MOLECULAR GENETICS AND METABOLISM **64,** 198–204 (1998)
ARTICLE NO. GM982716

Activating Mutations of the Ca^{2+}-Sensing Receptor

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Received March 27, 1998, and in revised form April 15, 1998

The Ca^{2+}-sensing receptor (CaR) is a member of the seven-transmembrane domain, G-protein-coupled receptor superfamily. It is expressed in parathyroid, kidney, and other tissues. In parathyroid, activation of the CaR by extracellular Ca^{2+} negatively regulates the secretion of parathyroid hormone. In the the thick ascending limb of Henle's loop, receptor activation decreases renal reabsorption of Ca^{2+}. Heterozygous inactivating mutations of the CaR cause familial benign hypocalciuric hypercalcemia while homozygous inactivating mutations cause neonatal severe hyperparathyroidism. Conversely, activating mutations of the CaR cause autosomal dominant and sporadic hypoparathyroidism. Affected individuals have hypocalcemia which ranges from mild and asymptomatic to life-threatening. They also show a greater tendency to hypercalciuria than do other patients with hypoparathyroidism. Most, but not all, of the reported activating mutations occur in the amino-terminal, extracellular domain of the receptor. When expressed in cultured cells, mutant receptors can show both increased receptor sensitivity to Ca^{2+} and increased maximal signal transduction capacity.

Key Words: **Ca^{2+}-sensing receptor; parathyroid hormone; autosomal dominant hypoparathyroidism; hypercalciuria.**

CALCIUM HOMEOSTASIS

Extracellular Ca^{2+} concentration is maintained within a narrow range by several homeostatic mechanisms. The most important of these mechanisms is a negative feedback loop involving parathyroid hormone (PTH), 1,25-$(OH)_2$ vitamin D, and Ca^{2+} (1,2). When extracellular Ca^{2+} concentration decreases below the system setpoint, PTH secretion from the parathyroid glands is stimulated. PTH increases osteoclastic reabsorption of mineralized bone matrix, thus bringing calcium phosphate out of the solid phase and into solution. PTH acts in the kidney to increase reabsorption of Ca^{2+} and decrease reabsorption of phosphate from the tubular lumen (3). PTH also acts on the proximal convoluted tubule of the kidney to stimulate 1α hydroxylation of 25-OH vitamin D. The resulting 1,25-$(OH)_2$ vitamin D, in turn, acts on the intestinal epithelium to increase absorption of calcium and phosphate from the intestinal lumen. Thus, PTH acts to recruit Ca^{2+} from skeletal, renal, and intestinal sources, restoring the extracellular Ca^{2+} concentration back to its setpoint. Conversely, when extracellular Ca^{2+} rises above its setpoint, PTH secretion decreases thus bringing the Ca^{2+} concentration down toward the setpoint.

Extracellular Ca^{2+} homeostasis is also maintained by a short feedback mechanism involving a direct effect of Ca^{2+} on the kidney. Increased extracellular Ca^{2+} acts on the medullary thick ascending limb to inhibit reabsorption of Ca^{2+} from the tubular lumen. Consequently, increased extracellular Ca^{2+} concentration leads to increased urinary Ca^{2+} excretion while decreased extracellular Ca^{2+} concentration leads to decreased urinary Ca^{2+} excretion.

Ca^{2+}-SENSING RECEPTOR

To complete the negative feedback loops, both of these homeostatic systems require a mechanism to

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1096-7192/98 $25.00

sense the extracellular Ca^{2+} concentration. In 1993, Brown and co-workers characterized a novel cell-surface receptor which responded to extracellular Ca^{2+} (4). The sequence of this receptor indicated that it is a member of the seven-transmembrane domain, G-protein-coupled receptor superfamily (Fig. 1). The human Ca^{2+}-sensing receptor (CaR) contains 1078 amino acids. The large amino-terminal extracellular domain includes clusters of acidic amino acids which resemble sites in other low affinity, calcium-binding proteins, such as calreticulin and calsequestrin (4). These acidic sites are thought be involved in the Ca^{2+} binding site. The CaR also contains seven transmembrane segments with three intervening extracellular loops, three intervening intracellular loops, and a cytoplasmic carboxy-terminal tail characteristic of G-protein-coupled receptors.

The CaR activates phospholipase C through the interaction with a G-protein, presumably a member of the Gq family (5–7). The stimulation of phospholipase C leads to increased levels of diacylglycerol and inositol phophates and secondarily increased intracellular Ca^{2+} concentrations. CaR activation is also able to inhibit cAMP accumulation in bovine parathyroid cells (8).

The CaR is expressed in parathyroid cells, C cells of the thyroid, and multiple cell types within the kidney and brain (9–11). In the kidney the CaR is present in the basocellular membrane of tubular cells in the medullary thick ascending limb of the loop of Henle. The CaR expressed by these cells presumably mediates the inhibitory effect of peritubular Ca^{2+} on reabsorption of Ca^{2+} and Mg^{2+} from the lumen (12). Thus, receptor expression in this portion of the tubule provides a molecular explanation for the physiological observation that increased serum calcium concentration causes increased urinary excretion of calcium. The CaR is also expressed in the apical membrane of the inner medullary collecting duct where it is thought to mediate the inhibitory effect of luminal calcium concentration on AVP-stimulated water permeability. Thus, receptor expression at this site provides an explanation for the polyuria associated with hypercalcemia. The teleological explanation for this mechanism may be to dilute urinary Ca^{2+} and thus prevent nephrolithiasis.

In the thyroidal C cells, Ca^{2+} may mediate the stimulatory effects of Ca^{2+} on calcitonin secretion. The exact role of the CaR in other tissues remains to be elucidated; in the GI tract the CaR may be involved in regulating motility (13), and in the brain it may be involved in the regulation of thirst and the secretion of hormones regulating water homeostasis (9).

INACTIVATING CaR MUTATIONS

In humans, heterozygous inactivating mutations in the CaR cause a syndrome known variously as familial benign hypercalcemia, familial hypocalciuric hypercalcemia, or familial benign hypocalciuric hypercalcemia (FBHH) (14–17). Because of the inactivating mutation in one allele, individuals with FBHH presumably have fewer normal CaR molecules per cell. As a result, these individuals have partial resistance to Ca^{2+} in tissues which express the CaR (16,17).

In parathyroid tissue, the extracellular Ca^{2+} concentration required to suppress PTH secretion is greater in individuals with FBHH than in normal individuals (16,18). As a result, patients with FBHH have hypercalcemia throughout life. Despite the elevated Ca^{2+} concentration, the circulating PTH levels are not suppressed, reflecting the partial resistance of the parathyroid gland to extracellular Ca^{2+}. Most individuals with FBHH have no symptoms (15).

In FBHH, the kidney also shows evidence of decreased responsiveness to Ca^{2+} (15). In other hypercalcemic disorders, the elevated extracellular Ca^{2+} concentration, acting through the CaR, inhibits reabsorption of Ca^{2+}, thus causing hypercalciuria. In contrast, patients with FBHH have normal or even low urinary calcium excretion, demonstrating their renal resistance to the hypercalcemia (19). Similarly, patients with FBHH do not show the decrease in urinary concentrating ability which is observed with other causes of hypercalcemia.

Almost all of the families studied to date show different mutations in the CaR. Most of the mutations detected involve the large amino-terminal, extracellular domain and thus may interfere with ligand binding (20). However, missense mutations have also been reported in and near transmembrane domains (20). Nonsense mutations, deletions, and insertions can also cause FBHH (20–23).

When expressed in a human embryonic kidney cell line, HEK 293, different mutations associated with FBHH show differing effects (24,25). Some of the mutations (including both missense and frame shift mutations) completely abolish signal transduction. Mutations in the amino-terminal, extracellular domain tend to increase the EC_{50} for extracellular

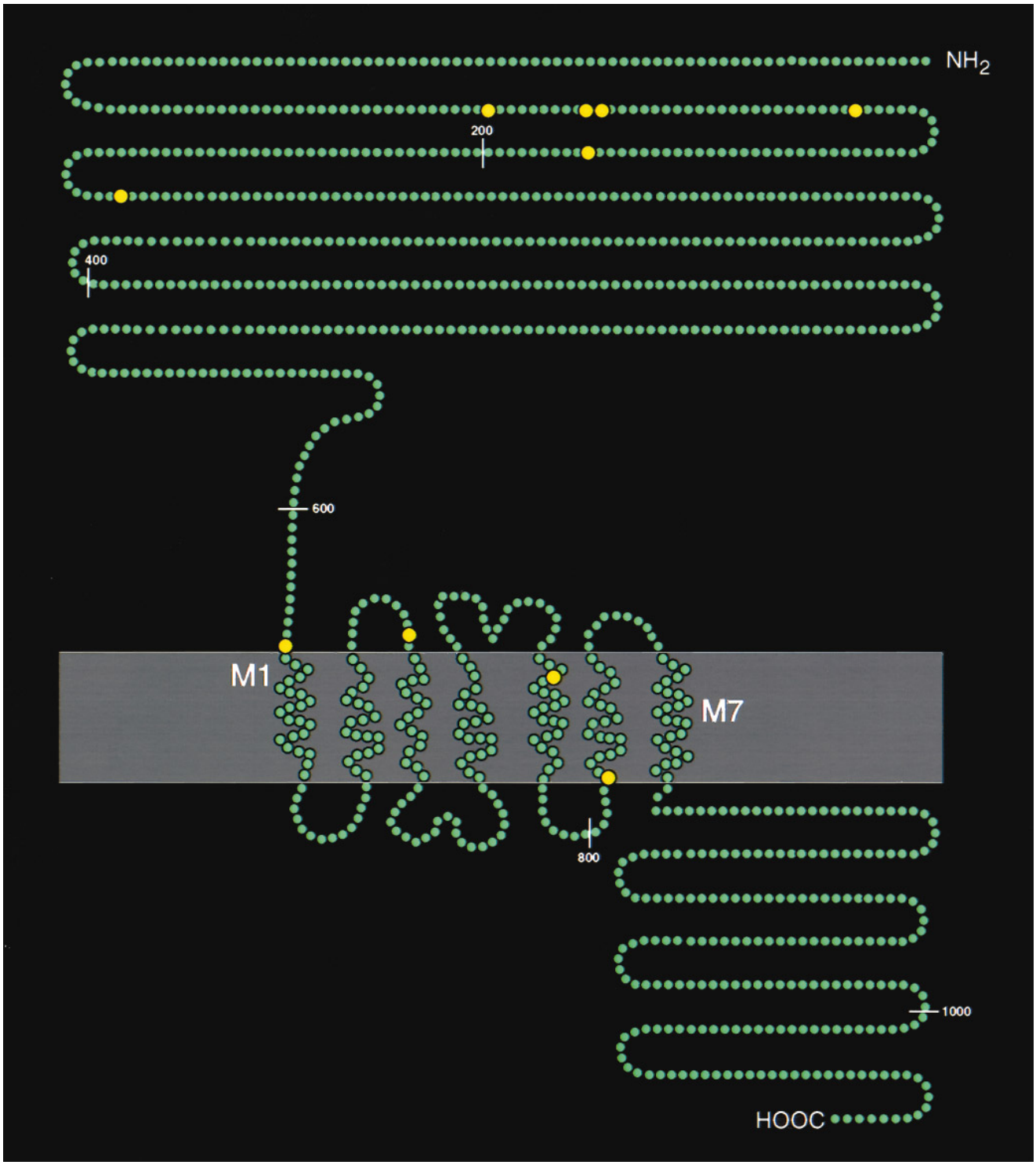

FIG. 1. Schematic representation of the human CaR. The gray area represents the cell membrane, with the extracellular space at the top. Circles represent amino acids. The amino acid substitutions identified in hypoparathyroidism are illustrated in yellow. All other amino acids are illustrated in green. M1 and M7 indicate the first and seventh transmembrane domains, respectively.

Ca^{2+}, suggesting a decrease in the affinity of the receptor for Ca^{2+} (24). One of the mutations in this region and another involving the third cytoplasmic loop affected both the EC_{50} and the maximal signal transduction capacity (24). Furthermore, one mutation in the sixth transmembrane domain increased the EC_{50} of the receptor for Ca^{2+} (25). Thus, the simple model that extracellular domain mutations affect ligand binding while transmembrane/cytoplasmic mutations affect signal transduction capacity cannot explain all of the data.

Homozygous (or compound heterozygous) inactivating mutations in the CaR cause neonatal severe hyperparathyroidism (NSHPT). Thus, NSHPT typically occurs when both parents of the affected infant have FBHH. Cells of affected patients have no normally functioning CaR and consequently are highly resistant to extracellular Ca^{2+}. In parathyroid, the normal restraining influence of the CaR is absent. As a result, PTH secretion is greatly increased, and interestingly the parathyroid glands also hypertrophy. This disorder presents shortly after birth with hypotonia, decreased skeletal mineralization, failure to thrive, and constipation. Serum Ca^{2+} levels are very high, often ranging from 14 to 20 mg/dl, and serum levels of PTH are inappropriately very elevated. NSHPT is usually fatal unless total parathyroidectomy is performed emergently (26,27).

HYPOPARATHYROIDISM

Hypoparathyroidism is caused by inadequate secretion of PTH which leads to hypocalcemia and hyperphosphatemia. Hypoparathyroidism may occur as part of a syndrome of congenital anomalies such as Di George, Kenny-Caffey, and Barakat syndromes or may result from immune destruction of the parathyroid glands as part of a polyglandular autoimmune disorder (28). Hypoparathyroidism also occurs in isolation. Isolated hypoparathyroidism can be inherited as an autosomal dominant, autosomal recessive, or X-linked trait, or it can occur sporadically (29–31).

Mutations in the preproPTH gene can cause isolated hypoparathyroidism. However, such mutations have only been detected in two families, one family with autosomal dominant hypoparathyroidism (30) and one family with autosomal recessive hypoparathyroidism (31). In most families studied, the hypoparathyroidism does not cosegregate with polymorphisms in the preproPTH gene (32). Thus, in most cases, familial hypoparathyroidism is probably not due to mutations in this gene.

CaR HYPERFUNCTION: CLINICAL MANIFESTATIONS

Hypoparathyroidism can also be caused by activating mutations in the CaR. The first evidence supporting this association was provided by Finegold *et al.* who described a family in which autosomal dominant, isolated hypoparathryoidism that cosegregated with markers on chromosome 3q, the region that contains the CaR gene (33). The authors suggested that an activating mutation in this gene might cause the disease by inhibiting PTH secretion at inappropriately low serum Ca^{2+} concentrations. This prediction was confirmed by Pollack *et al.* who identified a mutation in the CaR gene in a family with mild autosomal dominant hypocalcemia (34). The missense mutation, which involved the aminoterminal, extracellular domain of the CaR, showed increased signal transduction activity compared to the wild-type receptor when expressed in *Xenopus laevis* ooytes.

Mutations of the CaR have now been found in at least eight families with autosomal dominant hypoparathyroidism (34–38). These mutations can cause not only mild, asymptomatic hypocalcemia but also clinically significant hypoparathyroidism (36,37). In the most severe cases, patients present in infancy with hypocalcemic seizures (36).

Patients with activating mutations in the CaR are particularly prone to hypercalciuria (35,36). This tendency presumably reflects CaR activation in the medullary thick ascending limb which inhibits reabsorption of Ca^{2+} from the tubular lumen. When these patients are untreated and thus at their altered setpoint for Ca^{2+}, the urinary Ca^{2+} excretion is often within the normal range. However, when they are treated with oral vitamin D analogs and calcium, they typically develop hypercalciuria, even when the serum Ca^{2+} is still near the lower limit of normal (35). This greater tendency to hypercalciuria may predispose these patients to nephrocalcinosis and renal insufficiency (36). For this reason it has been recommended that patients with mild hypocalcemia not receive treatment (35) and that patients with more severe disease should receive therapy tailored to minimize the risk of hypercalciuria.

CaR HYPERFUNCTION: INHERITANCE

Hypoparathyroidism due to activating CaR mutations is inherited in an autosomal dominant fashion. Thus, as with many other genes, inactivating mutations in the CaR gene show an essentially recessive phenotype (in terms of clinical manifestations) whereas activating mutations show a dominant phenotype.

Hypoparathyroidism caused by activating CaR mutations also occurs sporadically. Five sporadic cases have been reported to date (36,39,40). None of the parents of the affected individuals carried the mutation, and, thus, the mutations must have arisen *de novo*. In one of these cases, the affected patient presented at 18 years of age. Thus, even patients presenting in adulthood with mild hypoparathyroidism who have no affected family members may still have a genetic etiology (39).

NOMENCLATURE

Various names have been used to describe the syndrome caused by activating CaR mutations. Some authors describe it as one form of autosomal dominant hypoparathryoidism. Other authors avoid the term hypoparathyroidism in order to emphasize the unique features of the syndrome, especially the altered setpoint for PTH secretion and the particular tendency to hypercalciuria (35). Thus, the term hypocalcemic hypercalciuria has been suggested (35), but may be confused with hypocalciuric hypercalcemia. We have suggested the term, CaR hyperfunction which emphasizes the molecular etiology.

ACTIVATING CaR MUTATIONS: MOLECULAR MECHANISMS

CaR mutations associated with hypoparathyroidism have been identified primarily in the amino-terminal, extracellular domain of the receptor. However, five of these mutations have been identified in other regions: the fifth transmembrane domain, the sixth transmembrane domain, the seventh transmembrane domain, and the first extracellular loop (Fig. 1).

To test the hypothesis that CaR mutations associated with hypoparathyroidism are activating and to elucidate the mechanism of activation, the function of several mutant receptors has been studied in cultured mammalian cells. Pearce *et al.* studied three point mutations which had been identified in kindreds with autosomal dominant hypocalcemia (25). Mutant receptors were expressed in HEK 293 cells and exposed to varying extracellular Ca^{2+} concentrations. Cells expressing the mutant receptors showed an increased response, assessed by intracellular Ca^{2+} measurement. All three mutations showed a left shift of their concentration–response curve. However, when the inositol phosphate (IP) response was assessed, these mutations produced an unusual biphasic concentration–response curve, with maximal response occurring at 1.5–2.0 mM Ca^{2+}, followed by a reduced response at higher Ca^{2+} concentrations (35).

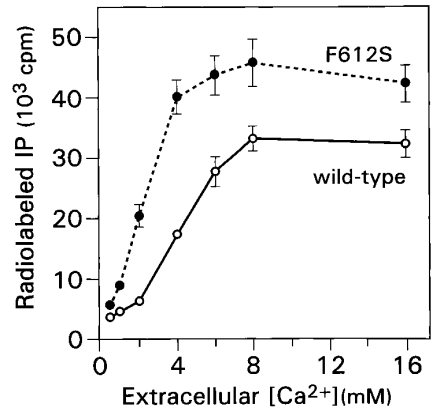

FIG. 2. Inositol phosphate (IP) response to extracellular Ca^{2+} in transfected HEK-293 cells. Cells were transfected with WT or mutant (F612S) CaR cDNA expression plasmids. After 48 h, cells were labeled with [myo-^{3}H] inositol for 16–24 h and then exposed to graded extracellular concentrations of Ca^{2+} for 30 min. IPs were isolated and counted by liquid scintillation. Nine separate transfections were performed at each Ca^{2+} concentration. (Reproduced by permission, from Ref. (37) International Pediatric Research Foundation, Inc.).

We recently studied four missense CaR mutations identified in three patients with sporadic hypoparathyroidism and in a family with autosomal dominant hypoparathyroidism (37,39). HEK 293 transiently transfected with WT and mutant receptors were exposed to graded Ca^{2+} concentratons from 0.5 to 16 mM, and IP accumulation was measured. Three of these mutations produced a concomitant increase in receptor sensitivity to Ca^{2+} and maximal IP response to high Ca^{2+} concentrations. The results for one of these mutations (F612S) is shown in Fig. 2. The mutant receptor curve was left shifted, with a significantly decreased EC_{50} compared to the WT receptor ($P < 0.001$). This finding suggests an increased affinity of the mutant receptor for Ca^{2+}. F612S also produced a greater maximal response at

high Ca^{2+} concentration than did the WT receptor ($P < 0.001$). The number of receptors on the surface of the cells transfected with the mutant receptor was not increased; thus the increased maximal activity was probably due to increased activity per receptor. A similar pattern of activation, with increased sensitivity and increased maximal signal transduction has been observed for other G-protein-coupled receptors, such as β2-adrenergic receptor, α1B-adrenergic receptor, and the platelet-activating factor receptor (41,42). This dual effect may be explained by the allosteric ternary complex model proposed by Lefkowitz *et al*. for G-protein-coupled receptors (41). In this model, receptors are assumed to exist in equilibrium between an inactive state and an active state. Agonists are presumed to have a higher affinity for the receptor in the active conformation. Agonist binding shifts the equilibrium toward the active state. We hypothesize that the mutations in the CaR shift the equlibrium toward the active conformation (37,39). Because the active confirmation is proposed to have a higher affinity for ligand, the observed decrease in EC_{50} would be explained. At high Ca^{2+} concentrations, these mutations would increase the proportion of receptors in the active state, thus causing the observed increase in maximal signal transduction.

SUMMARY AND CONCLUSIONS

The Ca^{2+}-sensing receptor is a member of the seven-transmembrane domain, G-protein-coupled receptor superfamily. It plays a critical role in Ca^{2+} homeostasis by allowing the parathyroid, kidney, and other tissues to sense the extracellular Ca^{2+} concentration. In the parathyroid gland, activation of the CaR by extracellular Ca^{2+} negatively regulates the secretion of parathyroid hormone. In the kidney, receptor activation decreases renal reabsorption of Ca^{2+}.

Inactivating mutations in the CaR gene cause hypercalcemia with relative hypocalciuria whereas activating mutations cause hypocalcemia with relative hypercalciuria. This hypocalcemia can range from mild and asymptomatic to life-threatening. Most of the activating mutations reported to date occur in the extracellular domain of the receptor. When expressed in cultured cells, mutant receptors can show both increased receptor sensitivity to Ca^{2+} and increased maximal signal transduction capacity.

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