

# Extracellular calcium-sensing receptor: structural and functional features and association with diseases

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## Abstract

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The recently cloned extracellular calcium-sensing receptor (CaR) is a G protein-coupled receptor that plays an essential role in the regulation of extracellular calcium homeostasis. This receptor is expressed in all tissues related to this control (parathyroid glands, thyroid C-cells, kidneys, intestine and bones) and also in tissues with apparently no role in the maintenance of extracellular calcium levels, such as brain, skin and pancreas. The CaR amino acid sequence is compatible with three major domains: a long and hydrophilic aminoterminal extracellular domain, where most of the activating and inactivating mutations described to date are located and where the dimerization process occurs, and the agonist-binding site is located, a hydrophobic transmembrane domain involved in the signal transduction mechanism from the extracellular domain to its respective G protein, and a carboxyterminal intracellular tail, with a well-established role for cell surface CaR expression and for signal transduction. CaR cloning was immediately followed by the association of genetic human diseases with inactivating and activating CaR mutations: familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism are caused by CaR-inactivating mutations, whereas autosomal dominant hypoparathyroidism is secondary to CaR-activating mutations. Finally, we will comment on the development of drugs that modulate CaR function by either activating (calcimimetic drugs) or antagonizing it (calcilytic drugs), and on their potential therapeutic implications, such as medical control of specific cases of primary and uremic hyperparathyroidism with calcimimetic drugs and a potential treatment for osteoporosis with a calcilytic drug.

### Key words

- Calcium-sensing receptor
- Familial hypocalciuric hypercalcemia
- Autosomal dominant hypoparathyroidism
- Calcimimetic drugs
- Calcilytic drugs

## Introduction: an overview of structural and functional features of the extracellular calcium-sensing receptor

The extracellular calcium-sensing receptor (CaR) was cloned by Brown et al. (1) in 1993 from bovine parathyroid glands. The cloning of this receptor provided valuable information regarding mechanisms related

to the control of extracellular calcium homeostasis. Until 1993, hormones such as parathormone (PTH), 1,25-dihydroxyvitamin D<sub>3</sub> and calcitonin, interacting with their respective target organs and tissues involved in the regulation of calcium homeostasis, were promptly recognized as major participants in the adequate maintenance of this delicate balance. However, the presumed common mechanism responsible for the sensing of

minor variations in extracellular calcium concentration could be only successfully identified with the cloning of the CaR (1).

In fact, many papers subsequently demonstrated the expression of the CaR in all the tissues directly related to the control of calcium homeostasis: human and chicken parathyroid glands; rat, human and rabbit kidney; thyroid rat and human C-cells; human and rat intestine, and also in diverse bone marrow-derived cells (2,3). With this in mind, it is currently accepted that, in addition to serving as an intracellular second messenger, calcium ion also plays a hormone-like

role as an extracellular first messenger (4). In this context, there is no doubt that the CaR plays an essential role in the regulation of extracellular calcium homeostasis, mainly by mediating the inhibitory actions of extracellular calcium on parathyroid hormone secretion by the parathyroid glands and by influencing the rates of renal tubular calcium reabsorption and secretion of calcitonin by thyroidal C-cells. In addition to all these tissues directly involved in the calcium homeostatic system, the CaR seems to be widely distributed, being expressed in tissues with apparently no evident role in the control of

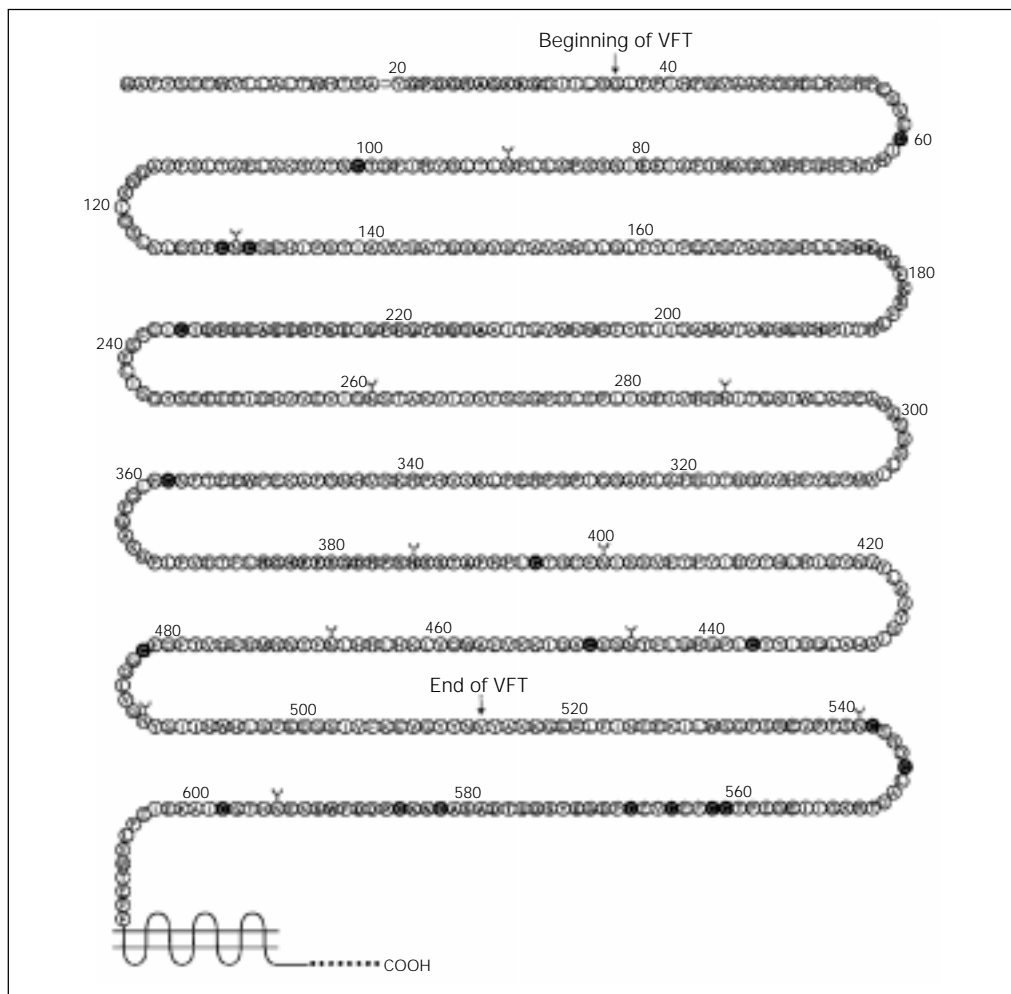


Figure 1. Schematic diagram of the human extracellular calcium-sensing receptor (hCaR) showing the amino acid sequence of the large hCaR extracellular domain. The location of the N-linked glycosylation sites is shown and all the 19 cysteines are shown inside black circles. The beginning and the end of the putative "venus flytrap" (VFT) domain are indicated. The cysteine-rich domain includes the nine cysteine residues located between C542 and C598.

extracellular calcium homeostasis, such as breast, keratinocytes, a variety of cells within the central nervous system, pancreas, and lens epithelial cells (2). The specific roles of the CaR in all these tissues still remain to be elucidated.

Structurally speaking, the human homologue of the CaR is a G protein-coupled receptor (GPCR) consisting of 1078 amino acid residues (3). The first 612 amino acids are included in a large extracellular domain (ECD), which is a feature of the subfamily to which the CaR belongs. In addition to the CaR, this subfamily of GPCRs (also called family C or family 3) includes the metabotropic glutamate receptors (5), putative pheromone receptors in the vomeronasal organ (6-8), putative taste receptors (9) and GABA<sub>B</sub> receptors (10).

Part of this large ECD (from amino acid residue 36 to amino acid residue 513) seems to be structurally related to the bilobed (“venus flytrap”) structure of bacterial periplasmic binding proteins (11-14) (Figure 1), and hypothetically the closing of the two lobes after binding of ligands triggers the transmission of signals from the ECD to the receptor’s cytoplasmic signaling loops. The CaR ECD is thought to be the site of Ca<sup>2+</sup> binding (15,16) and contains 11 potential N-linked glycosylation sites (17,18) and a total of 19 cysteines, nine of them being highly conserved and located at the end of the ECD, and constituting the so-called cysteine-rich domain which plays a critical role in signal transduction from the corresponding “venus flytrap” domain to the seven-transmembrane domain (19) (Figure 1). Recent studies have demonstrated that the CaR is expressed on the cell surface as an intermolecular disulfide-linked dimer (20,21) and cysteine residues which are critical for CaR dimerization have been identified (13,22). Moreover, heterodimerization of mutant CaRs and intermolecular interactions of the heterodimeric receptor have been shown to be functionally important for receptor-dependent signaling

(23,24) and recent findings have suggested that the CaR has at least two distinct types of motifs mediating dimerization and functional interactions: covalent interactions involving intermolecular disulfide bonds and noncovalent, possibly hydrophobic, interactions (22). Most of the natural activating and inactivating mutations described so far are located in the ECD (Table 1) and a cluster of natural and artificial activating mutations is located between residues Ala 116 and Pro 136, suggesting that this region is very important

Table 1. Natural activating and inactivating mutations of the human calcium-sensing receptor described to date.

Activating mutations	Site	Inactivating mutations	Site
K47N	ECD	C7-frameshift	ECD
A116T	ECD	Q27R	ECD
N118K	ECD	P39A	ECD
L125P	ECD	P40A	ECD
E127A	ECD	S53P	ECD
F128L	ECD	P55L	ECD
C129F	ECD	R62M	ECD
T151M	ECD	R66C	ECD
E191K	ECD	S137P	ECD
P221L	ECD	T138M	ECD
E228Q	ECD	G143E	ECD
Q245R	ECD	L174R	ECD
Q254R	ECD	N178D	ECD
F612S	ECD	R185Q	ECD
Q681H	1st EC loop	D215G	ECD
L773R	TM-5	Y218S	ECD
F788C	TM-5	R220W	ECD
F806S	TM-6	P221S	ECD
S820F	TM-6	R227L(Q)	ECD
A835T	3rd EC loop	E297K	ECD
A843E	TM-7	C395R	ECD
S895-V1075 (in-frame deletion)	C-tail	G549R	ECD
		G552R	ECD
		C582Y	ECD
		S607Stop	ECD
		R648X	1st IC loop
		S657Y	TM-2
		G670R	TM-2
		R680C	1st EC loop
		P747-frameshift	2nd EC loop
		P748R	2nd EC loop
		R795W	3rd IC loop
		V817I	TM-6
		T876-frameshift	C-tail
		F881L	C-tail

ECD, extracellular domain; EC, extracellular; IC, intracellular; TM, transmembrane domain; C-tail, carboxyterminal tail.

for the maintenance of the inactive conformation of the CaR (25).

CaR activation by extracellular calcium involves activation of phospholipase C $_{\beta}$  via the G $_q$  subfamily of G proteins, with a consequent transient increase of intracellular calcium. Actually, the CaR is thought to couple through its intracellular loops as well as its carboxyterminal intracellular tail (C-tail) to the various G proteins that mediate its biological actions (including G $_q$  for activating phospholipase C and G $_i$  for inhibiting adenylate cyclase) (2,4). The functional characteristics of the expressed wild-type CaR are included in the definition of the EC $_{50}$  for extracellular calcium, which is the effective concentration of extracellular calcium eliciting one half of the maximal intracellular calcium response. This corresponds to a value around 4.1 mM in transiently transfected HEK293 cells (26).

The seven-transmembrane domain, characteristic of the superfamily of GPCRs, is involved in the signal transduction mechanism from the CaR ECD to its respective G protein. In addition (see Table 1), natural activating and inactivating mutations have been identified in the seven-transmembrane domain, including the single constitutive activating mutation (A843E) of the CaR described to date (27). Within the intracellular domains of the human CaR, there are five predicted protein kinase C phosphorylation sites. The C-tail comprises over 200 amino acid residues and its role for cell surface CaR expression and for signal transduction has been described. Moreover, it has been demonstrated that the first 20 amino acid residues in the membrane-proximal portion of the C-tail are important for CaR function (28,29).

### **Extracellular calcium-sensing receptor: association with diseases**

CaR cloning was promptly followed by identification of inherited human hypercal-

cemic and hypocalcemic disorders, respectively resulting from loss and gain of function mutations in the CaR (2,30,31). In familial hypocalciuric hypercalcemia (FHH) (32-34) and neonatal severe hyperparathyroidism (NSHPT) (35), loss of function mutations in the CaR lead to generalized resistance to extracellular calcium. The opposite phenotype, hypocalcemia with relative hypercalciuria, is associated with a disorder termed autosomal dominant hypocalcemia (ADH) (36), which has been shown to be caused by gain of function mutations in the CaR leading to hyperresponsiveness to extracellular calcium.

A list of natural activating and inactivating mutations described so far (with their respective locations in the CaR) that are associated with these diseases is shown in Table 1. The single exception regarding phenotype is an inactivating mutation (F881L) located in the C-tail which leads to hypercalcemia and hypercalciuria (37).

FHH is inherited as an autosomal-dominant trait with a high penetrance of over 90% (33,34). The majority of families exhibit linkage of the disease gene to chromosome 3 (band q21-24) known to contain the CaR gene, but an exception can be illustrated by a family that showed linkage of a phenotypically indistinguishable disorder to the short arm of chromosome 19, band 19p13.3 (38). FHH is a rare disorder of mineral metabolism characterized by lifelong, mild to moderate but usually asymptomatic hypercalcemia. Another feature of this disease is the presence of inappropriately low rates of urinary calcium excretion (a calcium to creatinine clearance ratio of <0.01) and nonsuppressed circulating levels of PTH, regardless of the presence of hypercalcemia (33,34). In short, the diagnosis of FHH can be established by documenting the combination of a low urinary calcium to creatinine clearance ratio, a normal PTH level, and the autosomal-dominant inheritance of mild, asymptomatic hypercalcemia. Consequently and re-

enforcing the importance of differential diagnosis with primary hyperparathyroidism, surgery (parathyroidectomy) is not indicated and will not improve the hypercalcemia of patients with FHH. Impairment of extracellular calcium sensing by the CaR in FHH patients was proven by functional studies in which human embryonic kidney (HEK293) cells were transfected with CaRs bearing FHH mutations. These studies showed an increase in  $EC_{50}$  and many mutants also disclosed a reduction in maximal activity (26,29), supporting previous studies regarding formal testing of calcium-regulated PTH secretion that indicated an increase in set-point (39).

Due to severe primary hyperparathyroidism with enlargement of all four parathyroid glands, the degree of hypercalcemia in NSHPT is usually more severe than that observed in FHH, and this disorder can be fatal if parathyroidectomy is not carried out within the first weeks of life. Bone demineralization, often accompanied by multiple fractures of long bones and ribs may be present. Some infants with NSHPT represent the homozygous form of FHH (40), or, as seen in one case, a compound heterozygote in which a different inactivating CaR mutation was inherited from each parent (41). In some cases, NSHPT may be caused by the presence of heterozygous inactivating mutations of the CaR, either in a familial setting or as a *de novo* mutation in the offspring of normal parents, and possibly in these cases the mutant CaR can exert a “dominant” negative action, impairing the function of the normal receptor (42). Recently, mice heterozygous or homozygous for targeted inactivation of the CaR gene have been developed. These mice shared biochemical features of FHH and NSHPT, supporting the physiological importance of CaR in mineral ion metabolism (43).

A heterozygous germline activating missense mutation of the human CaR inhibits PTH secretion and reduces renal calcium

reabsorption at an inappropriately low serum calcium concentration, leading to hypocalcemia, relative hypercalciuria and an inappropriately low serum PTH that characterizes subjects with ADH or sporadic hypocalcemia (27,44). Pearce et al. (36) studied six families with ADH and verified that, in addition to having asymptomatic hypocalcemia with detectable PTH levels, affected family members also had hypomagnesemia and hyperphosphatemia. Differently from what occurs in hypoparathyroidism, there is resetting of the calcium homeostatic system downward, maintaining the extracellular calcium at subnormal concentrations. In addition, a reason for classifying these families as ADH instead of familial isolated hypoparathyroidism is their response to vitamin D treatment. When an attempt was made to normalize their serum calcium levels with calcitriol, marked hypercalciuria with resultant nephrocalcinosis was observed in some cases. Some of them apparently also developed nephrogenic diabetes insipidus. Therefore, it seems that the calcium homeostatic system of these individuals is adjusted to a lower than normal serum calcium concentration, and attempts to make them normocalcemic result in “hypercalcemic” manifestations, such as hypercalciuria and nephrogenic diabetes insipidus (35). CaR mutations in ADH (36,44), with one single exception (27), cause increased sensitivity to extracellular calcium rather than constitutive activation. Expression of CaRs harboring activating mutations in HEK293 cells revealed reductions in  $EC_{50}$  of the mutant receptors (26,44,45).

### **Extracellular calcium-sensing receptor: therapeutic implications**

The CaR responds to many polycationic ligands, including divalent and trivalent cations (i.e.,  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Gd^{3+}$ ) and polyamines (neomycin and spermine) when expressed in either *Xenopus laevis* oocytes or HEK293

cells (1,26,46). It has been recently shown that the CaR can be also activated by amino acids, especially aromatic amino acids, and this finding may help to explain some actions of dietary proteins on calcium metabolism (47).

Since the CaR represents a potential therapeutic target for disorders in which the receptor is inappropriately overactive or underactive (48), some compounds have been developed with the aim of either activating (calcimimetics) (49) or inactivating (calcilytics) (50) the CaR.

A so-called calcimimetic agent NPS R-568 (49) has been shown to actually increase CaR sensitivity to  $\text{Ca}^{2+}$  and this action has been demonstrated to successfully inhibit PTH secretion *in vitro* and in subjects with primary hyperparathyroidism (51), including a patient with parathyroid carcinoma (52), and also in rats and patients with uremic hyperparathyroidism (53,54). In primary hyperparathyroidism, oral NPS R-568 produces fast (within minutes) and substantial (>50%) decreases in circulating PTH, followed hours later by reductions in serum calcium concentration at higher doses. In this context, this calcimimetic resets the elevated set-point of pathological parathyroid glands toward normal (2). As expected, a short-term use of this calcimimetic was also associated with an increase in urinary calcium excretion in hypercalcemic patients with primary hyperparathyroidism (51), sug-

gesting that a CaR antagonist could be very useful for the treatment of calcium-containing renal stones. The site of action of R-568 appears to be within and specific for the seven-transmembrane domain of the CaR (45).

Based on previous studies (55,56) showing that PTH is a potential anabolic agent for the bone and consequently a promising drug for the management of osteoporosis, Gowen et al. (50) recently studied the effects of antagonizing the parathyroid calcium receptor with the use of a so-called calcilytic drug (termed NPS 2143) in osteopenic ovariectomized rats. These authors observed an increase in endogenous PTH secretion leading to a significant increase in bone turnover. These preliminary results suggest that this or future similar drugs may be potential candidates for the treatment of osteoporosis. Future understanding of the functions of the expressed CaR in tissues with apparently no kind of role in the maintenance of extracellular calcium levels may suggest additional therapeutic roles for CaR agonists or antagonists (2).

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