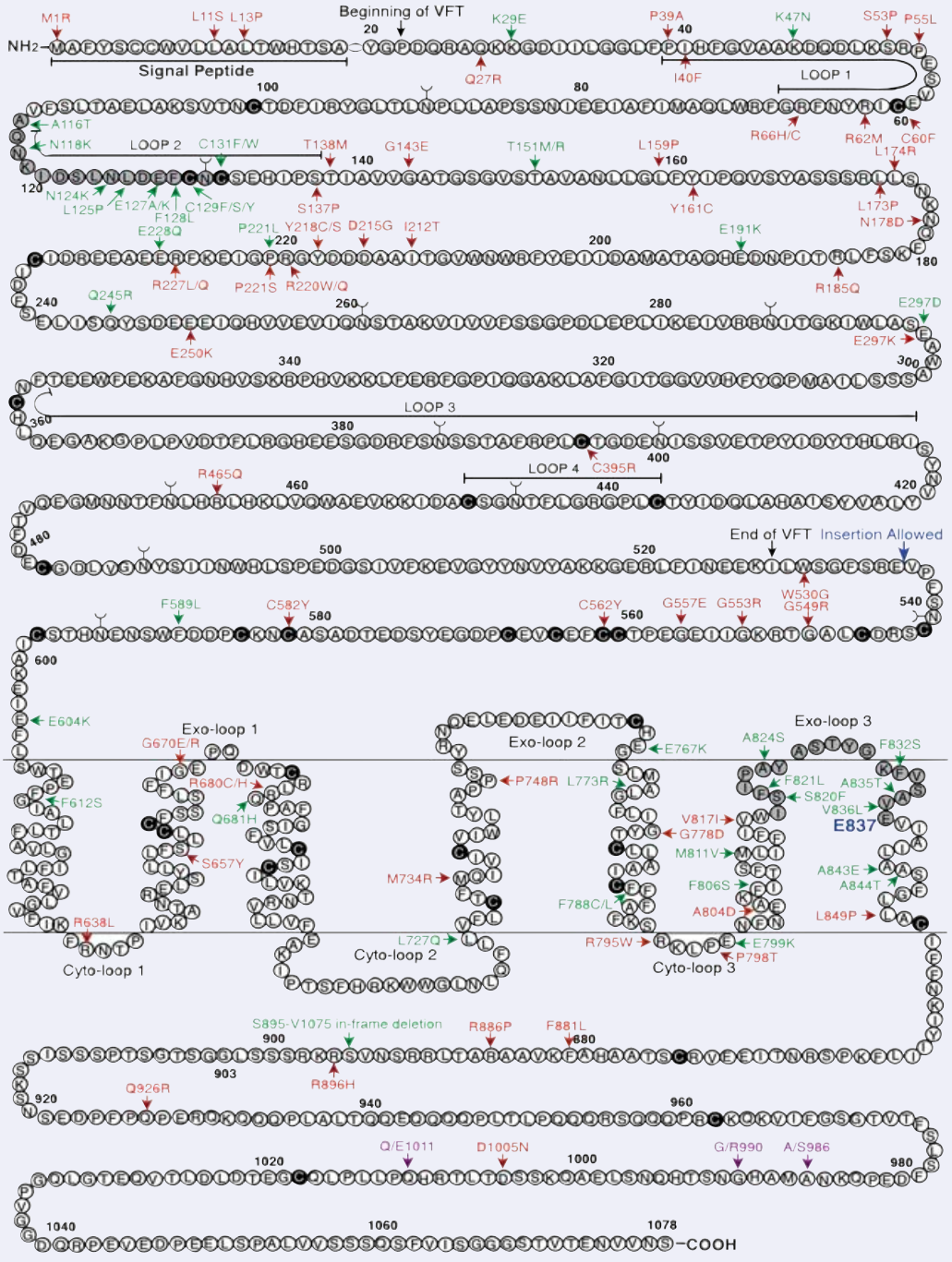

Fig. 1 The amino acid sequence of the hCaR (single letter code): cysteines (black), N-linked glycosylation sites (symbol), signal peptide, beginning and end of VFT domain, loops 1–4 of the VFT domain LB1, and the site between the end of VFT and the first cysteine in the Cys-rich domain that allow peptide insertion are highlighted. Inactivating mutations causing FHH/NSPHT are shown in red, and activating mutations causing ADH are shown in green. The three most common human CaR polymorphisms are shown in purple. E^{837}, shown to be involved in binding of allosteric modulators structurally related to NPS R-568, is highlighted in blue.

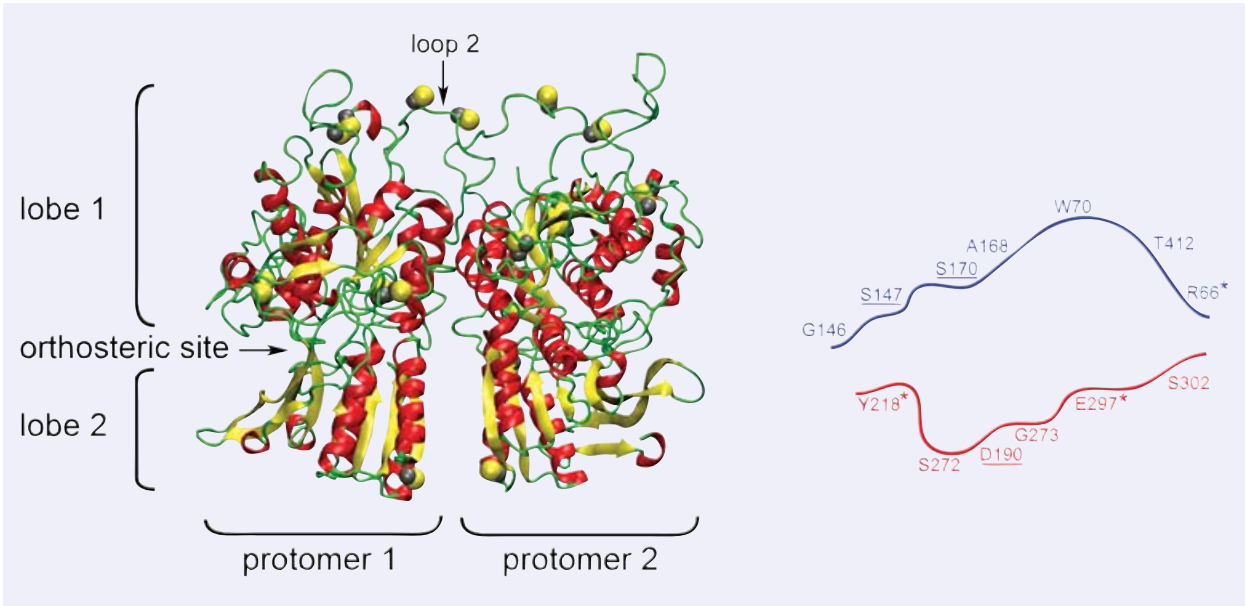

Fig. 2 *Left*, a model of the three-dimensional structure of the CaR VFT dimer: α helices in red, β sheets in yellow, loops and turns in green. Cysteines are shown. Both protomers and lobe 1, lobe 2, loop 2, and the putative orthosteric site of protomer 1 are labelled. Loop 2 is depicted arbitrarily in the model, since this loop was unstructured in all mGluR1 VFT crystal structures. The dimer interface runs along the vertical axis between the two protomers. *Right*, the CaR residues equivalent to those constituting the orthosteric site of the mGluR1. The residues in lobe 1 are shown in blue and the residues in lobe 2 are shown in red. An aster indicates the residue involved in naturally occurring inactivating CaR mutations (refer to Fig. 1), and artificial alanine mutations at the residues underlined impaired response of the receptor to extracellular Ca^{2+}.

CaR is also expressed widely at lower levels in many tissues that are not directly involved in calcium homeostasis, and the physiological and/or pathological significance of the CaR in those tissues remains speculative, but interesting potential roles such as in haematopoietic stem cell engraftment in the stem cell niche in bone marrow have been reported [19]. Other than the endogenous ligand Ca^{2+}, other polyvalent cations such as Mg^{2+}, Sr^{2+}, Gd^{3+}, La^{3+}, neomycin, spermine, etc. also activate the CaR *in vitro*. The physiological relevance of the interaction of the CaR with ions other than Ca^{2+} needs to be further investigated.

The CaR belongs to a unique subfamily, family 3 (or family C) [20], of GPCR which also includes receptors for the main neurotransmitters glutamate (eight subtypes of mGluR) and GABA (two subtypes of $GABA_B$ receptors), as well as receptors for basic amino acids (GPRC6A), and some taste receptors (T1R) and pheromone receptors (V2R). In addition to the signature 7TM characteristic for all GPCRs, they possess an unusually large N-terminal, extracellular domain (ECD) comprised of a Venus flytrap (VFT) domain and a cysteine-rich (Cys-rich) domain, except $GABA_B$ receptors which lack a Cys-rich domain.

Architecture of the CaR

Venus flytrap domain

The large N terminal ECD of the family 3 GPCRs share a low, but significant amino acid sequence similarity with a family of bacterial periplasmic amino acid-binding proteins [21], suggesting that these family 3 members might have evolved from an ancient family of cell-surface proteins binding essential extracellular solutes. The crystal structures of those bacterial proteins suggests a bi-lobed Venus-flytrap-like structure (VFT) in the ECDs of the family 3 receptors which oscillates between 'open' and 'closed' conformations and binding of the endogenous agonist in the cleft between the two lobes stabilizes a 'closed' conformation. Four insertions in the CaR sequence

that do not align with those bacterial proteins form protruding loops (designated loops 1–4 in Fig. 1) within lobe 1 of the VFT. Studies of mutant CaRs with deletions of parts of each of these loops revealed that a large part (365–385) of loop 3 could be deleted without impairing receptor function, but that deletions of loop 1 (50–59) or loop 4 (438–445), whilst not impairing receptor expression, reduced CaR activation. Interestingly, deletions of loop 2 (117–136) increased Ca^{2+} sensitivity of the mutant CaR [22].

The 3D structure of rat mGluR1 VFT from residue 33 to 522 (equivalent to 20–540 of the CaR) in its open state (ligand-free or antagonist bound) and in its closed state (agonist-bound) have verified the homodimerized bi-lobed VFT structure of this domain [6, 23]. In these crystal structures, the equivalent pairs of cysteines to CaR C^{60}–C^{101}, C^{358}–C^{395} and C^{437}–C^{449} form three intramolecular disulphides (Fig. 2). Earlier site-directed mutagenesis studies showed that these cysteines are crucial for CaR expression and function [24], indicating that they are probably crucial for the structural integrity of the VFT. mGluRs homodimerize through an intermolecular disulphide involving a conserved cysteine in loop 2 (residue 140 in the rat mGluR1 equivalent to C^{129} and C^{131} in the hCaR), consistent with earlier findings in site-directed mutagenesis experiments that both C^{129} and C^{131} are involved in intermolecular disulphide linkages [25].

The structure of loop 2 is of great interest as it is not only involved in intermolecular disulphide linkages, but also a hot spot for naturally occurring CaR activating mutations (described below). However, unfortunately this region is largely disordered in all mGluR1 VFT crystal structures.

The crystal structure of the mGluR1 VFT shows an extensive dimer interface involving residues in the amino-terminal portion of LB1, as well as within the proximal portion of LB2 [6, 23]. Homodimerization of the CaR, similar to the mGluR1, involves non-covalent interactions in addition to the two intermolecular disulphide bonds [26, 27]. The functional importance of CaR dimerization is demonstrated by complementation of function between two CaR mutants, with one carrying a loss-of-function mutation in the VFT and the other carrying a loss-of-function mutation in the 7TM or carboxyl terminus [28]. Further studies show that functional complementation requires existence of both Cys-rich and 7TM domains in the two defect CaR monomers to allow communication between the VFT and 7TM domains [29].

Due to the lack of a high-affinity ligand-binding assay for the CaR, the calcium-binding sites remain unknown. Ca^{2+} activates the CaR at mM concentrations, implying low-affinity Ca^{2+} binding. Meanwhile, activation of the CaR exhibits a pattern of positive cooperation, raising a possibility of multiple Ca^{2+}-binding sites within the VFT or elsewhere. As shown in the crystal structure, 13 residues in the mGluR1 VFT are involved in glutamate binding, six of which are identical or conservatively substituted in the CaR including S^{147}, S^{170} [30] and D^{190} [29] of the CaR (Fig. 2) which, when artificially mutated to alanine, impair CaR activation. Recent study further suggests that Ca^{2+} interacts with polar residues in the binding pockets in the ECD of the receptor, with residues S^{170}, D^{190}, Q^{193}, S^{296} and E^{297} directly involved in Ca^{2+} co-ordination and residues Y^{218}, F^{270} and S^{147} contributing to complete the co-ordination [31]. A recent study of a dimer between mGluR5-$GABA_{B1}$ and mGluR5-$GABA_{B2}$ chimeric receptors show that the closure of one mGluR5 VFT is sufficient for partial activation, but the closed state of both VFTs in the dimer is required for full activity [32]. Moreover, the most recently published mGluR3 ECD crystal structures with five different agonists show that, instead of altering the protein conformation, the receptor interestingly deals with different agonists by rearranging solvent molecules [33]. The structural differences between the glutamate-bound, 'active' *versus* antagonist-bound, 'inactive' forms of the mGluR1 VFT crystals revealed agonist-induced conformational changes [6, 23]. The VFT is closed in the glutamate-bound and open in the antagonist bound structures. Agonist-promoted VFT closure leads to a 70° rotation of one monomer relative to the other about an axis perpendicular to the dimer interface, bringing the C-termini of the two VFT monomers 26 Å closer. These important findings are likely relevant to other family 3 GPCRs including the CaR. We most recently reported that sensitivity of the CaR to extracellular Ca^{2+} was increased by binding one anti-CaR monoclonal antibody, but decreased by binding another antibody. Both antibodies bound to the lobe 2 of the VFT and presumably either enhance or impede agonist-promoted VFT closure and/or rotation [34].

Cysteine-rich domain

The CaR possesses a Cys-rich domain with nine highly conserved cysteines in an about 84 residue

long sequence as an adaptor region between the VFT and 7TM. The CaR Cys-rich domain allows some degrees of non-cysteine residue substitutions [35], however, mutation of any one of those nine cysteines to serine severely impairs expression and function of the CaR [24]. Deletion of the entire Cys-rich domain abolishes CaR activation, in spite of the preservation of some cell-surface expression [35], indicating a key role the Cys-rich domain plays in signal transmission between the VFT and 7TM domains.

Muto *et al.* most recently reported the crystal structures of mGluR3 ECD including both VFT and Cys-rich domain in agonist bound forms [33]. The Cys-rich domain contains three β–sheets, each composed of two short, antiparallel β–strands. Four intramolecular disulphide bridges, equivalent to the CaR C^{542}–C^{562}, C^{546}–C^{565}, C^{568}–C^{582} and C^{585}–C^{598}, are formed within this domain. The remaining cysteine (equivalent to C^{561} of the CaR) is bound to a cysteine in LB2 (equivalent to C^{236} of the CaR), explaining the importance of these residues found in experiments with cysteine mutant CaRs [24]. However, earlier TEV protease cleavage analysis of an engineered CaR with a unique cleavage site artificially inserted after residue E^{536} showed that the dimeric CaR VFT domain was released after TEV digestion and thus we concluded that it was not linked by a disulphide bond to the rest of the receptor [36]. The C^{236}–C^{561} disulphide bond in the CaR, if it exists, might be relatively labile compared to the intermolecular disulphide bonds involving C^{129} and C^{131}.

The structure of mGluR3 ECD in ligand free form is unfortunately not available to compare with those agonist-bound structures. The Cys-rich domain in one of the five mGluR3 ECD crystal structures was disordered, and surprisingly none of those five agonist-bound structures was in active form with agonist-induced VFT rotation seen in glutamate-bound mGluR1 VFT crystal. It is an enigma at this time and more studies are needed to further understand the interactions between the VFT and Cys-rich domains. It is also interesting to note that the short region between the end of the VFT, residue 528 and the initial cysteine 542 of the Cys-rich domain allows insertion of short peptides, such as the additional 10 residues in a splice variant of the human CaR found in a patient [12] and an engineered hexapeptide TEV protease recognition site following E^{536}, without impairing function [36].

How the signal of agonist-induced conformational changes in the VFT is transmitted *via* the Cys-rich domain to the 7TM remains unclear. From the evolutionary point of view, the first union of the VFT and the 7TM of totally different origins could be a random event, and a hybrid has evolved eventually into a precise machine [37]. The Cys-rich domain might have played a pivotal role in this process. The activation mechanism of the heterodimeric $GABA_B$ receptor, a family C GPCR lacking a Cys-rich domain is of interest in this regard.

Seven-transmembrane domain

All GPCRs share the signature seven transmembrane-spanning (7TM) domain. Agonist-induced GPCR activation presumably involves conformational changes of the membrane-spanning α helices, altering the disposition of intracellular loops and C-terminus, and thereby promoting activation of G proteins. Residues in intracellular loop 2 and 3 were found important for receptor function [38]. It was found recently that upon activation by glutamate, the second intracellular loops of the two 7TMs of a mGluR1 dimer move closer toward each other, while the first intracellular loops move further apart [39]. The CaR 7TM domain contains binding sites (allosteric sites) for a number of small molecule organic allosteric modulators and they are topologically distinct from the orthosteric site in the VFT domain. The presence of allosteric sites in the 7TM demonstrates that structural changes in the 7TM play a role in receptor activation.

Among over 1000 GPCRs identified, high-resolution 3D structure of only one receptor, bovine rhodopsin, in its inactive form has been solved. The rhodopsin crystal revealed interaction of covalently bound retinal with specific residues of the membrane-spanning helices [10]. The 7TMs of the CaR and other family 3 receptors display no significant amino acid sequence identity with the rhodopsin, a family 1 GPCR. None of the fingerprint motifs of the family 1 GPCR 7TM, except the two cysteines in exo-loop 1 and 2 (Fig. 1), can be found in the family 3 receptors. Moreover, the sizes and sequences of the extracellular loops and intracellular loops in the family 3 GPCR are different from those in the family 1 GPCR. Therefore, one should be cautious in extrapolating from the rhodopsin 3D structure to that of

family 3 GPCRs. Despite these obstacles, models of the 7TMs of CaR, mGluR1 and mGluR5 based on rhodopsin 3D structure have been constructed recently and shown to be useful in the study of binding sites for some allosteric modulators, suggesting overall structural similarity between the family 1 and 3 7TMs [40–43].

There are six prolines in the CaR 7TM which cause kinks in the transmembrane helices. The study of prolines and TM helix kinks in bacterial rhodopsin by Yohannon *et al.* suggested that P to A mutations in the 7TM often do not impair structure or function because of compensatory evolutionary changes in residues in other helices [44]. However, the prolines in the CaR 7TM remain critical. Missense mutation P748R in the TM4 was found in a patient with FHH suggesting this proline is important for receptor activation [45]. P^{823} in TM6 was also found to play a key role in receptor activation. Despite good expression of the P823A mutant CaR, its ability to be activated by Ca^{2+} is drastically reduced [46].

Intracellular carboxy-terminus

Much of the 216 residue carboxy-terminus of the receptor (residues 889–1078) can be truncated without impairing cell-surface expression and activation [47]. The carboxy-terminus, however, might be responsible for other properties of the CaR, such as its positively co-operative response to Ca^{2+} [48], and its binding to a scaffold protein, filamin-A [49]. Protein kinase C (PKC) phosphorylation of T^{888} inhibits CaR-mediated increases in cytosolic calcium *via* mobilization of intracellular Ca^{2+} stores. Truncating the CaR at residue 888 impairs the function of the receptor in a way similar to activating cellular PKC [50].

The three common CaR polymorphisms (A/S986, R/G990 and Q/E1011) were identified in the cytoplasmic tail of the CaR (Fig. 1). Their role in the pathogenesis of disorders of calcium metabolism is under investigation. The frequency of these polymorphisms varies in different populations and linkage dis-equilibrium exists between them, making it difficult to isolate the effects a single polymorphism. A study analysing serum calcium levels in samples from a normal population found that the homozygous polymorphism 986S was associated to higher serum calcium levels when compared to the heterozygous form, while the homozygous 986A had the lowest calcium levels [51].

Naturally occurring inactivating mutations associated with FHH and NSPHT

The CaR is one of just a few GPCRs where genetic mutations have been linked with actual disorders. The inherited disorders, familial FHH and neonatal severe hyperparathyroidism (NSHPT), are caused by inactivating mutations in the CaR gene, which right-shifts the set point for Ca^{2+} inhibition of PTH secretion and for stimulation of urinary calcium excretion. FHH is usually caused by heterozygous mutations, whereas NSHPT is usually caused by homozygous mutations or compound heterozygous mutations (*e.g.* one case with R680C and C60F compound mutations [52] and another case with R185stop and G670E compound mutations [53] were reported). If heterozygous missense or nonsense mutations prevent receptor expression at the cell surface, the remaining approximately 50% of the normal receptor provided by the wild-type allele would mediate parathyroid and renal calcium sensing. However, heterozygous mutations that permit CaR expression but impair function might cause severe FHH or NSPHT by acting as dominant negatives of the wild-type CaR, as the 25% mutant homodimers and 50% mutant-wild-type heterodimers would not contribute to receptor signalling, leaving only 25% wild-type homodimers to regulate parathyroid and renal function. Three of the best-characterized CaR mutations acting through a dominant-negative mechanism are R227L [54], R185Q [55] and I212T [56]. Truncation of the CaR proximal to residue 888 disrupts receptor function, thus frameshift and nonsense mutations causing such truncation (not shown in Fig. 1) are inactivating mutations (*e.g.* R648stop produces an inactive CaR with a single transmembrane domain [57]). Over 50 unique inactivating missense mutations in FHH/NSPHT have been identified to date. They are clustered in N-terminal portion of the VFT domain, the Cys-rich domain and the 7TM domain. Few inactivating mutations have been identified in the loop 3 region or distal C-terminal portion, consistent with the findings from *in vitro* experiments that deletion of most residues in these regions did not affect expression and function of the receptor [22, 47],

Three inactivating mutations have been identified within the signal peptide, which impairs normal CaR expression [58, 59]. Missense mutations that alter

critical cysteines, such as C60F, C395R, C562Y and C582Y, or introduce novel cysteines, such as R66C, Y161C, Y218C and R680C, might inactivate the receptor by interfering with formation of crucial disulphide bonds. Some inactivating mutations involve residues equivalent to those that constitute the orthosteric (agonist-binding) site in mGluR1, such as R66H, E297K and Y218S, and they might impair Ca^{2+} binding or agonist-induced dimer closure. Many other inactivating mutations in the N-terminal portion of the VFT involve residues at the dimer interface, such as S53P, P55L, S137P, T138M, G143E, L159P, N178D, D215G, R220W/Q, P221S and R227L/Q, and they might impair the crucial agonist-promoted dimer rotation. Mutations within the Cys-rich domain might impair normal folding of this domain. Inactivating mutations in the 7TM domain might also impair normal folding or interfere with activation-dependent conformational changes within the transmembrane helices. Inactivating mutations in the intracellular loops, such as R795W and P798T, and in the proximal portion of the C-tail, such as F881L and R886P, might prevent G protein coupling.

Naturally occurring activating mutations associated with ADH and Bartter's syndrome type V

Heterozygous, activating mutations in subjects with ADH cause a left-shift in the Ca^{2+} set point leading to relative hypocalcaemia and hypercalciuria. ADH mutations increase CaR sensitivity to extracellular Ca^{2+} rather than causing constitutive activation, except A843E, which is the only constitutive activating mutation described in the CaR [60]. This mutation and a few most potent ADH mutations, such as L125P and C131W cause type V Bartter's syndrome in subjects with hypocalcaemia and hypercalciuria [61, 62].

With the exception of an in-frame deletion, S895–V1075 [63], activating mutations in ADH are missense mutations. Such mutations presumably act by relieving inhibitory constraints that maintain the CaR in its inactive conformation. There are two clusters of ADH mutations in the CaR sequence. One locates in the loop 2 region of the VFT domain. There are 10 ADH mutations have been identified between residue 116 and 131 (A116T, N118K, N124K, L125P, E127A/K, F128L, C129F/S, C131W) [64]. *In vitro* random saturation mutagenesis in region A^{116}-P^{136} also identified the importance of this region for maintenance of the receptor in its inactive conformation [65]. As mentioned earlier, loop 2 is involved in intermolecular disulphide linkages and agonist-induced structural alterations occur in this loop even though large portion of this loop are disordered in mGluR1 VFT crystals. These loop 2 mutations and several other mutations at the dimer interface in our VFT model such as T151M, P221L, E228Q and Q245R, might enhance Ca^{2+} sensitivity of the CaR by facilitating agonist-promoted VFT closure and/or dimer rotation.

The second cluster of ADH mutations reside in transmembrane helices 5, 6 and 7 (Fig. 1) suggesting that movement of these helices relative to each other could be a crucial event in CaR activation. Out of 18 activating mutations in the CaR 7TM domain identified in ADH patients to date, 15 appear in the TM5-7 and six of which appear at the junction of TM helices 6 and 7 between residue I^{819} and E^{837}. Alanine-scanning mutagenesis revealed five additional residues in this region which when substituted by alanine led to CaR activation. While E837A was not activating, E837D and E837K mutations did activate the receptor. Therefore, region I^{819}–E^{837} of the 7TM domain represents a 'hot spot' for activating mutations [46].

As the available rhodopsin crystal structure is in inactive conformation, how photon-mediated retinal isomerization promotes the active rhodopsin conformation has not been clearly defined, but a recent NMR study suggests that 'rigid body' motion of TM6 is a key element in the activation mechanism [66]. Activating mutations in the hCaR 7TM might activate the receptor by facilitate such TM6 motion.

It is noteworthy that multiple naturally occurring activating mutations have been identified at some residues in the CaR, such as E127A/K, C129F/S/Y, C131F/W, T151M/R and F788C/L. In contrast, at a few other sites different mutations exerted opposite effects, such as P221L (activating) *versus* P221S (inactivating) [67, 68] and E297D (activating) *versus* E297K (inactivating) [31]. These observations are consistent with *in vitro* studies which showed that at residues such as N124 only a specific amino acid substitution activated the receptor [64] while at residues such as K^{29} a variety of amino acid substitutions activated the receptor [69]. The electrostatic property of the side chain of residue 29 appears

critical for receptor activation, suggesting a possible role of this residue in forming a salt bridge important for receptor function [69].

Allosteric modulators of the CaR and their therapeutic potentials

G protein-coupled receptors are the most common targets of drug action. Approximately 40% of all drugs currently on the market target GPCRs. Family 3 receptors have recently become a focus for the discovery of new allosteric modulators with therapeutic potential. Allosteric modulators offer advantages over classic orthosteric ligands as therapeutic agents, including the potential for greater GPCR-subtype selectivity and safety [70]. Novel allosteric modulators of the CaR are being vigorously explored in an effort to identify potential drugs for treatment of disorders of calcium metabolism. Positive allosteric modulators of the CaR increase CaR activation, thereby decreasing secretion of PTH, and thus are potentially useful for treatment of primary and secondary hyperparathyroidism [71, 72]. Among them, Sensipar (cinacalcet hydrochloride) is the first-in-class GPCR allosteric modulator approved by the FDA recently for treatment of secondary hyperparathyroidism in patients with chronic kidney disease on dialysis, and hypercalcaemia in patients with parathyroid cancer. On the other hand, negative allosteric modulators of the CaR decrease receptor activation, thereby stimulating endogenous PTH secretion. This potentially offers a novel method for treatment of osteoporosis [8].

In addition to their therapeutic potential, allosteric modulators of the CaR offer unique insights into the mechanisms of receptor activation. How the signal of conformational changes in orthosteric sites upon ligand binding is transmitted to the 7TM leading to receptor activation is a major unanswered question. It is speculated that movements of the helices within 7TM and/or between two 7TMs in dimeric CaRs are ultimately responsible for receptor activation and G protein coupling. Allosteric modulators bound to the allosteric sites in the 7TM domain of the CaR likely facilitate (positive modulators) or impede (negative modulators) these movements. Positive modulators might share a common mechanism of action with some activating CaR mutations in the 7TM.

Positive allosteric modulators

Type II calcimimetics NPS R-467 and NPS R-568 [71], Calindol [42], cinacalcet [9] act as positive allosteric modulators of the CaR, enhancing its sensitivity to Ca^{2+} without activating it by themselves [5]. They are selective for the CaR, failing to modulate closely related GPCRs such as mGluR1. NPS R-467 restores some function to inactivating FHH mutations such as R185Q [73], suggesting its potential for treatment of certain group of FHH patients as well. All above calcimimetic compounds are structurally related phenylalkylamines. NPS R-568 acts on the 7TM domain of the CaR; alanine substitution for E^{837}, at the top of TM 7, nearly abolishes the response to NPS R-568 without affecting CaR expression or response to Ca^{2+} [74]. Later it was reported that this same residue is also crucial for allosteric modulation by other phenylalkylamines including positive modulator Calindol and several negative allosteric modulators (described below) [42, 43]. It is speculated that a critical salt bridge is formed between the positively charged central group of these phenylalkylamines and the acidic side chain of residue E^{837}. Other than E^{837}, a nearby residue I^{841} in the TM7 was also found crucial for action by both NPS R-568 and Calindol. The partially overlapping allosteric sites in the CaR 7TM for phenylalkylamines including positive and negative modulators involve these two residues and possibly W^{818} and F^{821}. The lack of a 3D GPCR structure in active conformation as a template prevents modelling of the hCaR 7TM in calcimimetic bound active form.

Negative allosteric modulators

Negative modulators of the CaR, so-called calcilytic drugs such as NPS2143 [75], NPS89636 [76], Calhex-231 [42], Compound 1 [77] and certain derivatives of NPS2143 [78, 79] block Ca^{2+} activation of the CaR. These compounds, except Compound 1, are phenylalkylamines structurally related to those type II calcimimetics demonstrating that subtle differences in the configuration of compounds binding to the CaR 7TM might either prevent (negative modulators) or promote (positive modulators) helical motion required for receptor activation. Negative modulation of the CaR by NPS2143 and Calhex-231 also critically depend on residue E^{837} located in the TM7 (Fig. 3)

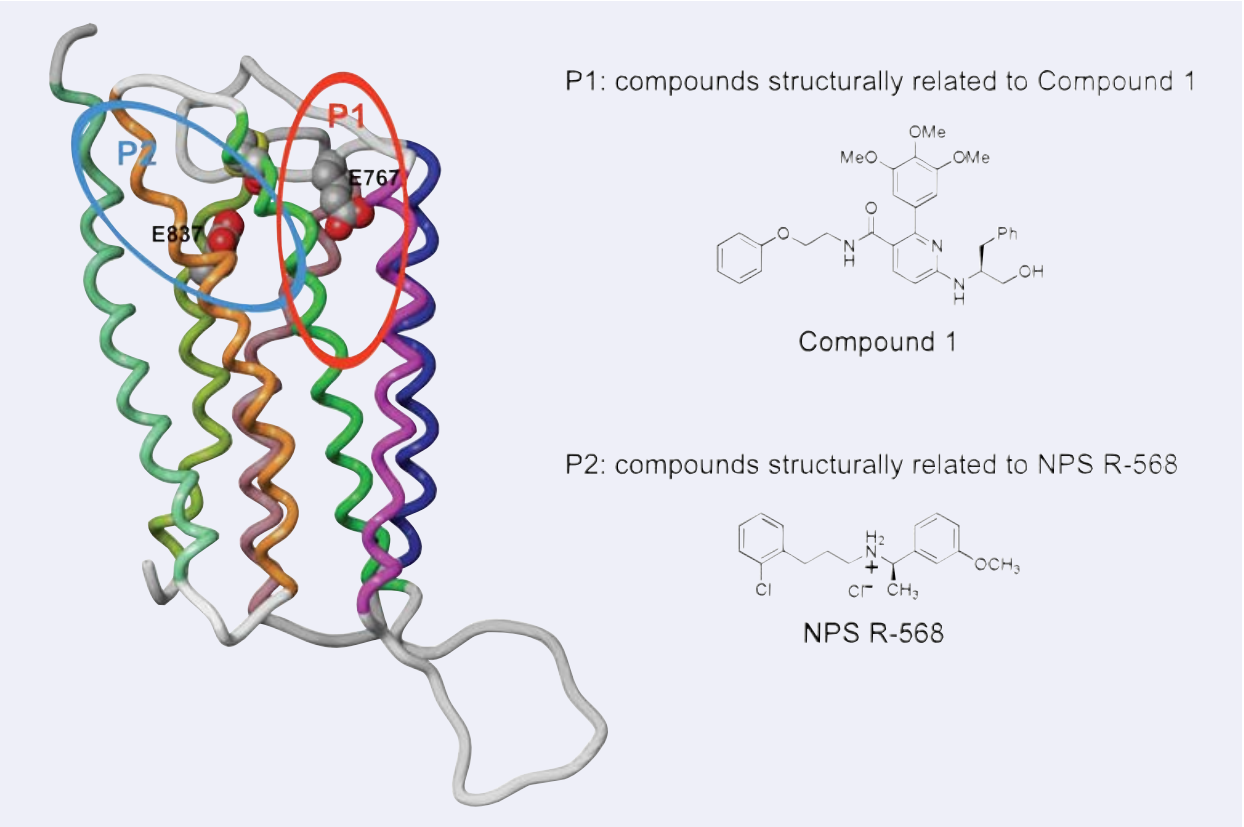

Fig. 3 Schematic representation of the CaR 7TM. Molecular docking results indicated two potential binding pockets for allosteric modulators within the upper part of the transmembrane helical bundle. The red circle indicates pocket P1, enclosed within TM3 (green), TM4 (purple), TM5 (blue), TM6 (brown) and exo-loop2; the blue circle indicates pocket P2, enclosed within TM1 (cyan), TM2 (orange), TM3, TM6, TM7 (yellow-green) and exo-loop 2. Residues E^{767}, E^{837}, and the conserved disulphide bond (C^{677}–C^{765}) connecting exo-loop 1 and 2 are shown as Corey-Pauling-Koltun. Compound 1 and its analogue JKJ05 are proposed to bind to pocket P1. JKJ05 is a negative modulator of the wild-type CaR and a positive modulator of E837A mutant CaR, and the positive modulation critically depends on the primary amine in JKJ05 which appears to interact with residue E^{767} in this model. Phenylalkylamines structurally related to NPS R-568 are proposed to bind to pocket P2, and they anchor to the pocket through a critical salt bridge between their positively charged central amino group and the acidic residue E837 in TM7.

[42, 43]. Both compounds are primarily anchored through an H-bond assisted salt bridge to this negatively charged residue.

In the rhodopsin crystal structure, 11-*cis*-retinal is covalently bound to K^{296} (7.43) in TM7 and is bracketed by residues in TM3 and TM6. This structure in its inactive state sheds light on homology modelling of the CaR 7TM with binding of calcilytics. Other than E^{837}, residues R^{680}, F^{684} and F^{688} in the TM3, and I^{841} in the TM7 were shown to be implicated in the recognition of both NPS 2143 and Calhex 231[42]. Among them, R^{680}, F^{684}, and F^{688} were shown not involved in recognition of calcimimetic compounds NPS R^{568} and Calindol [42].

Compound 1 (Fig. 3) is a unique negative allosteric modulator of the CaR that lacks a central amino group and retains activity against the E837A mutant [80]. It did not compete for receptor binding with an analogue of NPS2143 [77], suggesting a unique allosteric site for this compound in the CaR 7TM (Fig. 3).

NPS 2143 was found in *in vitro* experiments to inhibit the activity of all ADH mutations tested with the exception of constitutively activating mutation A843E [46]. Conventional treatment of ADH using vitamin D and calcium supplements is unsatisfactory because of the resultant hypercalciuria leading to nephrolithiasis. Negative allosteric modulators, such

as NPS 2143 offer the possibility of more 'physiologic' correction of the molecular defect in ADH by decreasing the sensitivity of the CaR to Ca^{2+}, enhancing PTH secretion and renal Ca^{2+} re-absorption. Negative allosteric CaR modulators are currently under study for possible use in treatment of osteoporosis, but further studies may be warranted to evaluate such agents for correction of hypocalcaemia in subjects with ADH.

We found that JKJ05, an analogue of Compound 1 with a positively charged primary amino group, acts as a negative allosteric modulator on the wild-type receptor, but as a positive modulator on the E837A mutant receptor [80]. This positive modulation critically depends on the primary amine in JKJ05. In our model of the hCaR 7TM, the primary amine in JKJ05 interacts with acidic residue E^{767} of the 7TM of the receptor (Fig. 3). Action of JKJ05, negative *versus* positive modulation, critically depends on a single residue in the CaR, suggesting the need for caution in therapeutic application of allosteric modulator-based agents. Further delineation of residues comprising allosteric-binding sites, elucidation of allosteric modulation mechanism and appreciation of potential sequence variation in the CaR 7TM are demanded.

Concluding remarks

Since the cloning of the CaR a little more than a decade ago, over 100 disease-causing naturally occurring CaR mutations have been identified. Study of these natural mutations and a much larger number of engineered mutations revealed regions and residues critical for receptor expression and function. Homology modelling of the CaR based on the 3D structures of mGluR1 VFT and rhodopsin provided insights into the folding of this large GPCR as well as the topologically distinctive orthosteric and allosteric sites in the receptor.

Several major questions concerning the structure and function of the CaR remain unanswered, including: (1) how many calcium ions bind to each receptor and which residues in the VFT constitute the orthosteric site; (2) what is the function of the Cys-rich domain and what conformational changes occur in this domain upon agonist binding to the VFT; (3) which residues in the 7TM constitute the allosteric sites for those allosteric modulators and what is the mechanism of allosteric modulation; and (4) a major question for all GPCRs 'what structural changes in the 7TM lead to activation of the G proteins coupled to the receptor'. Further studies by biochemical and structural biological approaches will certainly yield more information, which should enable us to make more informed designs of drugs targeting the CaR. With one allosteric modulator-based drug targeting the CaR already in the market, more drugs in this class can be expected to appear in the future for treatment of various disorders of calcium metabolism.

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