The calcium-sensing receptor in hereditary disorders of calcium homeostasis

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Summary

Inherited diseases of calcium homeostasis were described more than 30 years ago. Consecutively, the discovery of the calcium receptor (CaR) more than a decade ago, followed by the demonstration that familial diseases of hyper- and hypocalcemia, in some cases were caused by functionally important mutations in the CaR, highlighted the receptor's pivotal role in the maintenance of systemic calcium homeostasis. Mutations that change the receptor's affinity toward calcium induce illnesses of calcium homeostasis by changing the set-point for calcium-regulated PTH release as well as the renal handling of calcium. Gain-of-function mutations cause a form of autosomal dominant hypoparathyroidism (ADH); this rare disease exhibits a clinical spectrum from mild to much more severe and symptomatic hypocalcemia with relative or absolute hypercalciuria. Loss-of-function mutations produce PTH-dependent forms of hypercalcemia called familial hypocalciuric hypercalcemia (FHH) in their heterozygous state (one mutated allele) and neonatal severe primary hyperparathyroidism (NSPHT) in their homozygous or compound heterozygous forms (two mutated alleles). FHH is thought to occur with about 1% of the prevalence of primary hyperparathyroidism and is often a benign state of hypercalcemia with relatively low urinary calcium output. NSPHT, on the other hand, is a considerably more rare but severe form of hypercalcemia presenting in most cases during the first 6 months of life. Affected children become symptomatic early on in life with failure to thrive and severe hyperparathyroid bone disease and may even die if left untreated. Until recently treatment of this condition involved total or subtotal parathyroidectomy. Similar to the inherited diseases with mutations in the calcium-sensing receptor, autoimmune diseases with antibodies targeting the CaR have been recognized. This review will focus on the calcium-sensing receptor, including its molecular physiology, as well as its role in various diseases of calcium homeostasis that illuminate the receptor's role not only in pathophysiology but also in normal physiology. Lastly, we shall describe the CaR as a drug target with proven and potential applications.

KEY WORDS: seven transmembrane receptor, calcium homeostasis, calcimimetic, calcilytic, familial hypocalciuric hypercalcemia, autosomal dominant hypoparathyroidism, acquired hypoparathyroidism, osteoporosis, hyperparathyroidism.

Introduction

The calcium-sensing receptor (CaR) is a widely-expressed, seven transmembrane receptor (7TM receptor – also termed G protein-coupled receptor) (1). Its main function lies in the chief cells of the parathyroid gland. Here the CaR regulates the synthesis and secretion of parathyroid hormone (PTH). PTH is the most important hormone in the acute regulation of the extracellular ionic calcium concentration (Ca^{2+}_o). Ca^{2+}_o is maintained within a narrow range (1.1-1.3 mM). Both very high and very low levels of Ca^{2+}_o are dangerous and can be life-threatening. Minute changes in Ca^{2+}_o, in the range of a few percent, lead to immediate physiologic responses, including altered PTH secretion, that restore the level of Ca^{2+}_o to normal. Rapid changes in Ca^{2+}_o are more dangerous than slowly developing ones; therefore, the rapidity of the PTH response is essential. The CaR, cloned more than a decade ago, is a key player in this delicate process of calcium homeostasis (1). The CaR acts like a thermostat, but instead of measuring changes in temperature, it measures alterations in the level of Ca^{2+}_o, thereby functioning as a "calciostat" and informing the chief cells of the parathyroid glands of the exact level of Ca^{2+}_o. The parathyroid glands respond to changes in Ca^{2+}_o with CaR-mediated alterations in PTH secretion.

The calcium-sensing receptor was cloned using the widely exploited expression-cloning technique (1). Subsequent analysis of the receptor's structure and alignment of it with protein databases have situated the CaR within family C of the superfamily of seven transmembrane receptors. Other important receptors within this family are the metabotropic receptors for glutamate (mGluRs) and gamma-aminobutyric acid (GABA), as well as receptors for pheromones and odorants (in fish). And recently an orphan receptor, GPRC6A, has been found to resemble the CaR in several of its pharmacological properties (2). This receptor, like the CaR, is sensitive towards certain L-amino acids, although unlike the CaR, which senses aromatic amino acids most effectively, GPRC6A is a sensor of basic amino acids (3). It was subsequently shown that this receptor is also sensitive towards extracellular calcium (albeit at high concentrations) and calcimimetics (4), allosteric activators of the CaR, which may implicate GPRC6A as a second calcium-sensing receptor (CaSR2).

The physiological importance of the calcium-sensing receptor in humans was proven by the discovery of diseases caused by mutations in the receptor that lead to either loss-of-function or gain-of-function (5). Heterozygous (e.g., the mutation is present in only one allele and the other allele carries wild type CaR) gain-of-function mutations cause autosomal dominant hypoparathyroidism (ADH). Most patients have asymptomatic hypocalcemia with relative or absolute hypercalciuria. Heterozygous loss-of-function mutations give rise to familial hypocalciuric hypercalcemia – FHH, also termed familial be-

nign hypocalciuric hypercalcemia (FBHH) by some – in which most patients have an asymptomatic form of hypercalcemia with relative or absolute hypocalciuria. The homozygous or compound heterozygous variant of inactivating CaR mutations, on the other hand, produce neonatal severe primary hyperparathyroidism (NSHPT), a severe, sometimes deadly disease if left untreated. The mouse models of FHH (heterozygous inactivation of the CaR gene) and NSHPT (homozygous loss of the CaR) have phenotypes comparable to those of the respective human conditions, suggesting that the number of receptors on the cell surface is in some cases the determining factor for clinical expression of the disease. Thus one way in which the mutated receptors may exert their effects on calcium homeostasis is through a reduced level of expression of wild type receptors, as in the heterozygous knock out mice. Another way reflects the fact that the receptor normally functions as a dimer, such that heterodimerization of some mutant receptors with their respective wild type partner exerts a dominant negative action on the latter.

CaR expression is highest in the parathyroid glands, calcitonin-secreting C-cells of the thyroid gland, and kidney, but the CaR is also present in the two other organs involved in calcium homeostasis: gut and bone. This review will focus on the calcium-sensing receptor and its role in normal physiology as well as in disease states that illuminate not only pathophysiology but also normal calcium homeostasis as well.

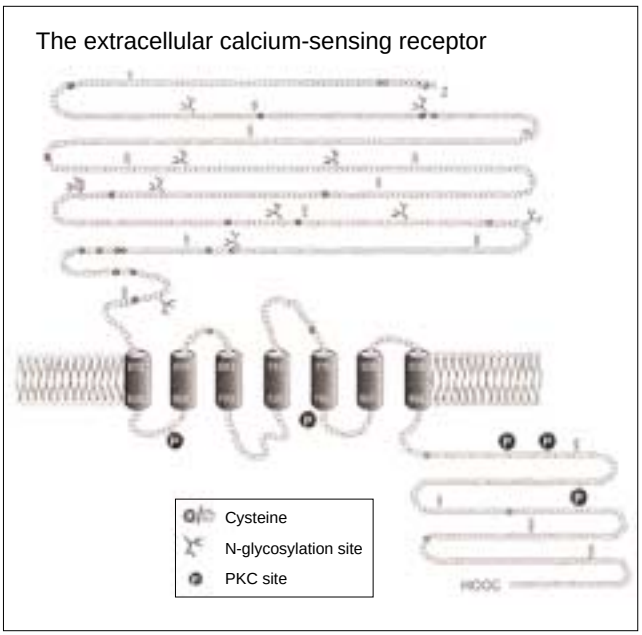

Figure 1 - Predicted topological features of the extracellular Ca^{2+}-sensing receptor cloned from human parathyroid glands in a schematic drawing. The extracellular domain contains approximately 600 amino acids, and the transmembrane domain and intracellular domain each contain approximately 200 amino acids. Also illustrated are the protein kinases C (PKC) phosphorylation sites, N-glycolysation sites and conserved cysteines.

Biochemical and physiological features of the CaR

To provide the reader of this review with sufficient background to understand how mutations in the CaR cause disease, a brief introduction to the structure of the receptor and its downstream signaling pathways is provided. Then, in this section of the review, we review the functions that the CaR plays in normal calcium homeostasis. Lastly we will provide an update on the topic of naturally occurring and pharmacologic ligands to the receptor.

Structure and signal apparatus

The CaR was isolated by utilizing the expression cloning technique in *Xenopus laevis* oocytes to secreen a bovine parathyroid cDNA library. A 5.3-kb clone was isolated in this manner that showed, when expressed in the oocytes, the same pharmacological properties as the Ca^{2+}_o-sensing apparatus in dispersed bovine parathyroid cells (1). Soon after, standard use of nucleic acid hybridization permitted the cloning of the CaR in humans (6, 7), rats (8), mice (9), and rabbits (10). The nucleic acid sequences of the receptors are at least 85% identical to that of the original bovine parathyroid CaR. The amino acid sequences exhibit even greater homology [>90% identity using http://www.ncbi.nlm.nih.gov/BLAST/]. Thus functionally important mutations in the receptor have most likely been discarded through evolution.

The CaR belongs to family C II of the superfamily of seven transmembrane (7TM) receptors, also termed G protein-coupled receptors (11). 7TM receptors are the largest group of cell surface membrane receptors. They are very important in clinical medicine, as the 7TM receptors are the targets of about 50% of currently available medications. The human CaR is 1078 amino acid residues long. The receptor has three structural domains, like all 7TM receptors (Fig. 1): The CaR has an unusually large extracellular domain (ECD), the hydrophobic N-terminal end of 612 residues; a transmembrane domain (TMD) of 250 amino acids containing the 7 membrane spanning helices, and an intracellular domain (ICD) 216 amino acids long, which is the hydrophilic C-terminal end of the protein. The receptor is modified by N-linked glycosylation; this is important for the cell membrane expression of the receptor. On the cell membrane the CaR is primarily in a dimeric form, and the two receptor monomers are linked by covalent disulfide bonds involving two cysteine residues in each monomer (cys129 and cys131) (12-14). The location(s) where Ca^{2+}_o binds to the CaR is yet not fully clear, but it has recently been suggested that one potential binding pocket resides within the cleft between the two lobes in each receptor monomer (15). Most likely the ECD of each CaR monomer contains more than one binding sites for Ca^{2+}_o, because the Hill coefficient for the activation of the CaR by calcium is 3-4, suggesting positively cooperative interactions among at least this number of binding sites within the dimeric CaR. The TMD seems also to be involved in Ca^{2+}_o-sensing, as a mutated CaR expressed without the ECD also responds to Ca^{2+}_o and other polyvalent cations (16, 17). The receptor carries two protein kinase A (PKA) and five protein kinase C (PKC) phosphorylation sites (Fig. 1) (18). The PKC sites participate in a negative feedback mechanism, as phosphorylation of the PKC sites inhibits activation of phospholipase C (PLC) by the CaR, a major downstream mediator of the receptor's biological responses. PLC is upstream of CaR-mediated activation of PKC. Before the CaR was cloned, it was recognized that activation of parathyroid chief cells with high calcium inhibited hormone-dependent cAMP production and induced PLC activity and thereby IP_3 production, suggesting that the CaR activated $G\alpha_q$ as well as $G\alpha_{i/o}$ (19, 20). The ICD binds the scaffolding proteins, filamin-A and caveolin-1 (the CaR is immunoprecipitated by anti-caveolin antibodies, but direct binding of caveolin-1 to the CaR has not been shown) (21, 22), both of which also bind to signaling partners activated by the CaR. Binding to filamin-A was recently report-

ed to induce protection of the CaR from degradation (23); this may explain why some authors have found that the CaR does not internalize secondary to ligand binding.

A plethora of intracellular signaling pathways of the CaR have been discovered utilizing a heterologous system of human embryonic kidney (HEK) cells with or without stably transfected CaR (HEK-CaR). Most of these intracellular signaling pathways have also been shown to be active in CaR-mediated signaling in other cells naturally expressing the CaR. In parathyroid cells and HEK-CaR cells, the CaR activates phospholipases (PL) A_2, C, and D (24). PLC produces IP_3, which in turn activates the IP_3 receptor in the membrane of the endoplasmic reticulum (ER), resulting in release of calcium from its internal stores within the ER; the resultant release of calcium into the cytosol produces spikes in the cytosolic free calcium concentration (Ca^{2+}_i). An initial step in the production of polyphosphoinositides is the activation of phosphoinositol 4-kinase, which converts PI to PI-4P. The CaR activates phosphoinositol 4-kinase through $G\alpha_q$ in parallel with activation of PLC in HEK-CaR cells (25). Another important class of intracellular signaling pathways linked to the CaR is the mitogen-activated protein kinases (MAPKs). MAPKs are activated by phosphorylation by their respective upstream kinases. MAP kinases are important intracellular signaling pathways that often act through changes in gene transcription, e.g., in the regulation of the cell cycle. But MAPKs can also regulate events close to the cell membrane, such as secretion of peptides and activity of potassium channels. We have shown that the CaR in HEK-CaR cells and parathyroid cells activates MAPKs (26). Soon after, Handlogten et al. also showed that the CaR promotes MAPK phosphorylation in HEK-CaR cells; as a control, they used HEK cells stably transfected with a dominant-negative CaR (Arg796Trp) (27). They also showed that the CaR activates PLA_2 through $G\alpha_q$, PLC, calmodulin, and calmodulin-dependent kinase, but not through Ga_s or MAPK in HEK-CaR cells. The reasons underlying the differences in the results of these two studies regarding the role of MAPK in PLA_2 activation remains to be clarified. In cells expressing the CaR at a lower level than parathyroid cells and HEK-CaR cells, such as testicular cancer cells, MAPK and phosphatidylinositol 3-kinase (PI-3kinase), a classical prosurvival pathway, have been found to be activated by the CaR (28, 29).

Agonists of the calcium-sensing receptor

The CaR is a promiscuous receptor with many ligands. CaR agonists are classified as type I or type II. Type I are direct agonists, whereas type II are allosteric modulators, i.e., they require the presence of calcium to activate the CaR; the type II modulators left-shift the calcium dose-response curve. The type I ligands are all polycations, both inorganic and organic. Inorganic di- and trivalent cations have been tested for their potency on the CaR, and they rank as follows: Gd^{3+}ŽLa^{3+}>>Ca^{2+}=Ba^{2+}>Sr^{2+}>Mg^{2+} (6, 30). The best known type I organic polycationic CaR agonists are neomycin, spermine, and amyloid β-peptides (31-33). Neomycin and gadolinium are often used to show that an effect of Ca^{2+}_o is likely to be mediated through the CaR, although they are by no means specific for the CaR. Slight changes in Ca^{2+}_o (50-100 micromolar) regulate CaR activity, but the affinity of the CaR for calcium is far lower than that of other GPCRs for their ligands. The low affinity of the CaR, when taken within the context of the millimolar levels of calcium within bodily fluids as well as the steepness of the relationship between Ca^{2+}_o and CaR activity, make the CaR a perfect "calciostat" for informing the cell of the exact concentration of Ca^{2+}_o within the immediate vicinity of its plasma membrane. The Hill coefficient, a measure of how well the receptor responds to small changes in agonist concentration, is 3 to 4 in HEK-CaR cells (34), as noted above. In dispersed parathyroid cells *in vitro*, the CaR is even more sensitive: PTH secretion is maximal at 0.75 mM and minimal just below 2 mM ionized Ca^{2+}_o (35). HEK-CaR cells as well as other cells that, in general, express the CaR at lower levels than the parathyroid chief cells, such as primary testis leydig cancer cells, have higher EC_{50} values (~3-4 mM) (34, 36).

As mentioned earlier, type II agonists are allosteric modulators of the CaR, i.e., they potentiate the effect of Ca^{2+}_o on the CaR, and comprise two groups: small molecule drugs and amino acids. Drugs allosterically activating the CaR are termed "calcimimetics" (37). NPS R-467, R-568 and AMG 073 have all been used in experimental studies and clinical trials and lately in the treatment of uremic secondary hyperparathyroidism (38-42). AMG 073 is the drug of choice in the clinic, because NPS R-467 and NPS R-568 are degraded by a cytochrome P-450 enzyme, CYP2D6 (Amgen, unpublished data). Five to seven percent of the population expresses CYP2D6 as an isoenzyme with reduced enzymatic activity, thereby resulting in higher blood level of these drugs in this segment of the population. The calcimimetic binds to the TMD and enhances the apparent affinity of the CaR for calcium. Some L-amino acids have also been found to be type II agonists of the CaR, whereas the respective D-amino acids are several-fold less potent in stimulating the receptor (43, 44). This CaR's capacity to respond to both extracellular calcium and L-amino acids may enable it to act as a receptor that senses nutrients in the gut, for example. Most likely, the amino acids bind to the CaR in the ECD, utilizing a binding pocket homologous to those binding glutamate and GABA in the mGluRs and $GABA_B$ receptors, respectively (45). This may be of pharmacological relevance, as the calcimimetic NPS R-467 and L-phenylalanine have synergistic effects in activating the CaR (46). Lastly, another type of pharmacological agent acting on the CaR is the calcilytics, which antagonize the action of Ca^{2+}_o on the receptor, which may be of use in osteoporosis treatment as it stimulates endogenous PTH secretion.

Physiology

For the body it is crucial that the level of Ca^{2+}_o is maintained within a narrow range (1.1-1.3 mM). Both very high and very low levels of Ca^{2+}_o are dangerous and can be life-threatening. Even minute changes in Ca^{2+}_o, in the range of a few percent, lead to immediate physiologic responses, including altered PTH secretion, that restore Ca^{2+}_o to its normal level. Rapid changes in Ca^{2+}_o are more dangerous than slowly developing ones; therefore the rapidity of the PTH response is essential.

Parathyroid hormone (PTH), calcitonin, and $1,25(OH)_2D_3$ are the three major Ca^{2+}_o-regulating hormones, the so-called "calciotropic hormones" (47, 48). As mentioned above, there is a crucial inverse relationship between Ca^{2+}_o and the calcium-elevating hormone, PTH – a relationship mediated by the CaR (Fig. 2) (1, 35). There is a positive relationship between Ca^{2+}_o and the release of calcitonin (CT), a Ca^{2+}_o-lowering hormone; this feedback loop of calcium on CT secretion is also mediated by the CaR (49). The rapidity of the secretory responses of PTH and CT to perturbations in extracellular calcium restores Ca^{2+}_o to its normal level within minutes to hours. The CaR is now known to be expressed not only in the calciotropic hormone-secreting organs (parathyroid gland and C-cells of the thyroid glands), but also in tissues that regulate the extracellular calcium concentration by translocating calcium ions into or out of the bodily fluids: kidney and, at lower levels, bone and intestinal cells. The presence of the CaR in these tissues involved in Ca^{2+}_o homeostasis enables Ca^{2+}_o to

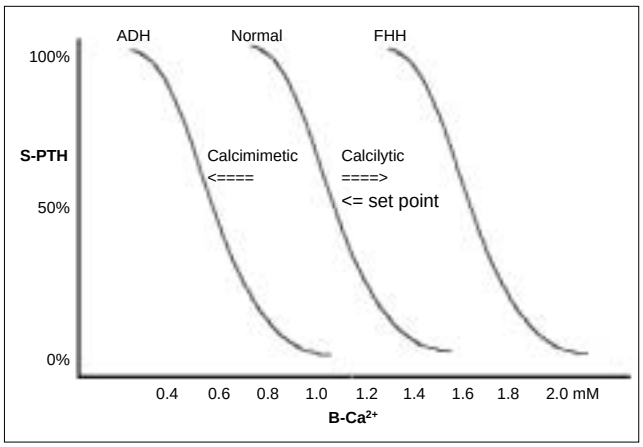

Figure 2 - Sigmoidal relationship between the blood Ca^{2+} and serum PTH in the normal state, in autosomal dominant hypoparathyroidism (ADH), and in familial hypocalciuric hypercalcemia (FHH) as well as the therapeutic effect of the calcimimetic (shifting set-point to the left) and the calcilytic (shifting set-point to the right). Set-point is defined as the calcium concentration causing half-maximal inhibition of secretion.

act, in effect, as another Ca^{2+}_o-regulating hormone, e.g., as a "first messenger". Increases in the extracellular ionized calcium concentration stimulate the CaR to lower Ca^{2+}_o by promoting CT secretion, increasing urinary calcium excretion and inhibiting PTH secretion. In the remaining parts of this section, we will briefly discuss the CaR's known and potential roles in three out of the four main organs involved in calcium homeostasis: parathyroid gland, kidney and bone. For a review of the localization and possible roles of the CaR along the gastrointestinal tract, please see review by Hebert et al., 2004 (50).

Parathyroid (PT) glands

The most important function of the CaR lies in the receptor's inhibitory effects on PTH secretion. The CaR regulates the release of PTH, a key calcium-elevating hormone, and the steep inverse sigmoidal relationship between Ca^{2+}_o and PTH was described long before the CaR was cloned. Studies of Ca^{2+}_o-regulated PTH release in dispersed parathyroid cells are largely limited to studies carried out within a matter of hours of isolation of the cells, since these cells lose their expression of the CaR on the cell membrane in a time-dependent manner (51, 52). The CaR is highly expressed on the cell surface of the chief cells of the PT cells. Not much is known about the regulation of its expression on the cell surface, but interestingly in rats, the expression of the CaR in parathyroid and kidney was upregulated by $1,25(OH)_2$vitamin D, while Ca^{2+}_o was without effect (53). Another instance in which there is regulation of CaR expression is in sheep that have undergone burn injury, where there was upregulation of the parathyroid CaR in the burned but not the sham animals. The authors also saw a related decrease in set-point for calcium suppression of PTH secretion. This may contribute to post burn hypocalcemia and hypoparathyroidism (54) as well as to the hypocalcemia with inappropriately normal levels of PTH observed in patients severely ill with inflammatory states.
In bovine parathyroid cells, the CaR is situated on the cell membrane within caveolin-rich membrane domains (21). Caveolae are recognized as message centers where a variety of signaling molecules are concentrated. A report found that CaR activation, through the PKC pathway and, to a lesser extent, the PI-3K pathway, increases ERK1/2 activity in normal parathyroid cells (55); these signaling pathways seem to be involved in the modulation of PTH secretion by Ca^{2+}_o.

Kidney: expression, function and regulation of the CaR

The kidney plays key roles in calcium homeostasis. The CaR is widely expressed along nearly the entire nephron. The cellular localization and apparent function(s) of the CaR seem to depend upon the site of the nephron in which it is expressed (56). We will now briefly describe the location of the CaR as well as its functions in selected parts of the nephron. The expression of the CaR along the nephron has been assessed using *in situ* hybridization and reverse transcriptase-polymerase chain reaction of micro-dissected nephron segments (8, 57). Subsequently, the cellular and regional distribution of receptor protein was assessed by immunofluorescence microscopy (58). One overall point is that the polarity of the CaR varies along the nephron. In the proximal tubule the CaR is expressed on the apical surface of proximal tubular cells. In contrast, in the cells of the cortical thick ascending limb (TAL), the CaR is expressed in the basolateral membrane. Likewise basolateral staining for the receptor was also detected in medullary thick ascending limb, macula densa, and the distal convoluted tubule. In the cortical collecting duct, CaR immunostaining is present on some intercalated cells, and in the inner medullary collecting duct the receptor has predominantly an apical distribution.
There have been relatively few studies addressing the factors regulating the expression of the CaR in the kidney. A recent report showed that in rat kidney, C-cell and parathyroid *in vivo* and in a human proximal tubule cell line *in vitro*, CaR gene transcription increased approximately two-old at 8 and 12 h after treatment with $1,25(OH)_2$vitamin D_3 (59). This study characterized vitamin D-responsive elements in each of the two promoters that lie upstream of the CaR gene. Another research group found no effect of a low phosphate diet on CaR expression along the nephron (60). In contrast, Riccardi et al. showed *in vivo* in rats that CaR protein in the proximal tubule was downregulated by a low phosphate diet as well as by treatment with PTH. Therefore, CaR expression in the proximal tubule of the rat kidney is regulated by vitamin D and PTH and, perhaps, by dietary phosphate (61). The only other study on the regulation of the expression of the CaR along the nephron showed that the level of CaR protein in purified apical membrane endosomes isolated from inner medullary collecting ducts was reduced in rats made hypercalcemic by administration of vitamin D (62). The functions of the CaR along the nephron are in brief: 1) to diminish PTH's inhibitory effect on renal phosphate reabsorption in the proximal tubule (63); 2) to inhibit renal calcium excretion in the cortical thick ascending limb of the loop of Henle (64); and 3) to reduce urinary concentrating capacity in the inner medullary collecting duct (65). For an extended review, please see Tfelt-Hansen et al., 2005 (66).

Bone

It is well described that Ca^{2+}_o inhibits osteoclast formation and activity and increases osteoblastic activity. The first evidence of a G protein-coupled, cation-sensing mechanism in osteoblasts came shortly after the cloning of the CaR (67). Subsequently, some (68-74), but not all (75, 76) reports have found the CaR to be expressed in osteoblasts. A very interesting study showed that osteoblasts from CaR knock-out mice still had a promitogenic response to Ca^{2+}_o (77), indicating the presence of a calcium-sensing mechanism other than the CaR. This mechanism could potentially be represented by the newly cloned GPRC6C

(4). One possible mechanism, besides the new receptor GPRC6C, underlying some of the calcium-induced effects in osteoblasts is the calcium-binding intracellular protein, calcyclin (78). The CaR is also expressed in articular and hypertrophic chondrocytes. Using a type II CaR agonist in organ culture (fetal rat metatarsal bones) for bone growth, Wu et al. (79) showed that the CaR regulates growth plate chondrogenesis and stimulates longitudinal bone growth.
The CaR is expressed on some osteoclasts (69, 80, 81) and their precursors (82). In addition to lowering osteoclast formation and activity, high calcium has been shown to augment the apoptosis of osteoclasts (83). The calcimimetic AMG 073, a type II agonist of the CaR, however, produced none of the effects of calcium on osteoblast proliferation or osteoclast formation and resorption in one study (84). One possible mechanism for calcium-sensing in osteoclasts other than the CaR is a plasma membrane, ryanodine-like receptor that couples to elevations in intracellular calcium (85). Thus, while the CaR and other calcium-sensing mechanisms may play a role in bone, further studies are clearly needed to resolve the discrepancies in the studies to date.

Genetic changes leading to FHH, NSHPT, and ADH

The CaR has been found to be responsible for the majority of cases of FHH, NSHPT, and ADH, and around 200 mutations have been reported in patients with these disorders (CaR Database at website http://www.casrdb.mcgill.ca/). The mutations are mainly missense, but also splice-site, nonsense, deletion, and insertion mutations. The mutations are mainly found in the extracellular domain and are found throughout exons 2 to 7 and in introns 2 and 4 of the CaR gene (5, 86-89).

Familial hypocalciuric hypercalcemia (FHH) [OMIM # 145980 (90)]

Clinical aspect of FHH

FHH is a benign state of hypercalcemia. The diagnosis of FHH is made in a patient with a family history of mild to moderate hypercalcemia averaging approximately 2.75 mM (total calcium), with an inappropriately low rate of urinary calcium excretion. While benign and asymptomatic hypercalcemia is typical of most FHH families, two families have been found to have calcium concentrations that average 3.13 and 3.35 mM, causing a neonatal severe hyperparathyroid-like state in some affected infants (34, 91). Since the condition is benign in most cases, patients with FHH are often undiagnosed until a routine blood sample shows an unexpectedly high serum calcium level or family screening is done due to the birth of a child with NSHPT (92). Patients with FHH usually have normal PTH levels, despite the hypercalcemia, although in some cases the PTH levels are elevated (86, 93).
The hypercalcemia in FHH, in the setting of a normal PTH that is inappropriately high compared to the serum calcium concentration, reflects a new, right-shifted set-point for Ca^{2+}_o-regulated PTH secretion (94, 95) (Fig. 2). A key finding is the relative hypocalciuria despite the hypercalcemia, which represents “resistance“ of the kidney to the normally hypercalciuric action of hypercalcemia and is analogous to the resistance of PTH secretion to the suppressive effect of high calcium in this condition. Interestingly, treatment with a loop diuretic enhances renal calcium excretion in hypoparathyroid patients with FHH (96). This finding points towards an important role of the TAL – the site of action of this class of diuretics – in the abnormal renal handling of calcium in FHH. The best distinction between FHH and other forms of hypercalcemia is often made by determining the ratio of calcium clearance to creatinine clearance (Ca/Cr). A value below 0.01 is found in about 80% of cases of FHH, while a similar proportion of cases of primary hyperparathyroidism caused by hyperplasia or parathyroid adenoma have values higher than this (97). Another clinical hallmark is the ability of patients with FHH to concentrate their urine normally, in contrast to patients with primary hyperparathyroidism, in whom maximal urinary concentration in response to dehydration is modestly diminished (98), although this finding is not used diagnostically. Patients with FHH typically have serum magnesium levels that are in the upper part of the normal range or mildly elevated. Lastly, while distinguishing FHH from primary hyperparathyroidism can in many cases be straightforward, a recent study investigating the genetic background of familial isolated hyperparathyroidism found that four of 22 unrelated probands had an inactivating mutation in the CaR, while five had mutations in the multiple endocrine neoplasia 1 gene (99). Thus the clinician should keep FHH in mind as an important and underdiagnosed cause of isolated familial hyperparathyroidism (99, 100), Furthermore this study may implicate a new less stringent perception of the range of phenotypes that may be encountered in patients with functional significant mutations in the CaR.
The clinical presentation of FHH, however, is not always straightforward. A family has been described that was serendipitously found to have hypercalcemia caused by an inactivating FHH mutation, and in whom hypercalciuria or even renal stone formation was found in some family members (86). Subtotal parathyroidectomy appeared to provide long-term remission of the hypercalcemia and hypercalciuria in this family, indicating that parathyroid surgery may be appropriate in an occasional FHH family. In two recent papers, single and multiple parathyroid adenomas were found in several patients with FHH (86, 101). Some authors have suggested that a histological finding of parathyroid lipohyperplasia, which has been found in some cases of FHH subjected to parathyroidectomy, is indicative of inactivating mutations of CaR (102, 103).
Thus overall FHH is an asymptomatic form of hypercalcemia, but in rare cases it may present as more severe hypercalcemia. Since the condition is generally so benign and, except in rare cases (see above), only total parathyroidectomy is curative, the great majority of these patients should be followed without intervention. In FHH patients with symptomatic hypercalcemia, the new calcimimetics might in theory be the optimal treatment; this approach, however, has not yet been reported.

Genetic aspects of FHH

Functionally important mutations in the CaR gene lead to changes in the amino acid sequence of the CaR protein. The altered amino acid sequence in FHH or NSHPT causes a loss-of-function of the CaR that shifts the set-point of Ca^{2+}_o-regulated PTH secretion to the right (Fig. 2). In 1972, Foley et al. (104) first characterized the hereditary condition later defined as familial hypocalciuric hypercalcemia (FHH). The predominant locus of the FHH disease gene was mapped by linkage analysis to the long arm of chromosome 3 (band q21-24) with the use of the hypercalcemic (90) phenotype in four large FHH families (105). FHH is not always linked to chromosome 3q. Interestingly, in two families, a clinical picture similar to FHH was linked to the short and long arms of chromosome 19 (106, 107), respectively, one called the Oklahoma variant (107). This may explain a minority of the ~30% of FHH cases in which no mutation in the CaR gene is identified. The remainder of these latter cases are presumed to harbor mutations in regulatory regions of the gene controlling its expression, but this has not been directly shown.

The first three different missense mutations in the CaR gene in the affected members of three unrelated families were reported during the same year as the cloning of the receptor in 1993 (108). Since the initial discovery of these mutations in the CaR gene, around 200 additional mutations have been characterized. Most are unique to individual families, although some unrelated kindreds have identical mutations (e.g., gly552arg) (5, 108). The majority of the mutations are missense and reside in the ECD or TMD of the receptor. An unusual type of mutation was discovered in a family with FHH, where the mutation was found in the acceptor splice site at position-1 of intron 2 of the CaR gene, causing a frame shift. The frame shift produced a truncated protein of 153 amino acids (87). The mRNA for the CaR was stable, but the truncated protein was never found on the cell membrane, in large part because of lacking any transmembrane domains. It is likely that the mutant CaR protein is degraded due to abnormal intracellular trafficking. Different mutations can lead to distinct phenotypes: a mutant receptor can exert a dominant-negative effect on the remaining normal CaR receptor, making the phenotype more severe (91). The normal CaR functions as a homodimer; accordingly, the heterodimerization of a non- or poorly functional mutant protein, which nevertheless reaches the cell surface, with the wild-type CaR can partially inactivate the heterodimeric receptor complex (14, 109, 110). A dominant-negative action reflects the specific properties of the mutant receptor protein on the cell membrane. With a mutation producing a dominant negative action, normally functioning, wild type CaR homodimers would comprise only about one-fourth of all the receptors (there would be a theoretically expected ratio of 1:2:1 of mutant homodimer, wild-type-mutant heterodimer, and wild-type homodimers, respectively). Inactivating mutations (e.g., deletions) in the CaR gene may not form heterodimers but simply reduce the number of normally functioning CaRs expressed on the cell surface, producing so-called haploinsufficiency, which results from a decrease in receptor expression/activity owing to the loss of one CaR allele. Two mutant CaRs with mutations at amino acid residues 11 and 13, respectively, have been characterized to have low cell surface expression in human embryonic kidney cells, most likely because the missense mutations were situated within the signal peptide of the ECD, which is required for proper targeting of the nascent receptor polypeptide to the lumen of the ER (111). One thing that should be kept in mind when evaluating the literature where different mutations have been characterized *in vitro* is that the cells used for the *in vitro* experiments are very different from the chief cells of the parathyroid glands or the kidney cells. Thus the experimental data should be seen as representing only an approximation of how the receptors function when expressed endogenously in the cells that are involved in maintaining calcium homeostasis.

Neonatal severe primary hyperparathyroidism (NSPHT) [OMIM 239200 (90)]

Clinical aspects of NSPHT

Neonatal severe primary hyperparathyroidism in most cases presents within the half year of life. The infant has severe, symptomatic hypercalcemia driven by PTH, as well as bony changes characteristic of hyperparathyroidism. Infants with NSHPT can display hypotonia, polyuria, dehydration, and failure to thrive. The hallmark of the disease is the associated hyperparathyroid bone disease. This bone disease often leads to multiple fractures. Fractures in the ribs may produce a “flail chest” syndrome that causes respiratory difficulties, since the affected infant will have decreased ability to expand its chest wall and thereby generate the negative intrathoracic pressure needed for inspiration. The mass of the parathyroid glands in NSHPT is often increased dramatically. Pathological investigation reveals chief cell hyperplasia. Biochemical analysis reveals hypercalcemia, hyperparathyroidism, and relative hypocalcuria (112). Levels of serum total calcium range from moderately elevated (e.g., 3-3.25 mM) to as high as 7.7 mM in the most severe cases (97). PTH levels are often observed to be 10-fold higher than the upper limit of normal. Early diagnosis is key, as untreated NSHPT can be a devastating neurodevelopmental disease that is lethal when not treated surgically (112). As mentioned above, patients with the most severe forms of NSHPT develop ribcage deformities, rachitic changes, skeletal undermineralization, and fractures (113, 114). There is a substantial mortality described in the literature; therefore, treatment for the disorder in its severe form is total parathyroidectomy. However, lately a wider clinical spectrum for the condition has become apparent; and the growing availability of genetic testing of the CaR gene has documented that some infants have milder hyperparathyroidism and a distinctively less severe clinical presentation and natural history. This latter form of the disease has been termed neonatal hyperparathyroidism (NHPT), and parathyroidectomy may not be the only treatment of choice in infants with this condition. Reports have documented that patients who would have been expected to have the clinical presentation of NHPT or NSHPT have survived with their condition undetected into adulthood, when they were found by routine screening to have PTH-dependent hypercalcemia. This may be due to heterozygous or homozygous FHH mutations, e.g., a 35-year-old woman with two copies of the missense mutation pro39ala from related parents. She was clinically asymptomatic, with a serum calcium concentration of 3.75 to 4.25 mM (115). Another patient with homozygous inactivating mutations was likewise not diagnosed until adulthood (102). In these patients the use of the calcimimetics in theory might represent a means of lowering the serum calcium concentration and determining whether the patient derived any symptomatic benefit, thereby potentially providing a long term, effective medical therapy.

Genetic aspects of NSHPT

NSHPT is an autosomal recessive disease, which means that the CaR gene from both the parents is mutated (e.g., homozygous FHH). Pollak et al. reported that in 11 families with FHH consanguineous unions produced four children with NSHPT (116). NSHPT, however, is quite uncommon among FHH families as a whole. In one case of NSHPT, two different mutations – one a mutation in exon 4 from the father and the second a mutation in exon 7 from the mother – were reported to cause the disease, e.g., owing to compound heterozygosity in the proband (117). Theoretically NSHPT can be the result either of a mutated allele of the CaR gene arising from two existing FHH kindreds or from a de novo mutational event, with or without the inheritance of a parental mutant allele (89). Furthermore a genetic study of a girl with phenotypic NSHPT and her family showed a single abnormal allele (present in exon 6, Gly552Arg) in her CaR gene, while her sister, who had the same genotype, had phenotypic FHH (110). Therefore, the factors contributing to the development of NSHPT are still only partly understood.

Autosomal dominant hypoparathyroidism (OMIM) [#601298 (90)]

Clinical aspects of ADH

Patients with this inherited form of hypocalcemia are often clini-

cally asymptomatic, similar to most patients with FHH. Some patients can have seizures, neuromuscular irritability and calcification of the basal ganglia. A biochemical hallmark of the disease is, of course, the mild to moderate hypocalcemia, with serum PTH levels that are inappropriately within the lower half of the normal range or frankly subnormal (e.g., rather than responding vigorously to the hypocalcemia, as would a normal parathyroid gland) (118). They often have relative or absolute hypercalciuria, with normal or elevated levels of urinary calcium excretion, respectively, despite the low serum calcium. Their renal calcium excretion has been found to be higher than that of patients with typical hypoparathyroidism in some studies, and, analogous to the diagnosis of FHH, a high urinary Ca/Cr ratio may be better than hypocalcemia alone for differentiating ADH from primary hypoparathyroidism. However, not all studies showed this difference in the level of urinary calcium excretion between idiopathic hypoparathyroidism and ADH (119, 120). Febrile episodes may unmask the disease; hence in some cases, and not infrequently during febrile episodes, patients with ADH may present with symptoms of hypocalcemia as well as seizures. It is important to prevent renal complications such as nephrocalcinosis, nephrolithiasis, and renal impairment. These renal complications are caused primarily by the hypercalciuria. The renal complications are usually seen in the setting where the clinician has tried to fully correct the low serum calcium with calcium and vitamin D treatment. Therapy with calcium supplements and vitamin D metabolites should be administered only to patients with symptomatic ADH, and the aim should be to increase the serum calcium concentration only to a level that renders the patient asymptomatic. Renal output of calcium should be monitored in treated patients to lower the risk of urinary complications (121). The development of the calcilytic could, in theory, provide an optimal treatment of symptomatic ADH patients. These agents would be expected to shift the set-point for calcium-regulated PTH secretion to the right and to reduce the renal excretion of calcium at any given serum calcium concentration.

Genetic aspects of ADH

ADH is a rare diagnosis, although in index cases it may represent a sizeable proportion of cases of idiopathic hypoparathyroidism, accounting for as many as a third of such cases (122). The mechanism behind the disease is the presence of an activating or gain-of-function mutation of the CaR gene that changes the set-point of Ca^{2+}_o-regulated PTH secretion to the left and lowers renal reabsorption of calcium (Fig. 2). Soon after the cloning of the CaR in 1994, Finegold et al. (123) showed that the disease is linked to a locus on chromosome 3 q13 – the same region on the chromosome that contains the gene for the CaR. In the same year, a heterozygous missense mutation, Glu127Ala, was identified as a cause of ADH in an unrelated family (118). Since these first reports, around 60 missense mutations have been characterized in families with ADH (CaR Database at website http://www.casrdb.mcgill.ca/). Most of these missense mutations are present within the ECD and TMD of the CaR. Expression of several of the known activating mutations of the CaR in HEK293 cells has shown that the mutations cause a clear left-shift in the activation of the CaR protein by Ca^{2+}_o and only rarely produce constitutive activation of the receptor (34, 121, 122, 124, 125). A recently described and novel type of activating mutation has been identified in France (122). The affected family members have a large deletion of 181 amino acids within the C-terminus of the CaR. Surprisingly, one family member was homozygous for the mutation but showed a phenotype similar to that of the heterozygous family members. These studies reveal that one mutated allele may be sufficient to induce a maximal shift in the set-point of Ca^{2+}_o-regulated PTH secretion, and that a second mutated allele does not change the biochemical properties of the receptor dimers any further (e.g., perhaps the mutant CaR monomers exert a “dominant positive” effect in mutant-wild type heterodimers vs. mutant homodimers). It should be emphasized, however, that the biochemical phenotype of a patient homozygous for an activating mutation has only been shown in this one report. Examination of the family in another novel study revealed a mutation that had occurred de novo in the proband. The heterozygous mutation was at codon 129 (TGC to AGC), causing a change of a cysteine to a serine (Cys129Ser) (126). This cysteine is involved in dimerization of the CaR, and these studies may suggest that this cysteine constrains the receptor in its inactive state.

Autoimmune diseases

Autoimmune acquired forms of hypo- and hypercalcemia analogous to ADH and FHH, respectively, have recently been described. While these conditions are rare, we will briefly review this literature, as the diseases are important to recognize in the differential diagnosis of the diseases of calcium homeostasis described above.

Anti-CaR antibodies and PTH-dependent hypercalcemia

Kifor et al. (127) identified autoantibodies to the CaR in four patients with other autoimmune conditions (e.g., Hashimoto’s thyroiditis and sprue), who had a clinical picture resembling that of FHH. In this study, the patients’ sera were found to stimulate PTH secretion and to inhibit high calcium-stimulated inositol phosphate accumulation and MAPK activation, most likely by antibody-mediated inhibition of the CaR, since the patients were shown to harbor anti-CaR antibodies. Further studies in larger cohorts of patients are needed to determine the incidence of autoimmune FHH with various types of autoimmunity and PTH-dependent hypercalcemia.

Anti-CaR antibodies and hypoparathyroidism

Acquired hypoparathyroidism (AH) is a disease with hypocalcemia caused by low PTH secretion with no known cause (e.g., hypomagnesemia or prior neck surgery). AH was the first disease that was linked to autoimmunity to the CaR. In an early study, Blizzard et al. reported autoantibodies to the parathyroid glands in 1967. They found that 38% of 74 patients with AH were positive for autoantibodies to the parathyroid, as compared with only 6% of 245 healthy control subjects (128). The autoantibodies to the parathyroid glands in patients with sporadic, adult-onset hypoparathyroidism were directed at the cell surface of human parathyroid cells and were reported to inhibit PTH release in a later study (129). Li et al. (130) subsequently reported that 14 of 25 patients with AH had antibodies directed at the CaR, whereas none of the control group of 50 patients with various other autoimmune diseases and 22 normal controls had antibodies to the receptor. In a recent study, Kifor at a. described two patients with hypoparathyroidism, who had anti-CaR antibodies that activated the receptor as evaluated by simulation of inositol phosphate accumulation and MAPK activity and inhibition of PTH secretion (131). Two additional studies have found conflicting results regarding the prevalence of anti-CaR antibodies among patients with autoimmune hypoparathyroidism. A recent study examining 90 patients with autoimmune polyen-

docrine syndrome type 1 found no autoantibodies against the CaR (132). In another study of 51 patients with idiopathic hypoparathyroidism – most of whom had isolated hypoparathyroidism – and 45 healthy controls, 49% of the patients had serologic evidence of organ-specific autoimmunity to the CaR protein (133). The paper showed an association between autoimmune antibodies to the CaR and the HLA-DR, implying an autoimmune component to the disease. Of note in this regard, the two patients in the study of Kifor et al. who had activating autoantibodies to the CaR also had Addison's and Graves' disease, respectively, further supporting the hypothesis of an autoimmune disease. Whether the difference between these studies reflects a difference in the incidence of anti-CaR antibodies between patients with type 1 APS and those with isolated hypoparathyroidism or is due to other factors remains to be clarified.

Summary and Future Issues

A significant component of the hereditary diseases of calcium homeostasis, particularly those with evidence of abnormal Ca^{2+}_o-sensing, have been shown to be caused by mutations in the calcium-sensing receptor. The CaR is a membrane bound 7TM receptor expressed in all of the tissues regulating extracellular calcium homeostasis. The CaR "senses" even minute (on the order of a few percent) changes in the level of calcium in the blood and acts, therefore, as the body's "calciostat". The CaR, in turn, modulates the functions of the cells expressing it so as to restore the level of blood calcium to normal. CaR-mediated regulation of the secretion of PTH plays a particularly important role in calcium homeostasis, because it directly or indirectly modulates the functions of all of the tissues involved in regulating blood calcium. Patients with loss-of-function mutations in the CaR gene display a form of hypercalcemia that is accompanied by relative hypocalciuria. In its heterozygous form, with one mutated allele, it is a benign form of hypercalcemia. In its homozygous form, with both CaR alleles mutated, the hypercalcemia may be fatal if left untreated. Gain-of-function mutations leads to an often benign state of hypocalcemia with relative hypercalciuria, called ADH. In the future it would be interesting to collect detailed clinical data in large cohorts in multicenter studies to investigate possible implications of the altered sensitivity of the CaR towards calcium in these patients as well as the resultant altered levels of serum calcium. This may be even more important than previously thought, as the CaR is now known to be functionally expressed in many organs, such as the brain, breast, cardiovascular system and intestine (66, 134), that are not known to participate in systemic calcium homeostasis. Lastly, symptomatic patients may benefit from the new CaR modulators, calcimimetics and calcilytics.

Abbreviations

ADH, autosomal dominant hypoparathyroidism; AH, acquired hypoparathyroidism; AKT, protein kinase B; Ca, calcium; Ca^{2+}_i, cytosolic frees calcium; Ca^{2+}_o, extracellular calcium; CaR, calcium-sensing receptor; CT, calcitonin; CNS, central nervous system; Cr, creatinine; ECD, extracellular domain; ERK1/2, extracellular signal-regulated kinases 1 and 2; FHH, familial hypocalciuric hypercalcemia; HEK, human embryonic kidney; HPT, hyperparathyroidism; ICD, intracellular domain; IP_3, inositol trisphosphate; JNK, c-jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; mGluR, metabotropic glutamate receptor; NSHPT, neonatal severe primary hyperparathyroidism; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol bisphosphate; PK; protein kinase; PL, phospholipase; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; TAL, thick ascending limb of Henle's loop; TMD, transmembrane domain; 7TM receptor, seven transmembrane receptor.

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