Calcium-Sensing Receptor and Associated Diseases

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The calcium-sensing receptor (CASR) is expressed in parathyroid hormone (PTH)-secreting cells of the parathyroid gland and cells lining the renal tubule. The activated CASR modulates intracellular signaling pathways altering PTH secretion and renal cation and water handling. Inherited abnormalities of the *CASR* gene give rise to a variety of disorders of mineral ion homeostasis. Heterozygous loss-of-function mutations cause familial (benign) hypocalciuric hypercalcemia (FHH) in which the lifelong mild hypercalcemia is generally asymptomatic. Homozygous inactivating mutations give rise to neonatal severe hyperparathyroidism (NSHPT) with extreme hypercalcemia and marked skeletal changes. Heterozygous activating mutations of the CASR cause autosomal dominant hypocalcemia (ADH) that may be asymptomatic or present with seizures in the neonatal period or childhood or later in life. Phenocopies of FHH or ADH are due to circulating CASR inactivating or activating autoantibodies, respectively. The CASR is the target of small molecule allosteric modifiers, either activators, calcimimetics, or inhibitors, calcilytics.

I. Calcium Homeostasis

Maintenance of blood levels of ionized calcium within a narrow normal range is of critical importance for control of neuronal excitability, muscle contraction, hormone secretion, and coagulation. The cell-surface calcium-sensing receptor (CASR) is the "calciostat" that orchestrates this systemic calcium homeostasis.[1] The CASR is expressed abundantly in the parathyroid hormone (PTH) producing chief cells of the parathyroid gland, the calcitonin-

producing C-cells of the thyroid, and the cells lining the kidney tubule. The seven-transmembrane (7-TM) G-protein-coupled receptor (GPCR) senses small changes in circulating calcium concentration and modulates intracellular signaling pathways that alter PTH and calcitonin secretion or renal cation handling, thereby restoring blood mineral ion levels to normal.

The relationship between extracellular ionized calcium and PTH concentration is represented by an inverse sigmoidal curve. The activity and/or expression level of the CASR dictates the so-called calcium set-point, defined as the extracellular calcium concentration at which PTH secretion from the parathyroid gland (or calcium reabsorption across the kidney tubule) is half-maximal.[2] Increases in extracellular calcium directly stimulate calcitonin secretion[3–5] and the CASR on breast ductal cells regulates calcium transport into milk.[6]

Other tissues in which the CASR is expressed where it is likely to play a mineral ion homeostatic role include, skeletal tissues[7–9]—osteoclasts and their precursors,[10] osteoblasts and their precursors, osteocytes, chondrocytes[11–13]—and placenta.[14] Tissues in which the CASR is expressed at lower levels and is likely to play roles unrelated to mineral ion homeostasis include neurons and glia of the brain,[15] keratinocytes,[16] vascular smooth muscle cells,[17] hematopoietic stem cells (in blood and bone marrow),[18] stomach,[19] intestine,[20] colon,[21] liver,[22] pancreas,[23] and others.[24]

II. CASR and Diseases

Inactivating mutations of the *CASR* gene cause familial hypocalciuric hypercalcemia (FHH: MIM# 145980) in which the lifelong hypercalcemia is generally asymptomatic—the disease is also known as familial benign hypercalcemia; while homozygosity manifests as neonatal severe hyperparathyroidism (NSHPT: MIM# 239200).[25–27] Infants with NSHPT may develop severe, symptomatic hypercalcemia with skeletal changes of unremitting hyperparathyroidism and the disorder can be life-threatening without treatment. Heterozygous activating mutations in the CASR cause autosomal dominant hypocalcemia (ADH: MIM# 601198), autosomal dominant hypoparathyroidism (MIM# 241400), or hypocalcemic hypercalciuria (MIM# 146200).[28–30] Some individuals with ADH may have mild hypocalcemia and relatively few symptoms. However, in some cases seizures can occur, and these often happen during febrile episodes due to intercurrent infections.[31,32]

III. CASR is a Family C GPCR

The CASR is a member of family C of the superfamily of GPCRs that comprise three different subfamilies that have ≥20% amino acid identity over their transmembrane domain (TMD) comprising the 7-TM-spanning region.[33] The GPCRs are also referred to as 7-TM receptors. Group 1 comprises the metabotropic glutamate receptors, mGluR 1–8, that are widely expressed in the central nervous system that bind and are activated by the excitatory neurotransmitter, glutamate. Group II contains the CASR, the vomeronasal receptors (VRs), and taste and odorant receptors. The VRs are expressed exclusively in the rodent vomeronasal sensory organ (VNO), which responds to pheromones to direct instinctive behavior. Group III comprises the gamma-aminobutyric acid ($GABA_B$) receptors that bind and activate the neuro-inhibitor, $GABA_B$. The widely expressed orphan receptor, GPRC6A, also belongs in family C, and is most similar to the CASR. GPRC6A binds and is activated by some L-α-amino acids and other ligands including calcium.

IV. Human CASR

The human *CASR* gene that maps to 3q13.3–21[34] spans ~103 kb and has eight exons.[35] Six exons (exons 2–7) encode the CASR protein of 1078 amino acids. In exon 7, there is usage of two different polyadenylation signal sequences (AATAAA) yielding either a short (177-nucleotide) or long (1304-nucleotide) 3′-untranslated region (UTR).[36,37] Exon 2 encodes 242 nucleotides of the 5′-UTR, followed by the translation initiation codon. Exons 1A and 1B encode alternative 5′-UTRs that splice to the common portion encoded by exon 2.[36,38] The transcriptional start sites (+1) of promoters P1 and P2 have been precisely mapped.[39] Promoter P1 has a TATA box at nucleotide −26 and a CCAAT box at −110 relative to the start site. Promoter P2 has Sp1 sites at the transcriptional start site. Functional vitamin D response elements (VDREs),[39] NF-κB elements,[40] and Glial Cells Missing (GCM) elements[41] are present in both promoters. Functional Stat1/3 elements are in promoter P1 and Sp1/3 elements are in promoter P2.[42]

The NH_2-terminal signal peptide that directs the nascent CASR polypeptide into the endoplasmic reticulum (ER), and is then removed, has 19 amino acids.[43,44] The extracellular domain (ECD) of the mature protein has ~600 amino acids comprised of a bilobed Venus-flytrap-like domain (VFT) that is connected by a cysteine-rich region and a peptide linker to the seven-transmembrane domain (TMD) with an intracellular COOH-terminal tail of 216 amino acids.[45] The CASR functions as a dimer with both covalent and non-covalent interaction between the VFTs of each monomer.[46] Ca^{2+} binds in the

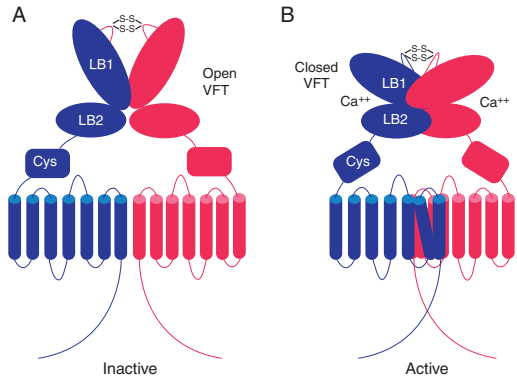

FIG. 1. Model of ligand activation of the CASR dimer at the cell surface. (A) Intermolecular disulfide bonds (in loop 2) link lobes 1 of each protomer (monomer) and in the absence of ligand maintain the VFT formed by lobes 1 and 2 in an open (inactive) conformation. (B) Ca^{2+} binds within the cleft between lobes 1 and 2 causing VFT closure and a rotation about the dimer interface with a change in configuration of loop 2 (the intermolecular disulfides). The conformation of the cysteine-rich region alters bringing about reconfiguration of some TM-helices such that intracellular loops contact G proteins triggering cell signaling.

cleft of each VFT causing the lobes to close on each other and the VFT to rotate and transfer a conformational change to the cysteine-rich region (Fig. 1). This results in movement of the membrane α-helices relative to one another thereby allowing G proteins to interact with intracellular loops and instigate cell signaling. The CASR couples largely via $G_{q/11}$ and G_i proteins although coupling via G_s has also been described[24,47,48] and activates or inhibits multiple intracellular signaling pathways.[49]

V. Orthosteric Agonists

The CASR is promiscuous and is activated by polycations, Ca^{2+}, Mg^{2+}, Ba^{2+}, Gd^{3+}, La^{3+}, Sr^{2+},[24] charged polyvalent molecules such as spermine and spermidine,[22,50] and β-amyloid peptides[51] and aminoglycoside antibiotics.[52] These agonists act by binding to sites in the ECD of the CASR.[53] Ionic strength,[54] pH and acid–base status,[55] and the L-α-amino acids, phenylalanine, tryptophan, and histidine[56–58] positively modulate sensitivity to Ca^{2+}. The CASR is likely to integrate diverse physiological signals, for example, those important for protein and mineral metabolism.[59]

VI. Allosteric Modifiers

A. Positive Allosteric Modulators (Calcimimetics)

In contrast to orthosteric agonists, small orally active compounds have been developed that bind within the TMD.[60,61] First-generation positive allosteric modulators are phenylalkylamine compounds derived from voltage-sensitive calcium channel blockers (Fig. 2). The compounds, NPS R-467 and R-568 act in a stereoselective way to enhance the sensitivity of the CASR to its orthosteric agonists while having no intrinsic activity of their own.[62,63] Second-generation calcimimetics such as cinacalcet have been developed with improved pharmacokinetic properties.[64] Calindol is similar in its properties to NPS R-568.[65,66] Calcimimetics act on parathyroid cells *in vitro* and *in vivo* to inhibit PTH secretion. Cinacalcet is in clinical use for treatment of uremic secondary hyperparathyroidism and parathyroid cancer.

B. Negative Allosteric Modulators (Calcilytics)

The first reported negative allosteric modulator was NPS 2143[67] and other calcilytics of different structure to NPS 2143 are Calhex 231[68] and Compound 1[69] (Fig. 2). Calcilytics antagonize the parathyroid cell CASR *in vitro* and *in vivo* and stimulate PTH secretion.[70]

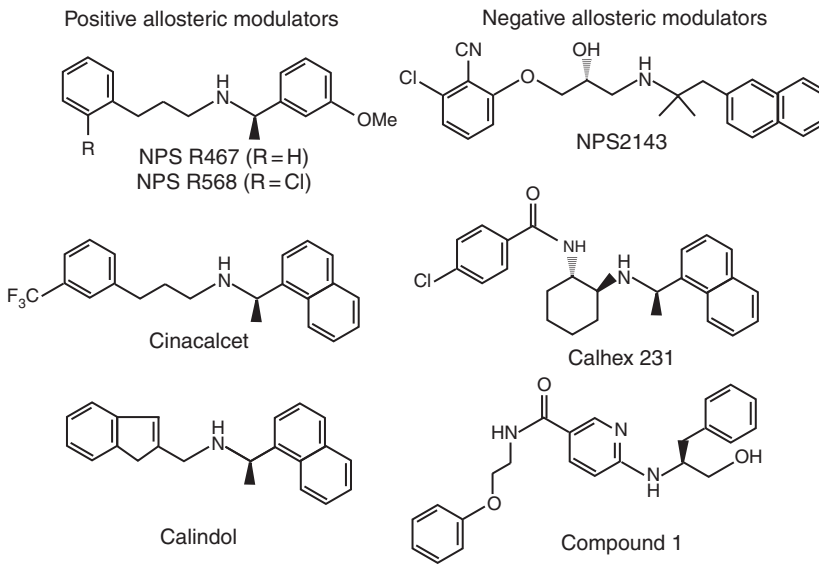

FIG. 2. Allosteric modulators of the CASR. Positive modulators: NPS R-467 and NPS R-568, Cinacalcet and Calindol. Negative modulators: NPS 2143, Calhex 231, and Compound 1.

VII. Structure and Function

Functional characterization of the CASR with respect to important structural features has been achieved most often by use of so-called heterologous systems in which a mammalian expression vector with a cDNA insert (often with an epitope tag) representing either wild-type or mutant CASR is transfected into human embryonic kidney (HEK) 293, or in a few cases, COS, cells.[71] In this review, HEK293 cells transfected with the CASR are designated as CASR-HEK cells. A variety of analyses are used to assess overall expression, expression at the plasma membrane, glycosylation and dimerization status, and signaling capability via several pathways.[46,72–75] Relative binding affinities of ligands are inferred indirectly by the responsiveness of cell signaling pathways. There is no Ca^{2+}-binding assay.

A. Asparagine-Linked Glycosylation

Western blot analysis of extracts of HEK293 cells transfected with CASR run under standard SDS–PAGE conditions reveals a variety of molecular species and the nature of these has been demonstrated by use of tunicamycin and endoglycosidase H (endo H) and peptide-*N*-glycosidase F (PNGase F) to interrogate the overall and the immature and mature N-linked glycosylation status, respectively, as well as more robust denaturation conditions to evaluate dimerization.[71] The species are 120 kDa (nonglycosylated monomer), 140 kDa (immature glycosylated monomer), 160 kDa (mature glycosylated monomer), and other species >280 kDa (dimers). N-linked glycosylation is required for expression of the CASR at the cell surface.[76] Of the 11 potential N-linked glycosylation sites on the ECD of the human CASR, eight sites (N-90, N-130, N-261, N-287, N-446, N-468, N-488, and N-541) are used, whereas the three remaining sites (N-386, N-400, and N-594) are not (Fig. 3). Glycosylation of at least three sites is critical for cell-surface expression, but glycosylation is not critical for signal transduction.[77]

B. Venus-Flytrap Domain

The ECDs of the CASR and the other Group C GPCRs have limited amino acid homology to bacterial periplasmic amino acid-binding proteins for which the crystallographic structures are known.[78] Like these binding proteins, the ECDs have a so-called VFT or clam shell structure consisting of two lobes each with α-helices and β-sheet folds connected by a hinge region of three interwoven strands. This has been confirmed by 3D analysis of crystals of the mGluR1 ECD in open (ligand-free) and closed (ligand-bound) conformations[79,80] and similar structures deduced by homology modeling for the CASR ECD (amino

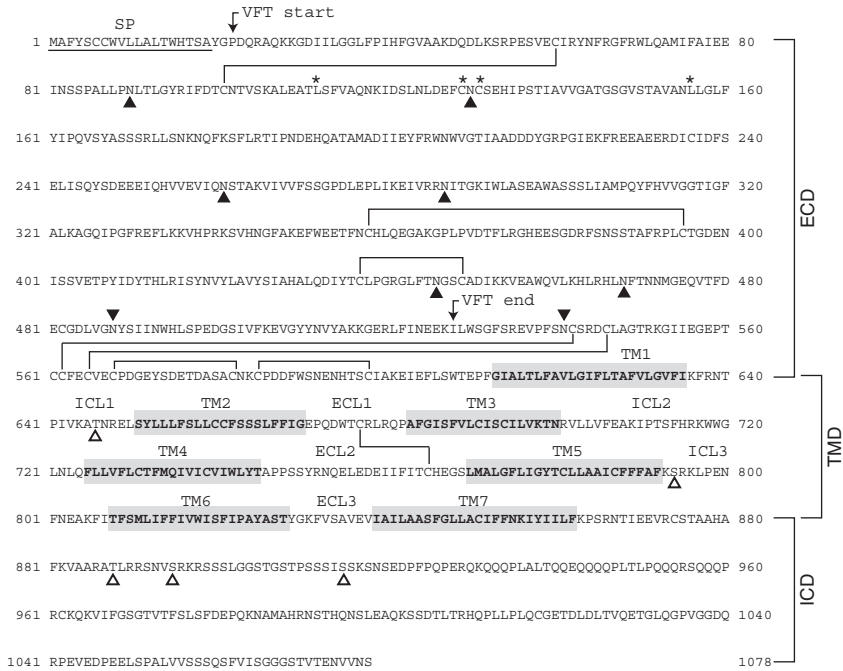

FIG. 3. Amino acid sequence (1–1078) and key features of the human CASR: SP, signal peptide (1–19) that is underlined; ECD, extracellular domain (20–612); TMD, transmembrane domain (613–862); ICD, intracellular domain (863–1078). In the ECD: VFT, Venus-flytrap domain, arrows indicate start (22) and end (528); intramolecular disulfide bonds, cysteine (C) residues joined by lines; functional N-linked glycosylation sites, filled arrowheads; asterisks (∗), leucine (L) residues and cysteine (C) residues contributing to hydrophobic and covalent intermolecular linkages, respectively, for dimer formation. In the TMD: transmembrane α-helices (TM1–TM7), bolded residues on shaded boxes; intracellular loops (ICL1–ICL3); extracellular loops (ECL1–ECL3); disulfide bond between ECL2 and ECL3, cysteine (C) residues joined by a line. PKC sites, open arrowheads (S or T) in TMD and ICD.

acids 23–528).[45,46,81] Some parts of the CASR ECD do not align with the bacterial protein structure and are represented by four protruding loops: loop 1 (50–59), loop 2 (117–136), loop 3 (365–385), and loop 4 (438–445). By study of engineered mutants, deletion of loop 1 leads to reduced activity of the receptor.[82] The VFT model predicts a rotation of one lobe relative to the other upon ligand binding which directly or indirectly modifies the conformation of the TMD leading to receptor activation. A cysteine-rich region from amino acids 542 to 598 may play a role in signal transduction, after ligand binding, from the more NH_2-terminal part of the ECD to the TMD.[83,84]

C. Dimerization

The CASR exists in a functional form as dimers on the cell surface of transfected cells and this is promoted in part through intermolecular disulfide bonds involving C129 and C131 in the ECD.[85,86] These cysteine residues are proposed to be located in loop 2 (117–136) that protrudes from one lobe of the VFT of each monomer (or protomer).[87] However, these covalent linkages are not needed for functional interactions between each CASR protomer within the dimer. In fact, removal of loop 2 leads to increased sensitivity to Ca^{2+}.[82] The CASR has at least two types of motifs mediating dimerization (and functional interactions); covalent interactions (intermolecular disulfide bonds) and noncovalent (hydrophobic) interactions.[88] Two leucine residues, L112 and L156, in the ECD are important for noncovalent dimerization. A receptor in which the leucines are mutated (L112S and L156S) still exists as a covalently linked dimer with higher affinity for calcium than the wild-type receptor. A combination of the four mutations, L112S, L156S, C129S, and C131S, reduces receptor dimerization and markedly inactivates the CASR. Dimerization through the ECD is essential for formation of a functional tertiary structure of the CASR.[89] With respect to the other cysteines in the VFT, it can be predicted, by homology modeling of the CASR structure on the mGluR ECD, that intramolecular disulfide bonds form between C60–C101, C358–C395, and C437–C449 (Fig. 3). Site-directed mutagenesis studies showed that each of these cysteines are critical for CASR expression and function and are likely to be critical for the VFT structure.[90] Intracellular CASR dimers were identified in rat kidney medulla extracts[86] and in CASR-HEK cells.[91] Constitutive dimerization was demonstrated within the ER by photobleaching fluorescence energy transfer microscopy.[92]

D. Ligand-Binding Sites in the VFT

Ca^{2+} activates the CASR at millimolar concentrations consistent with low-affinity binding and there is positive cooperativity suggesting multiple Ca^{2+} binding sites. Several of the 13 amino acids in the VFT of the mGluR1 involved in glutamate binding are identical or conservatively substituted in the CASR.[58] In one study, it was proposed that the Ca^{2+}-binding site in the CASR comprises polar residues—S170, D190, Q193, S296, and E297—directly involved in Ca^{2+} coordination, and an additional set of residues that contributes to the "coordination sphere" of the cation (F270, Y218, S147).[93] Another study identified three potential Ca^{2+}-binding sites in a modeled CASR structure.[94] To probe the intrinsic Ca^{2+}-binding properties of predicted sequences, two predicted continuous Ca^{2+}-binding sequences were individually engineered into a

scaffold protein, the non-Ca^{2+}-binding protein, CD2. One of these sites is the same or similar to that described previously.[93] In further studies, three globular subdomains in the intact CASR structure have been identified each of which is predicted to contain two to three Ca^{2+}-binding sites[95] (Fig. 4).

Studies of the related mGluRs provide evidence that the VFT domains may function somewhat differently in different mGluRs. For heterodimers of mGluR5-$GABA_{B1}$ while closure of one mGluR VFT leads to partial activation, closure of both VFTs in the dimer is needed for full activity.[96] mGluR3 ECD crystal structures with ligands show that rather than changes in protein conformation the different agonists rearrange solvent molecules.[97] This contrasts with the VFT being closed versus open under glutamate-bound versus not bound conditions for mGluR1[79,80]—the model presently proposed for the CASR. There is a rotation of one monomer relative to the other about an axis perpendicular to the dimer interface bringing the COOH-termini of the two VFT monomers closer. Consistent with this model being relevant for the CASR, two different monoclonal antibodies against lobe 2 of the VFT either increase or decrease sensitivity to extracellular Ca^{2+} presumably by enhancing or inhibiting agonist-promoted VFT closure or rotation.[98]

E. Cysteine-Rich Domain

Between the VFT and the start of the TMD, the CASR has a Cys-rich region with nine conserved cysteines and if any one of these is mutated, expression and function of the CASR is impaired.[90] Deletion of the entire Cys-rich region preserves some cell-surface expression but Ca^{2+} activation is lost.[84] By homology modeling based upon the crystal structure of the mGluR3 ECD,[97] the Cys-rich region of the CASR has three β-sheets, each comprised of two antiparallel β-strands. Four pairs of cysteines, C542–C562, C546–C565, C568–C582, and C585–C598, form disulfide linkages. The remaining cysteine, C561, has been reported to be unpaired[90,99] although extrapolation from data of the mGluR3 structure suggests potential linkage between C236 (in lobe 2 of the VFT) and C561. Perhaps, in the CASR, this disulfide bond is particularly labile relative to the others. The exact role that the Cys-rich region plays in transmitting agonist-induced conformational changes in the VFT to the TMD is not known. Also, not all family C GPCRs have a Cys-rich domain; $GABA_B$ receptors do not.

The short region (529–541) between the end of the VFT and the Cys-rich region can be variable in length as a fully functioning splice variant having 10 additional amino acids has been identified[36] and engineered alterations in that region do not affect function.[99]

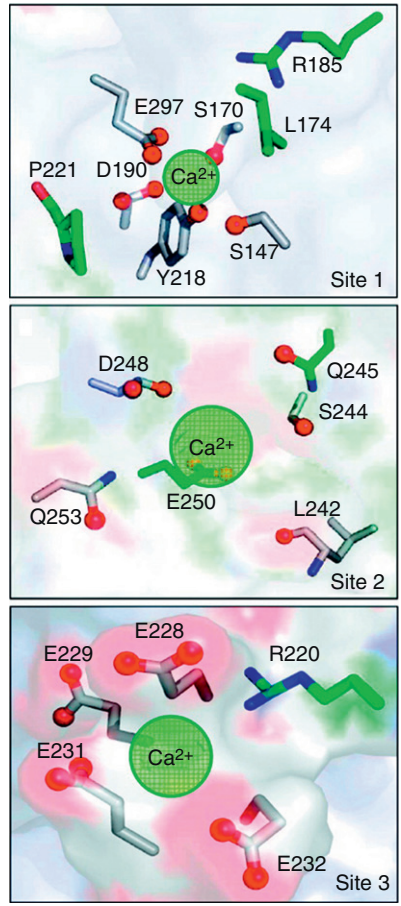

FIG. 4. Location of three predicted Ca^{2+}-binding sites in a subdomain (amino acids 132–300) of the ECD of the CASR. Site 1 is in the hinge region of the VFT structure formed by lobes 1 and 2 (see Fig. 1). Sites 2 and 3 are in the first half of lobe 2 (amino acids 215–253). Sites 4 and 5 (not shown) are clustered in the second half of lobe 1 (amino acids 350–400). (Redrawn with permission from Ref. 95.)

F. Peptide Linker

A 14 amino acid linker (599–613) connects the Cys-rich region to the TMD. A similar linker is found in all family C GPCRs with the exception of the $GABA_B$ receptor. Small alterations in the length and composition of the linker impaired cell-surface expression and abrogated signaling. Alanine substitution of four conserved residues identified L606 as being critical for cell-surface expression

and signaling. However, substitution of L606 with either Ile or Val led to activation. Therefore, it was suggested the linker region, in particular L606, plays a critical role in transmission of the active signal from ECD to TMD.[100]

G. Extracellular Loops

Mutation of any of three acidic residues in extracellular loop 2 (ECL2), D758, E759, and E767, increased sensitivity to Ca^{2+} activation of both full-length receptor and one lacking the ECD. Mutation of E837 in ECL3 reduced sensitivity to the calcimimetic NPS R-568. Mutation of other acidic residues had no effect. It was suggested that the three acidic residues in ECL2 maintain an inactive conformation of the TMD.[101]

The roles of charged amino acids and cysteines in the three extracellular loops were examined by alanine-scanning mutagenesis.[102] Mutation of C677 and C765 in ECL1 and ECL2, respectively, led to altered glycosylation, reduced cell-surface expression to 5% of wild type, and completely ablated phosphoinositol hydrolysis (PI). In these studies, replacement of charged amino acids produced only minor changes in receptor activation except for E767A and K831A in ECL2 and ECL3, respectively, that showed gain of function by increased apparent Ca^{2+} affinity. A double mutant E767K/K831E in which the positions of charged residues were exchanged had impaired cell-surface expression and no PI response to Ca^{2+}. Hence, the two cysteines form critical disulfide links and the side chains of E767 and K831 are likely to be involved with ionic interactions with other amino acids. It was suggested that the interactions are important for receptor folding and maintaining the TMD in an inactive conformation.

H. Transmembrane Domain and Allosteric Modifiers

Agonists and antagonists of rhodopsin-like family A GPCRs bind within the TMD. Similarly, the allosteric modulators of the CASR (a family C GPCR) bind within the TMD. Models of the CASR TMD have been constructed based on the crystal structure of bovine rhodopsin in the inactive form with retinal bound.[103] It should be noted that the TMDs of the family A GPCRs like rhodopsin and family C GPCRs like CASR have no significant sequence identity. The sizes and sequences of the extracellular loops and intracellular loops of family A and C receptors are different with the exception of the two cysteines that form a disulfide bond between ECL1 and ECL2. However, the models have apparently been useful in identifying contact sites between residues in the CASR TMD and allosteric modulators.[104–108] It is important to note that assignments of the residues that comprise the TM-helices changed somewhat after the introduction of the rhodopsin model. This had the effect of reducing the sizes of some of the intracellular and extracellular loops. This was

most apparent for ICL1, ECL1, ICL3, and ECL3. An instructive comparison can be made by reference to Fig. 2[109] for the original TM assignments and to Fig. 1[45] for the rhodopsin-type assignments. The present review is using the original TM assignments.

Two calcimimetic allosteric modulators, NPS R-568 and Calindol, that bind the TMD of the CASR have been reported to potentiate Ca^{2+} activation without independently activating the wild-type receptor. The ability of Calindol to activate a CASR construct (T903-Rhoc) in which the ECD and carboxyl-terminal tail have been deleted to produce a rhodopsin-like TMD was tested. Although Calindol has little or no agonist activity in the absence of extracellular Ca^{2+} for the ECD-containing wild-type or carboxyl-terminal deleted receptors, it acts as a strong agonist of the T903-Rhoc. Ca^{2+} alone displays little or no agonist activity for the CASR TMD, but potentiates the activation by the calcimimetic.[104]

The docking of Calhex 231, a negative allosteric modulator, has been evaluated with the 3D model of the CASR TMD. In the model, Glu-837 (TM7) anchors the two nitrogen atoms of Calhex 231 and locates the aromatic moieties in two adjacent hydrophobic pockets delineated by TMs 3, 5, and 6 and TMs 1, 2, 3, and 7, respectively. Two receptor mutations, F684A (TM3) and E837A (TM7) caused a loss of the ability of Calhex231 to inhibit Ca^{2+}-induced cell signaling. Three other mutations, F688A (TM3), W818A (TM6), and I841A (TM7), produced a marked increase in the IC_{50} of Calhex 231 for the Ca^{2+}-response whereas L776A (TM5) and F821A (TM6) led to a decrease in the IC_{50}. The data validate the proposed model for interaction of Calhex 231 with the TMDs of the CASR. Residues at the same positions have been shown to delimit the antagonist-binding cavity of diverse GPCRs.[105]

The same group modeled the binding pocket of the two calcimimetics, NPS R-568 and Calindol, and compared the findings with those for calcilytics. There are (subtle) differences between the binding of calcimimetics and calcilytics. Some mutations have no effect on calcimimetics but affect the binding of calcilytics in TM3 and TM5 suggesting that the binding pockets of the positive and allosteric modulators are partially overlapping but not identical.[106] Residues W818, F821, E837, and I841 that are located in transmembrane helices, TM6 and TM7, were involved in the binding pocket for both calcimimetics and calcilytics. Additional residues located in TM3 (R680, F684, and F688) were involved in the recognition of calcilytics.[106]

A different negative allosteric modulator, compound 1, retains activity against the E837A mutant that lacks a response to other positive and negative modulators.[108] The related compound, JKJ05, acts as a negative modulator on the wild-type receptor but as a positive modulator on the E837A mutant. This activity is dependent upon the primary amine in JKJ05 that interacts with the acidic E767 in the 7-TM model generated in this study.[108]

The TMD has six prolines that cause kinks in the transmembrane helices that are likely to be functionally important. An engineered P823A mutation in TM6 was well expressed but demonstrated markedly reduced activation by Ca^{2+}.[110]

I. Intracellular Loops

Mutagenesis of residues within either ICL2 or ICL3 led to reduced cell signaling monitored by inositol trisphosphate (IP_3) production in response to elevations in extracellular Ca^{2+}. A signaling deficient phenotype was observed for F707A in ICL1, and L798A, F802A, and E804A in ICL2.[111] These studies were conducted with bovine CASR (that has 1085 residues compared to the 1078 of the human) and the equivalent residues in the human CASR would be F706, L797, F801, and E803.

J. COOH-Terminal Tail

The COOH-terminal intracellular tail contributes to several properties of the receptor; intracellular signaling, level of cell-surface expression, and rate of desensitization.

Deletion of the majority of the carboxyl terminus (889–1078) is compatible with normal processing, cell-surface expression, and signal transduction.[112] However, mutants truncated at 706 and 802 within the second and third intracellular loops, respectively, lack a signaling response to extracellular Ca^{2+} and are not properly glycosylated and fail to reach the cell surface. Although these mutants did not exhibit a mature glycosylated monomeric species, it can be noted that dimers were formed. Mutants truncated at 888 and 903 within the carboxyl terminus were equivalent to the wild type in all assays. A small region between residues 874 and 888 is critical for normal signal transduction. Mutants truncated at 865 and 874 had no response to extracellular Ca^{2+} despite only a slight ($\sim$25%) reduction in cell-surface expression. Full-length CASR mutants with residues between 874 and 888 substituted by alanines showed either no (875A, 876A, 879A) or reduced (881A–883A) calcium response at levels of cell-surface expression equivalent to wild type.

In a separate study, there were no phospholipase C (PLC) responses to high extracellular Ca^{2+} in cells expressing CASR 1–866 (with a COOH-tail of three residues) although receptors were expressed at the plasma membrane.[113] The residues between S866 and V895 were scanned with tandem-Ala and single site mutagenesis. Two point mutants H880A and F882A showed 50–70% reductions in high extracellular Ca^{2+}-induced IP_3 production. Levels of expression and glycosylation of the mutants were comparable with wild-type CASR but the mutant receptors were retained in intracellular organelles and colocalized with an ER marker. It was suggested that the signaling defects were likely due to defective trafficking to the cell surface. Modeling indicated a

putative α-helical structure (15 amino acids) between 877 and 891. The data suggested that specific amino acids, and possibly unique secondary structure, are required for efficient targeting of the CASR to the cell surface. Note that as these studies were done with bovine CASR cDNA, the equivalent residue numbers for human CASR are one less than those given earlier.

VIII. Receptor Downregulation and Protein Kinase C

The CASR is desensitized to a limited extent by repeat exposure to agonists and phosphorylation either by GPCR kinases and recruitment of β-arrestin[114] or by protein kinase C (PKC) and reduced capacity to activate PLC.[115] The activated CASR stimulates PLC that mobilizes intracellular Ca^{2+} stores, on the one hand, and activates PKC, on the other. The CASR has five potential PKC sites in its intracellular domains (IC loops and intracellular tail) and phosphorylation of T888 (the most important site), S895, and S915 in the intracellular tail maximally inhibits Ca^{2+} mobilization.[116] Truncating the receptor at T888 has a negative effect on release of intracellular Ca^{2+} stores without affecting the activation of Ca^{2+} influx. Although not shown directly, the PKC phosphorylation of the CASR was suggested to prevent interaction with G protein subtypes critical for releasing Ca^{2+} stores and this was independent of activation of Ca^{2+} influx.[117]

IX. Receptor-Activity-Modifying Proteins and CASR Trafficking

Receptor-activity-modifying proteins (RAMPs) associate with some of the family B GPCRs, and this can be required for their functional expression at the cell surface. The particular RAMP (1, 2 or 3) can dictate the ligand specificity of particular receptors. A transfected pH-sensitive GFP superecliptic pHluorin (SEP)-CASR was retained in the ER in COS7 cells that do not contain endogenous RAMPs. SEP-CASRs were delivered to the plasma membrane in HEK293 cells that do express RAMP1.[118] Coexpression of RAMP1 or RAMP3, but not RAMP2, in COS7 cells was sufficient to target the CASR to the cell surface. These and further experiments supported the notion that association of RAMPs is necessary and sufficient to transfer the immature CASR from the ER to the Golgi where it becomes fully glycosylated. The basis for these studies is that while HEK293 cells widely used for CASR transfection studies do express the required RAMPs, COS7 cells do not. However, it can be noted that other investigators have successfully used COS7 cells for CASR transfection studies.[119,120] Further studies are required to assess the physiological significance of these findings.

X. Ubiquitination and Conformational Checkpoint in CASR Processing

CASR is ubiquitinated by the E3 ligase dorfin and degraded via an ER-associated degradation pathway[121] and evidence was provided for conformational or functional checkpoint in CASR biogenesis.[122] The finding of stabilization of a subset of the loss-of-function CASR mutants tested but not gain-of-function CASR mutants by the proteasome inhibitor MG132 suggested that receptor sensitivity to calcium influences susceptibility to proteasomal degradation. The allosteric activator NPS R-568 and antagonist NPS 2143 were used to promote the active and inactive conformations of the CASR, respectively. Overnight treatment with NPS R-568 or NPS 2143 differentially regulated maturation and cell-surface expression of wild-type CASR, directly affecting maximal signaling responses. NPS R-568 rescued expression of loss-of-function CASR mutants, increasing plasma membrane expression and ERK1/2 phosphorylation in response to 5 mM Ca^{2+}.[122] Further studies have confirmed that a subset (but not all) of additional missense CASR inactivating mutants, including some that are already expressed at the cell surface, show enhanced responsiveness to extracellular Ca^{2+} after treatment with the "pharmacochaperone," NPS R-568.[123,124] Disordered calcium homeostasis caused by some CASR mutations may result from altered receptor biogenesis independent of receptor function (a protein folding disorder). Allosteric modulators may not only alter CASR sensitivity to calcium (and signaling) but also modulate receptor expression.

XI. CASR and Overview of Signaling Pathways

The CASR is a pleiotropic GPCR that can couple to more than one type of G protein. The most well-characterized interactions are with $G_{q/11}$ and G_i, but coupling to $G_{12/13}$, G_o, and G_s occurs in some cell types. Stimulated CASR couples to G_q (pertussis toxin-insensitive) causing PLC-mediated 1,2-diacylglycerol (DAG) formation, and IP_3 formation with intracellular Ca^{2+} mobilization and couples to G_i (pertussis toxin-sensitive) causing inhibition of cAMP formation (Fig. 5). By increasing the intracellular Ca^{2+} concentration and phospholipid metabolites such as DAG, the activated CASR activates the serine/threonine kinase PKC. Both conventional and atypical isoforms of PKC can be involved. Via PLC and PKC activation, the activated CASR also stimulates phospholipase A_2 (PLA_2) with arachidonic acid production and phospholipase D (PLD) with phosphatidic acid formation. The mitogen-activated protein kinase (MAPK) family includes the proline-directed serine/threonine

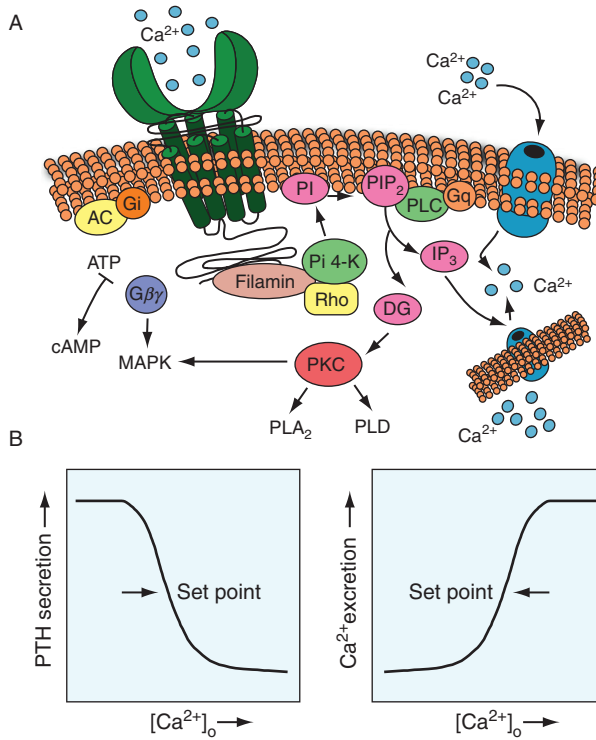

FIG. 5. (A) Signaling pathways activated by the CASR. (B) The CASR controls the relationship between the extracellular calcium concentration $[Ca^{2+}]_o$ and PTH secretion on the one hand and urinary calcium excretion on the other. The set-point is the $[Ca^{2+}]_o$ at which PTH secretion or calcium excretion is half-maximal.

protein kinases ERK1 (p44) and ERK2 (p42), p38 and stress-activated c-Jun N-kinases (JNK). Both $G_{i/o}$ and $G_{q/11}$ coupling have been implicated in ERK1 and ERK2 phosphorylation by the activated CASR. The stimulated CASR can also activate p38 and JNK.[49]

When the cytoplasmic calcium responses of individual cells are examined by single cell fluorescence recording, small increments in extracellular calcium sustain intracellular Ca^{2+} oscillations that decay to a nonoscillatory plateau.[125] Besides the so-called type 1 agonists (e.g., calcium itself), the allosteric type 2 agonists including the calcimimetic NPS R-568 and aromatic amino acids, L-phenylalanine and L-tryptophan, trigger CASR-activated intracellular Ca^{2+} oscillations although there are some quantitative and qualitative differences between the two type of agonists. Aromatic amino acids produce transient oscillations whereas extracellular Ca^{2+} produces sinusoidal oscillations perhaps

because calcium is both agonist and permeates the cell to influence the oscillations. In some cells, the extracellular calcium activation of oscillations in intracellular Ca^{2+}.[126–128] occurs through PLC activation and the Ca^{2+} permeable channel, TRPC1. The signaling determinants involved in CASR-generated intracellular Ca^{2+} oscillations appear to lie within amino acids 868–888 of the intracellular tail.[126,127]

XII. CASR and the Parathyroid

The precise mechanisms whereby activation of the CASR inhibits PTH secretion and synthesis and parathyroid cell proliferation are not known. Activation of the $G_{q/11}$ pathway appears critical as mice with parathyroid knockout of both G_q and G_{11} have a phenotype of severe hyperparathyroidism mimicking that of mice (and humans) with complete deletion of the *Casr* (*CASR*).[129] Confirmation that the G_q-coupled pathway is important in part comes from the finding that transgenic mice specifically overexpressing a dominant-negative $G\alpha_q$ loop minigene in parathyroid chief cells had increased PTH mRNA and serum PTH levels and parathyroid gland size. The abnormalities that reflected reduced sensitivity of the parathyroid gland to calcium were similar to those of heterozygous *Casr* knockout mice.[130]

In parathyroid cells, agonist activation of CASR stimulates activation of phospholipases, PLC, PLA_2, and PLD.[131] CASR activation of PLC involves a pertussis toxin-insensitive pathway via the G_q and G_{11} proteins and also a pertussis toxin-sensitive pathway via G_i isoforms. Activation of PLC leads to PLA_2 and PLD activation mediated by DAG stimulated PKC. At the parathyroid cell membrane, the CASR is bound directly or indirectly to calveolin-1 and resides within caveolae, flask-like invaginations of the plasma membrane.[132] Filamins are a family of nonmuscle actin binding proteins that cross-link actin into a three-dimensional structure. Filamins interact with a very large number of cellular proteins of diverse functions and act as signaling scaffolding molecules. Filamin-A interacts with caveolin-1 and also binds the COOH-terminal tail of the CASR.[133–136] This appears to facilitate the ability of the CASR to activate MAPKs such as ERK1/2. In heterologous systems, the interaction between CASR and filamin-A stabilizes the CASR and protects it from desensitization.[137]

Increases in extracellular Ca^{2+} cause transient increases in intracellular Ca^{2+} in parathyroid cells by activation of PLC[131] resulting in IP_3-mediated release of intracellular Ca^{2+} stores.[73] Sustained increases in intracellular Ca^{2+} occur through an influx pathway that may involve a nonselective cation channel.[138,139] High extracellular Ca^{2+} inhibits agonist-stimulated cAMP in parathyroid cells, an action thought to involve inhibition of adenylate cyclase by

an isoform of G_i.[140] The CASR stimulates the activity of MAPK (ERK1/2 and p38) through both a PKC-dependent pathway downstream of G_q-mediated activation of PLC then PKC, and via tyrosine kinases that utilize $\beta\gamma$ subunits released upon activation of G_i.[141,142] Activated MAPK stimulates PLA_2, releasing free arachidonic acid that is then further metabolized.[141] Previous studies showed that the lipoxygenase pathway of arachidonate metabolism is critical for inhibition of PTH secretion.[143] Addition of 12- and 15-hydroxyeicosatetranoic acid (HETE) and the hydroperoxyeicosatetranoic acid (PHETE) inhibits PTH secretion from parathyroid cells.[144] While there is evidence for a pathway starting with activated CASR, MEK/MAPK, and arachidonate metabolites, the further links to PTH release are not known.

There is an accumulation of F-actin, caveolin-1, filamin-A, PTH-containing vesicles, and the CASR at the apical secretory pole of parathyroid cells under high extracellular Ca^{2+} concentrations.[145] Actin polymerization is critical for inhibition of PTH release as incubation with actin depolymerizing agents promoted PTH secretion at both low and high extracellular Ca^{2+} concentrations. It is suggested that activated CASR may influence the phosphorylation status of filamin-A, thereby controlling actin polymerization and PTH release. Further studies are required to identify the critical intermediary signaling molecules activated by the CASR in this mechanism.

Calpains are a family of proteases that recognize bonds between domains and cleave at nonspecific amino acid sequences. Calpains hydrolyze target proteins in a restricted manner to produce large fragments retaining intact domains of the original protein. In caveolae preparations from bovine parathyroid cells incubated with low extracellular Ca^{2+} (0.5 mM), inactive heterodimeric forms of *m*-calpain are present and raising the extracellular Ca^{2+} to 3 mM decreases the amount of *m*-calpain with an increase in CASR protein and phosphorylated PKC-α and -β.[146] Localization of *m*-calpain within caveolae may maintain the enzyme in an inactive state whereas in the long term the CASR may be degraded by *m*-calpain.

Elevations in extracellular Ca^{2+} inhibit PTH mRNA levels[147] and calcimimetics that sensitize the parathyroid cell CASR to calcium have the same effect.[63] The regulation of PTH synthesis by the activated CASR occurs by a posttranscriptional mechanism involving destabilization of PTH mRNA.[148] The importance of the CASR in the tonic control of parathyroid cell proliferation is exemplified by the loss of this control in humans or mice lacking the *CASR* gene in which marked parathyroid hyperplasia ensues.[149] Treatment of rats with renal impairment with calcimimetics prevents the secondary parathyroid hyperplasia that would normally occur.[150,151] Supplementation of mouse models lacking components of the vitamin D endocrine system (either ligand or receptor) with calcium reduces the parathyroid hyperplasia.[152] The pathways involved whereby CASR controls parathyroid proliferation are not known.

XIII. CASR and the Renal Tubule

CASR is expressed along the length of the kidney tubule. The CASR is present on the basolateral side of the cortical thick ascending limb (CTAL), at high levels, and the distal convoluted tubule (DCT), that play critical roles in hormone-regulated calcium and magnesium reabsorption.[153] The CASR is also expressed at the base of the microvilli of the proximal tubular brush border, the basolateral side of the medullary thick ascending limb (MTAL) tubular cells, and the luminal side of the epithelial cells of the inner medullary collecting duct (IMCD).[154] In the proximal tubule, the activated CASR inhibits PTH-stimulated phosphaturia and also is likely to mediate the direct calcium control of the proximal tubular 25-hydroxyvitamin D-1α-hydroxylase.[39,155]

In the CTAL, high levels of peritubular but not tubular calcium inhibit calcium and magnesium reabsorption in isolated tubule segments,[156] an effect likely to be mediated by the basolateral CASR on paracellular ion transport. Mechanistically, this occurs via CASR inhibition of the hormone-stimulated cAMP levels thereby reducing the activity of the Na, K, 2Cl cotransporter on the one hand and stimulating arachidonic acid that is metabolized by a P450 pathway on the other[157] to inhibit other channels and cotransporters critical for calcium and magnesium reabsorption (see later section on Bartter's syndrome type V). Extracellular calcium modulates the expression of key genes in the DCT responsible for transcellular calcium uptake such as those for the apical uptake channel, TRPV5, calbindin D, the basolateral calcium pump, PMCA2b, and exchanger, NCX1.[158–160] While these changes occur in part via alterations in circulating PTH and $I,25(OH)_2D$, direct effects of calcium also play a role.[161] The DCT is difficult to investigate *in situ* but studies with a mouse distal convoluted tubule (MDCT) cell line demonstrated expression of the CASR and that its activation with either calcium or magnesium inhibited hormone (PTH, calcitonin, glucagon, or arginine vasopressin)-stimulated cAMP levels.[162] In addition, the magnesium/calcium sensing inhibited the hormone-stimulated cation (magnesium) uptake in these cells.[163]

XIV. Disorders Associated with CASR (Table I)

A. Familial Hypocalciuric Hypercalcemia

Reports of a syndrome called familial benign hypercalcemia appeared some 40 years ago.[164,165] The clinical features were documented in several large kindreds in which not only the generally asymptomatic nature of the lifelong hypercalcemia[166] but also the abnormal renal handling of calcium[167] was noted. In this familial (benign) hypocalciuric hypercalcemia (FHH) syndrome the

hypercalcemia is usually mild, with serum calcium levels no more than 10% above the upper limit of normal, although a few families exhibit higher serum calcium concentrations.[168] The symptoms and complications seen in patients with other forms of hypercalcemia are not a part of FHH. Circulating vitamin D metabolite levels are usually normal and intestinal absorption of calcium is normal or slightly reduced. Skeletal consequences of FHH are minor with the suggestion of a slightly increased bone turnover than normal being matched by increased bone formation.[169,170] Affected individuals from some FHH kindreds may experience pancreatitis, gallstones, or chondrocalcinosis.[166,167,171] The CASR is expressed at low levels in pancreas,[23] liver,[22] and chondrocytes[12,172] and decreased activity of the CASR could have a functional effect in these tissues.

The degree of hypercalcemia in most FHH patients is similar to that of patients with mild primary hyperparathyroidism (PHPT).[168] In contrast, serum magnesium levels are at the upper end of the normal range or slightly above.[173] The serum PTH levels are inappropriately normal (given the hypercalcemia) pointing to a derangement of the sensing of the blood calcium level by the parathyroid gland.[174,175] Diagnostic difficulties can arise in the ~15% of PHPT patients who have serum PTH levels at the upper limit of normal.[176] Because of this, FHH patients often underwent parathyroidectomy.[177–179] However, in the vast majority of the cases, the patients remained hypercalcemic because the defect in renal calcium sensing was uncorrected[180–182] and the consensus is that surgery should be avoided in this benign disorder.

There is unusually high renal tubular reabsorption of calcium and magnesium for the prevailing blood calcium concentration. In FHH patients, the renal calcium/creatinine clearance ratio is less than 1% while in PHPT and other hypercalcemic disorders it is usually much higher.[167] However, there is some overlap and some families with FHH have affected members with hypercalciuria and/or nephrolithiasis.[183–185] In FHH, unlike in PHPT, urinary concentrating ability is normal.[186] The renal CASR may mediate the polyuria and diminished urinary concentrating ability characteristic of the hypercalcemia in PHPT. One proposed mechanism is that in the renal IMCD the apically expressed CASR regulates (counteracts) vasopressin-controlled water permeability.[187]

B. Neonatal Severe Hyperparathyroidism

PHPT is extremely rare in childhood.[188,189] In reviewing several kindreds with FHH, it was noted that three patients had severe hyperparathyroidism in the neonatal period.[167] These and other cases involved multiglandular parathyroid hyperplasia rather than adenoma.[190–192] Neonatal severe hyperparathyroidism (NSHPT) occurs in children under the age of 6 months who have marked symptomatic hypercalcemia with the bony changes of hyperparathyroidism. In its most severe form, NSHPT can be a devastating neurodevelopmental

disorder and is often fatal unless treated by total parathyroidectomy.[193,194] However, some cases (and in most instances not associated with FHH kindreds) present with hypercalcemia that is less marked and/or is transient (self-limited) and respond well to medical management.[195]

NSHPT can result in the following way: (1) The neonate is homozygous for the CASR mutation with each parent of a consanguineous union having one copy of the mutated allele and thus each having FHH.[194,196] (2) The neonate is a compound heterozygote carrying two different mutations each one coming from the individual parents.[197] (3) The neonate is heterozygous for the mutation and the mother is unaffected. This situation may arise either with a *de novo* mutation[198] or in the case of a paternal mutation.[81] Gestation of the FHH fetus in a normal mother will induce fetal secondary hyperparathyroidism because the fetal parathyroid glands will perceive the normal calcium level set by the mother as low.[199] These are the cases that are often self-limited.

Rare cases have been noted in which an infant (having a wild-type CASR genotype) and born of a mother with ADH may present neonatally with low-normal serum calcium and high-normal PTH levels. With a normal CASR, the fetal parathyroid glands respond *in utero* to the maternal hypocalcemia with an increased output of PTH.[200]

C. Molecular Genetics of FHH and NSHPT

In FHH, the inheritance is autosomal dominant with virtually 100% penetrance but variable expressivity. The disease locus maps in most cases to chromosome 3q (FHH type 1).[201–203] In four families with FHH and NSHPT, in which there was evidence of consanguinity, haplotype analysis with chromosome 3q markers linked to the FHH locus was consistent with NSHPT being the homozygous expression of the FHH disorder.[26] The *CASR* gene, itself, was shown by hamster–human hybrid cell hybridization analysis to reside on chromosome 3.[25] The *CASR* gene was mapped to 3q13.3–21 by fluorescence *in situ* hybridization (FISH) analysis.[34] However, the FHH trait exhibits genetic heterogeneity. In one family the disorder was mapped to 19p13.3 (FHH type 2)[202] and in another kindred with atypical features such as osteomalacia in some affected members and increased circulating PTH with age the condition mapped to 19q13 (FHH type 3).[204–206] At present, the genes responsible for the trait at the chromosome 19 loci are unknown.

D. Autosomal Dominant Hypocalcemia

Primary hypoparathyroidism encompasses a heterogeneous group of conditions in which hypocalcemia and hyperphosphatemia occur as a result of deficient PTH secretion. Familial isolated hypoparathyroidism (FIH; MIM# 146200) shows multiple modes of inheritance. Autosomal recessive FIH occurs with homozygous inactivating mutations of the PTH gene or the glial cells

missing-2 (GCM2) gene that encodes a transcription factor expressed in the PTH-secreting cells of the parathyroid gland and is essential for their development in terrestrial vertebrates (41, see Ref. 207 and references therein). Autosomal dominant inheritance occurs with heterozygous inactivating mutations of the PTH gene (MIM# 168450)[208] or the GCM2 gene[41,209] that act in a dominant-negative fashion.

Hypoparathyroidism segregated in a large family as an autosomal dominant trait and was linked to chromosome 3q.[29] In this family, and others, heterozygous activating mutations of the *CASR* gene were identified.[28,30] The descriptor, ADH (MIM# 601198), rather than hypoparathyroidism, is now more commonly used for this familial hypocalcemic disorder.[210]

In ADH, the hypocalcemia can be mild to moderate and patients have relatively few symptoms. Seizures can occur, especially in younger patients, and these often occur during febrile episodes due to intercurrent infections. Parathesias, tetany, and laryngospasm occur but are uncommon. There is a tendency toward hyperphosphatemia, although serum phosphate levels may be normal. The renal CASR can function as a magnesium sensor[162,163] and serum magnesium levels can be at the lower end of normal or even below normal in untreated ADH patients. Serum intact PTH levels although low, are usually within the normal range. Urinary calcium excretion is higher than in hypoparathyroid patients of other etiologies, despite serum PTH levels being lower in the latter patient group.[31]

Renal tubular responsiveness to vitamin D and its metabolites is much more exuberant in ADH patients than in other hypoparathyroid patients. With the marked hypercalciuria, there is a greater risk of renal complications such as nephrocalcinosis, nephrolithiasis, and impaired renal function. The active metabolite of vitamin D, $1,25(OH)_2D$, enhances renal CASR expression.[39] Promotion of expression of the activated CASR excessively inhibits the reabsorption of calcium by the renal tubular cells sustaining the hypercalciuria. Therefore, ADH patients should be treated sparingly with vitamin D or calcium supplements.[31]

E. Bartter's Syndrome Subtype V

Although Bartter's syndrome subtype V (MIM# 146200) is represented by only a handful of cases due to heterozygous severe activating mutations in the CASR,[211–213] they provide special insight into the functioning of the CASR in the thick ascending limb of the nephron.[160] The Bartter syndrome encompasses a heterogeneous group of electrolyte homeostasis disorders, the common features of which are hypokalemic alkalosis, hyperreninemia, and hyperaldosteronism.[214] Bartter syndrome subtypes I–IV are autosomal recessive disorders due to inactivating mutations in the following ion transporters or channels active in the thick ascending limb of the loop of Henle; type I, the

sodium–potassium–chloride cotransporter (NKCC2 or SLC12A2); type 2, the outwardly rectifying potassium channel (ROMK); type III, the voltage-gated chloride channel (CLC-Kb); type IV, Barttin, a β subunit that is required for trafficking of CLC-Ka and CLC-Kb. Patients with Bartter's syndrome subtype V have, in addition to the classic features of the syndrome, hypocalcemia and may exhibit neuromuscular manifestations, seizures, and basal ganglia calcifications.

In the cells of the renal thick ascending limb of the loop of Henle, the NKCC2 cotransporter is situated in the apical membrane (luminal side) and facilitates entry of Na^+, K^+, and Cl^-. Luminal K^+ is rate limiting for NKCC2 activity. The K^+ that enters the cell is recycled to the lumen via the apical potassium channel, ROMK. At the basolateral (blood side) membrane, exit of Na^+ occurs via the Na^+–K^+-ATPase and Cl^- exits via the voltage-gated chloride channel, CIC-Kb. CIC-Kb function requires interaction with the subunit Barttin. The transepithelial electrochemical gradient that is set up drives paracellular transport of Na^+, Mg^{2+}, and Ca^{2+} from the lumen to the blood. The CASR is situated in the basolateral membrane and when activated increases intracellular 20-HETE and decreases cAMP concentrations that inhibit ROMK and NKCC2 activities.[214] Thus, severe activating mutations of the CASR lead to the phenotype of Bartter's syndrome in addition to the hypocalcemic hypercalciuria of ADH (Table I).

XV. CASR Mutation Repertoire

Over 200 unique mutations in the CASR have been identified, 135 of the inactivating, FHH/NSHPT type, and 68 of the activating, ADH type (Fig. 6). There are 20 recurrent inactivating and 14 recurrent activating mutations found in apparently unrelated families. There are over 300 reports of families or individuals presenting independently with a CASR mutation (see http://www.casrdb.mcgill.ca).[215,216]

A. CASR-Inactivating Mutations (Table II)

The majority of the inactivating mutations are missense, single amino acid substitutions, and some insertion, deletion, frame shift, truncation, and splice-site mutations have been described. The mutations are scattered throughout the protein sequence with some clustering in the first half of the ECD (amino acids 137–250 within the VFT), the latter part of the ECD (amino acids 549–595 within the cysteine-rich region), and parts of the TMD. The scattering of mutations is consistent with the notion of the CASR having multiple functional components that collectively contribute to activity and that a critical mutation in any one of them can cause major impairment in function.[217,218]

TABLE I
DISORDERS ASSOCIATED WITH THE CASR

Familial (benign) hypocalciuric hypercalcemia (FHH)
- Mild hypercalcemia and hypermagnesemia
- PTH inappropriately normal
- Parathyroidectomy ineffective in normalizing hypercalcemia
- Renal calcium clearance ratio <0.01
- Renal concentrating ability normal

Neonatal severe hyperparathyroidism (NSHPT)
- Marked symptomatic hypercalcemia
- Bony changes of hyperparathyroidism
- Neurodevelopmental deficits if untreated
- Parathyroidectomy recommended
- Some less severe (self-limiting) forms

Autosomal dominant hypocalcemia (ADH)
- Mild to moderate hypocalcemia and hypomagnesemia
- Symptoms: seizures in younger patients
- Parathesias, tetany, and laryngospasm uncommon
- Urinary calcium excretion relatively increased
- Bartters subtype V in a few severe cases

Autoimmune hypocalciuric hypercalcemia (AHH)
- Phenocopy of FHH
- Inactivating CASR autoantibodies

Acquired hypoparathyroidism (AH)
- Phenocopy of ADH
- Activating CASR autoantibodies

Several mechanisms can account for impairment in CASR function: (1) Impairment in biosynthesis of the receptor and/or increased degradation.[44] (2) Normal biosynthesis and expression of the receptor within the cell but defective trafficking of the receptor from the ER to the plasma membrane.[92] (3) Normal maturation and trafficking of the receptor and expression on the plasma membrane but reduced affinity for calcium or other defect in the coupling of activation of the VFT to conformational changes in the TMD.[81] (4) Inability of the cell-surface CASR to couple normally to G proteins required for activation of cell signaling pathways. (5) An additional consideration applies

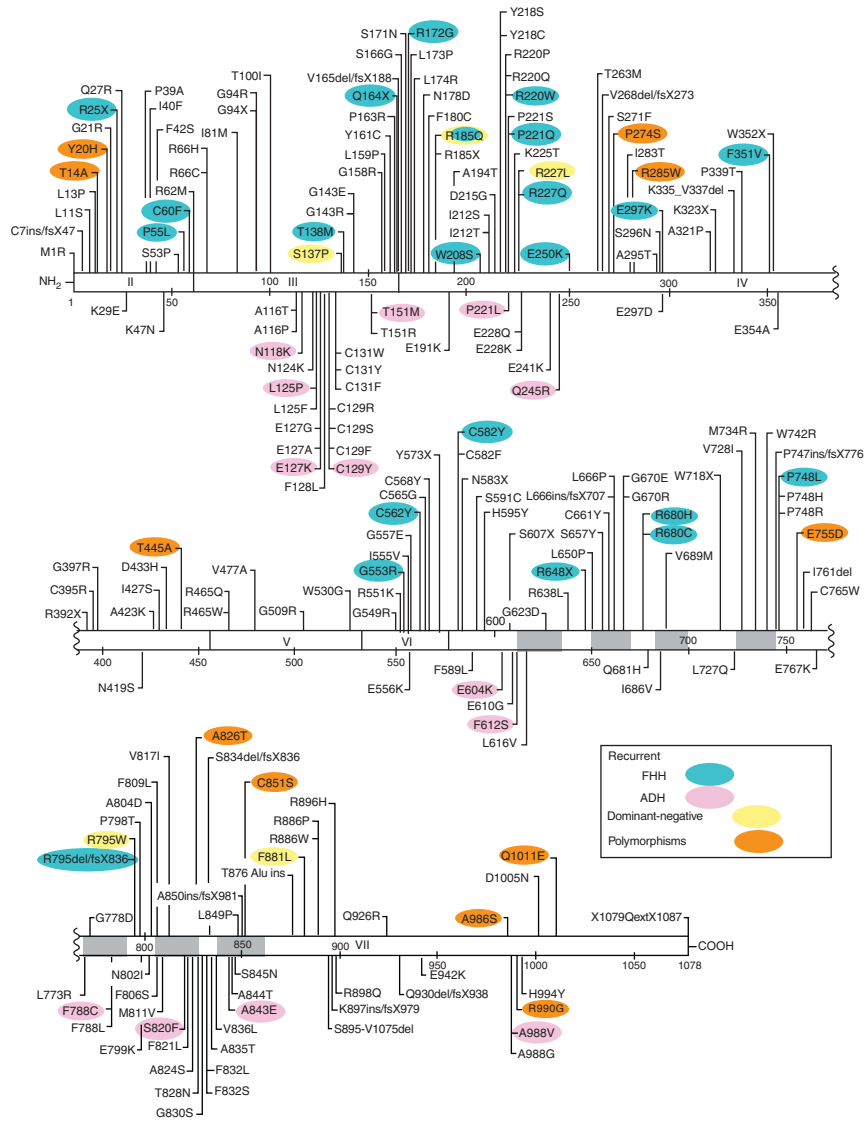

FIG. 6. Mutations and polymorphisms in the CASR. Schema of the CASR (which has 1078 amino acids) showing the relationship between gene exons (II–VII) and the portions of the protein they encode. Exons II–VI and the beginning of exon VII encode the ECD of ~600 amino acids, exon VII encodes the TMD of ~250 amino acids including membrane-spanning helices TM1–TM7 (indicated by the shaded boxes), ECL1–ECL3, ICL1–ICL3, and the ICD of ~200 amino acids. The locations of the inactivating mutations found in patients with FHH and/or NSHPT (as well as some polymorphisms) are shown above and of the activating mutations found in patients with ADH (as well as polymorphism R990G) are shown below the bars representing the protein-coding exons.

to those mutants that by virtue of their ability to dimerize with the normal CASR encoded by the wild-type allele (in FHH), may exert a dominant-negative effect reducing the function of the wild-type CASR.[198,219,220]

One mutation, M1R, would disrupt the initiation codon altering the protein start site (AXX**ATG**G), with three potential start sites upstream of the authentic site that would generate short peptides in a different reading frame and one downstream and in frame that would generate a protein beginning at amino acid 74. The precise consequences of the mutation have not been examined although expression levels are much reduced.[221]

The CASR has an NH_2-terminal 19-amino acid-signal peptide that directs the nascent polypeptide chain as it emerges from the ribosome, into the ER. Two mutations, L11S and L13P, lie within the signal peptide hydrophobic core.[44,222] When transiently transfected into kidney cells, the L11S and L13P mutants demonstrated markedly reduced intracellular and plasma membrane expression and signaling via the MAPK pathway in response to elevations in extracellular calcium relative to wild-type CASR. In cotranslational processing assays, which test the functionality of the signal peptide in the early secretory pathway, in contrast to the wild type, the mutants failed to be inserted into microsomes (representing the ER) and undergo core *N*-glycosylation. Hence, these mutants did not achieve proper biosynthesis and when coexpressed with the wild-type CASR did not influence the ability of the wild-type CASR to activate the MAPK signaling pathway at all.[44]

The mature CASR protein begins at amino acid 20. The three-dimensional globular lobes 1 and 2 of the VFT domain are formed by discontinuous protein sequence starting at amino acid 23 and ending at amino acid 528. A hinge region is formed by three strands of the sequence that weaves back and forth between the two lobes. Between amino acids 21–100, several inactivating missense mutations are present. Mutations S53P and the recurrent P55L occur in the unstructured loop 1 (amino acids 50–59) that when deleted leads to reduced receptor activity.[82] The C60F mutation would break an intramolecular disulfide bond with C101 critical for formation of the VFT structure.[90] The R66H and R66C mutations represent a class of mutant that undergoes dimerization as normal but does not exit the ER and never achieves localization at the plasma membrane.[92] From amino acids 115–131, inactivating mutations are absent but rather there is a very critical clustering of activating mutations in this region.

From amino acids 137–227, there are two dense clusters of inactivating missense mutations and then further mutations at a lower density extending to 339 and onwards. Many of these are likely to be involved (either directly or indirectly) in the putative ligand-binding domains that have been modeled. One study proposed a Ca^{2+}-binding site comprising polar residues—S170, D190, Q193, S296, and E297—directly involved in Ca^{2+}-binding with an

additional set of residues contributing to a so-called coordination sphere of the cation (F270, Y218, S147).[93] The cluster of missense mutations from amino acids 158–185 as well as those from 271 onwards could contribute to alterations in the Ca^{2+}-binding site while other groupings of mutations (137–143, 208–227, and 252–352) could have an impact upon the coordination sphere of the cation. Another group has proposed that amino acids 132–300 be designated as a subdomain of the VFT having three putative Ca^{2+}-binding sites.[95] Site 1 is in the hinge region of the VFT and involves residues S147, S170, L174, R185, D190, Y218, P221, and E297. This would appear to overlap with the Ca^{2+}-binding site proposed by the previous study. Naturally occurring mutations of a selection of these residues (L174R, R185Q, Y218S, Y218C, P221Q, P221S, and E297K), some of which are recurrent, have been identified. The three adjacent serines, residues 169–171, have been proposed to be a binding site for aromatic amino acids such as phenylalanine and tyrosine that function as allosteric activators[223] and there is a naturally occurring S171N mutation. Ca^{2+}-binding site 2 involves residues L242, S244, Q245, D248, E250, and Q253. The recurrent inactivating mutation, E250K, has been reported. Site 3 involves residues R220, E228, E229, E231, and E232 and the recurrent inactivating mutation, R220W, has been described. For some of the residues within the Ca^{2+}-binding sites activating mutations have also been described and at some residues the resulting mutation can be either inactivating or activating depending upon the nature of the replacement amino acid.

Some of the missense mutations are located in the hinge region and could affect mobility of the hinge. The F180C mutation while expressed normally at the cell surface potentially can form a disulfide bond with the normally unlinked C482 residue thereby inhibiting closure of the lobes leading to markedly reduced cell signaling.[81] From amino acids 250–530, missense mutations are found but at much lower density than in the regions already discussed. The C395R mutation would disrupt a critical disulfide bond with C358 contributing to the VFT domain structure.[90] From amino acids 549–595, a dense clustering of mutations occurs within the critical cysteine-rich region. Several of these involve the cysteine residues themselves (C562Y, C565G, C568Y, C582Y, and C582F).

Many mutations are found within the TMD with several in α-helices TM2, TM3, and TM6. Although valuable information has come from modeling how the allosteric modifiers might interact with the TMD (see earlier section), to a large extent, it is still unclear how specific mutations within the TMD modify function. Further studies are needed in this area. With respect to mutations in the extracellular loops, there is C765W in ECL2 that would disrupt the critical disulfide bond formed with C677 in ECL1. Recurrent mutations of P748 at the junction of TM4 and ECL2 (P748H, P748L, and P748R) have been recorded. P748H was well expressed at the cell surface but exhibited reduced

responsiveness to extracellular Ca^{2+}.[120] Several mutations are in ECL3 (and the extracellular part of TM6) potentially pointing to critical interaction of the ECD with this extracellular loop. Rather few inactivating mutations occur in the ICD, the F881L and R886P mutations were identified in cases of familial isolated hyperparathyroidism (FIHP) perhaps pointing to this part of the receptor being important in the parathyroid cell and less so in the nephron (Table II).

B. CASR-Activating Mutations (Table III)

Within the ECD, an important clustering of activating mutations is found from amino acids 116–131 in the loop of each protomer critical for intermolecular bonding to form the CASR dimer. Several mutations are found at C129 (C129F, C129R, C129S, C129Y) and C131 (C131F, C131Y, C131W). The intermolecular covalent linkages—129-S–S-129 and 131-S–S-131—contribute to the maintenance of the receptor in an inactivate state. Consistent with this notion, deletion of loop 2 (amino acids 114–126) in an engineered mutant led to increased sensitivity to Ca^{2+}.[82]

From amino acids 151–297, there is a scattering of activating mutations and these are within the Ca^{2+}-binding sites described above under inactivating mutations. The T151M mutation was found in a very large ADH kindred, one branch of which also had neoplasms such as pituitary adenoma, prostate carcinoma, or medulloblastoma, tissues that express the CASR.[224] Cadmium, an environmental carcinogen, was shown to transform NIH3T3 cells expressing the mutant but not wild-type CASR. This particular branch of the family came from a heavily industrialized area of Texas and it was postulated that the combination of toxic metal exposure and this particular CASR mutant stimulated oncogenesis in some family members.[224] E297D is in Ca^{2+}-binding site 1, Q245R is in site 2, and E228Q and E228K are in site 3. Of interest is that while P221L is activating, different substitutions at the same residue, P221S and P221Q are inactivating. Likewise, while E297D is activating, E297K is inactivating. For the changes at P221, an altered effect on the α-helical structure has been proposed.[95] For E297, 297D could promote enhanced Ca^{2+}-binding whereas the oppositely charged 297K could repulse Ca^{2+} binding.

Few other activating mutations occur in the remainder of the ECD but some mutations are present from amino acids 589–612 within the peptide linker region described earlier. Studies with engineered mutants had suggested this region is important for transmitting the activation signal from the ECD to the TMD. Within the TMD, the E767K in ECL2 mutation is of particular interest given that attention had been focused upon this residue by studies of engineered mutants with the change from a negatively charged amino acid to either a positive or neutral amino acid being activating. A few other activating mutations are scattered along the TMD but there is a striking clustering at ECL3 and the extracellular parts of TM6 and TM7. The recurrent A843E

TABLE II
INACTIVATING MUTATIONS IN THE *CASR*

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E2-5′UTR	c. − 10 acg>atg	FHH	Christie *et al.* (2007)	302
E2-SP	M1R	FHH/NSHPT	De Andrade *et al.* (2006)	221
E2-SP	C7ins/fsX47	FHH	D'Souza-Li *et al.* (2002)	218
E2-SP	L11S	FHH	Pidasheva *et al.* (2005)	44
E2-SP	L13P	FHH/NSHPT	Miyashiro *et al.* (2004)	222
E2-SP	T14A	Poly	Pidasheva *et al.* (2005)	44
E2-ECD	G21R	FHH	Nissen *et al.* (2007)	344
E2-ECD	R25X	NSHPT	Despert *et al.* (2005)	308
E2-ECD	R25X	FHH	Ward *et al.* (2006)	368
E2-ECD	R25X	NSHPT	Christie *et al.* (2007)	302
E2-ECD	Q27R	NSHPT	Chikatsu *et al.* (1999)	298
E2-ECD	P39A	FHH/NSHPT	Aida *et al.* (1995a)	292
E2-ECD	I40F	FHH	Vargas-Poussou *et al.* (2002)	211
E2-ECD	F42S	FHH	Cole *et al.* (2009)	225
E2-ECD	S53P	FHH	Heath *et al.* (1996)	217
E2-ECD	P55L	FHH	Pearce *et al.* (1995)	203
E2-ECD	P55L	FHH	Heath *et al.* (1996)	217
E2-ECD	P55L	FHH	Fukumoto *et al.* (2001)	316
E2-ECD	P55L	FHH	Cetani *et al.* (2003)	119
E2-ECD	P55L	FHH	Speer *et al.* (2003)	358
E2-ECD	P55L	FHH	Cole *et al.* (2009)	225
E2-ECD	C60F	NSHPT	Waller *et al.* (2004)	365
E2-ECD	C60F	NSHPT	Christie *et al.* (2007)	302
E2-ECD	R62M	FHH/NSHPT	Chou *et al.* (1995)	300
IVS2	+ 1g>c	FHH	Cetani *et al.* (2008)	120
IVS2	− 1g>t	FHH/NSHPT	D'Souza-Li *et al.* (2001)	310
E3-ECD	R66C	FHH/NSHPT	Chou *et al.* (1995)	300
E3-ECD	R66H	NSHPT	Pidasheva *et al.* (2006)	92
E3-ECD	I81M	FHH	Cole *et al.* (2009)	225
E3-ECD	G94R	FHH	Defrance-Faivre *et al.* (2008)	305
E3-ECD	G94X	NSHPT	Ward *et al.* (2004)	367
E3-ECD	T100I	FIHP	Warner *et al.* (2004)	269

(*Continues*)

TABLE II (*Continued*)

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E3-ECD	S137P	FHH	Soie *et al.* (1999)	267
E3-ECD	T138M	FHH	Chou *et al.* (1995)	300
E3-ECD	T138M	FHH	D'Souza-Li *et al.* (2002)	218
E3-ECD	G143E	FHH	Chou *et al.* (1995)	300
E3-ECD	G143R	FHH	Cole *et al.* (2009)	225
E3-ECD	G158R	FHH	Cole *et al.* (2009)	225
E3-ECD	L159P	FIHP	Simonds *et al.* (2002)	268
E3-ECD	Y161C	NSHPT	Rajguru *et al.* (2001)	350
E3-ECD	P163R	TCP	Murugaian *et al.* (2008)	341
E3-ECD	Q164X	NSHPT	Waller *et al.* (2004)	365
E3-ECD	Q164X	NSHPT	Christie *et al.* (2007)	302
E3-ECD	Q164del/fsX188	NSHPT	Ward *et al.* (2006)	366
E3-ECD	S166G	FHH	Cole *et al.* (2009)	225
E4-ECD	S171N	FHH	Nissen *et al.* (2007)	344
E4-ECD	R172G	FHH	Christie *et al.* (2007)	302
E4-ECD	R172G	FHH	Henn *et al.* (2008)	320
E4-ECD	L173P	FHH	Felderbauer *et al.* (2005)	313
E4-ECD	L174R	FHH	Ward *et al.* (1997)	369
E4-ECD	N178D	FHH	Pearce *et al.* (1996)	184
E4-ECD	F180C	FHH	Zajickova *et al.* (2007)	81
E4-ECD	R185X	FHH/NSHPT	Kobayashi *et al.* (1997)	197
E4-ECD	R185Q	FHH	Pollak *et al.* (1993)	25
E4-ECD	R185Q	FHH	Heath *et al.* (1996)	217
E4-ECD	R185Q	NSHPT	Bai *et al.* (1997)	198
E4-ECD	R185Q	FHH	Sarli *et al.* (2004)	353
E4-ECD	R185Q	FHH	Christie *et al.* (2007)	302
E4-ECD	R185Q	NSHPT	Obermannova *et al.* (2009)	346
E4-ECD	A194T	FHH	Sinha *et al.* (2008)	357
E4-ECD	W208S	FHH	Christie *et al.* (2007)	302
E4-ECD	W208S	FHH	Demedts *et al.* (2008)	307
E4-ECD	I212S	NSHPT	Christie *et al.* (2007)	302
E4-ECD	I212T	FHH	Marcocci *et al.* (2003)	337
E4-ECD	D215G	FHH	Heath *et al.* (1996)	217

(*Continues*)

TABLE II (*Continued*)

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E4-ECD	Y218C	FHH	Cetani *et al.* (2003)	119
E4-ECD	Y218S	FHH	Pearce *et al.* (1995)	203
E4-ECD	R220P	FHH	Ryan *et al.* (2005)	351
E4-ECD	R220W	FHH	Fukumoto *et al.* (2001)	316
E4-ECD	R220W	FHH	Schwarz *et al.* (2000)	355
E4-ECD	R220W	FHH	D'Souza-Li *et al.* (2002)	218
E4-ECD	R220W	FIHP	Simonds *et al.* (2002)	268
E4-ECD	R220W	FHH	Christie *et al.* (2007)	302
E4-ECD	R220W	NSHPT	Fox *et al.* (2007)	315
E4-ECD	R220W	FHH	Festen-Spanjer *et al.* (2007)	291
E4-ECD	R220W	FHH	Cole *et al.* (2009)	225
E4-ECD	R220Q	FHH	Pearce *et al.* (1996)	184
E4-ECD	P221S	FHH	Pearce *et al.* (1996)	184
E4-ECD	P221Q	FHH	Christie *et al.* (2007)	302
E4-ECD	P221Q	FHH	Nissen *et al.* (2007)	344
E4-ECD	K225T	FHH	Nissen *et al.* (2007)	344
E4-ECD	R227Q	FHH	Chou *et al.* (1995)	300
E4-ECD	R227Q	FHH	Wystrychowski *et al.* (2005)	220
E4-ECD	R227L	NSHPT	Pearce *et al.* (1995)	203
E4-ECD	E250K	FIHP	Simonds *et al.* (2002)	268
E4-ECD	E250K	FHH	Nissen *et al.* (2007)	344
E4-ECD	V268del/fsX273	FIHP	Simonds *et al.* (2002)	268
E4-ECD	S271F	FHH	Nissen *et al.* (2007)	344
E4-ECD	A295T	FHH	Defrance-Faivre *et al.* (2008)	305
E4-ECD	S296N	FHH	Christie *et al.* (2007)	302
E4-ECD	E297K	FHH/NSHPT	Pollak *et al.* (1993)	25
E4-ECD	E297K	FHH	Woo *et al.* (2006)	371
E4-ECD	A321P	FHH	Ono *et al.* (2008)	348
E4-ECD	K323X	FHH	Ward *et al.* (2006)	368
E4-ECD	K335_V337del	FIHP	Warner *et al.* (2004)	269
E4-ECD	P339T	FHH	Hannan *et al.* (2007)	318
E4-ECD	W352X	FHH	Nissen *et al.* (2007)	344
E4-ECD	R392X	NSHPT	Christie *et al.* (2007)	302

(*Continues*)

TABLE II (*Continued*)

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E4-ECD	C395R	FHH	Vigouroux *et al.* (2000)	364
E4-ECD	G397R	FHH	Nissen *et al.* (2007)	344
E4-ECD	A423K	FHH	Livadariu *et al.* (2008)	332
E4-ECD	I427S	TCP	Murugaian *et al.* (2008)	341
E4-ECD	D433H	TCP	Murugaian *et al.* (2008)	341
E4-ECD	T445A	Poly	Simonds *et al.* (2002)	268
E5-ECD	R465Q	FHH	Leech *et al.* (2006)	328
E5-ECD	V477A	TCP	Murugaian *et al.* (2008)	341
E5-ECD	G509R	FHH	Nissen *et al.* (2007)	344
E5-ECD	W530G	FHH	Rus *et al.* (2008)	123
E6-ECD	G549R	FHH	D'Souza-Li *et al.* (2002)	218
E6-ECD	R551K	NSHPT	Toke *et al.* (2007)	362
E6-ECD	G553R	FHH/NSHPT	Schwarz *et al.* (2000, family 1)	355
E6-ECD	G553R	FHH	Schwarz *et al.* (2000, family 2)	355
E6-ECD	G553R	FHH	Nissen *et al.* (2007, family 1)	344
E6-ECD	G553R	FHH	Nissen *et al.* (2007, family 2)	344
E6-ECD	I555V	FHH	Nissen *et al.* (2007)	344
E6-ECD	G557E	FHH	Nakayama *et al.* (2001)	343
E6-ECD	C562Y	FHH	Burski *et al.* (2002)	297
E6-ECD	C562Y	FHH	Nissen *et al.* (2007)	344
E6-ECD	C562Y	FHH	Nissen *et al.* (2007)	344
E6-ECD	C562Y	FHH	Cole *et al.* (2009)	225
E6-ECD	C565G	FHH	Cole *et al.* (2009)	225
E6-ECD	C568Y	FHH	Rus *et al.* (2008)	123
E6-ECD	Y573X	FHH	Nissen *et al.* (2007)	344
E7-ECD	C582Y	NSHPT	Pearce *et al.* (1995)	203
E7-ECD	C582Y	FHH	Nissen *et al.* (2007)	344
E7-ECD	C582Y	FHH	Cole *et al.* (2009)	225
E7-ECD	C582F	FHH	Nissen *et al.* (2007)	344
E7-ECD	N583X	FHH	Pidasheva *et al.* (2006)	92
E7-ECD	S591C	NSHPT	Nyweide *et al.* (2006)	345
E7-ECD	H595Y	FHH	Cetani *et al.* (2008)	120
E7-ECD	S607X	FHH	Pearce *et al.* (1995)	203

(*Continues*)

TABLE II (*Continued*)

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E7-TM1	G623D	FHH	Nissen *et al.* (2007)	344
E7-ICL1	R638L	FHH/NSHPT	D'Souza-Li *et al.* (2002)	311
E7-ICL1	R648X	FHH	Jap *et al.* (2001)	325
E7-ICL1	R648X	FHH	Yamauchi *et al.* (2002)	373
E7-ICL1	R648X	FHH	Defrance-Faivre *et al.* (2008)	305
E7-ICL1	R648X	NSHPT	Ward *et al.* (2004)	367
E7-TM2	L650P	FIHP	Warner *et al.* (2004)	269
E7-TM2	S657Y	FHH	Heath *et al.* (1996)	217
E7-TM2	C661Y	FHH	Cole *et al.* (2009)	225
E7-TM2	L666ins/fsX707	FHH	Nissen *et al.* (2007)	344
E7-TM2	L666P	FHH	Defrance-Faivre *et al.* (2008)	305
E7-TM2	G670R	FHH	Pearce *et al.* (1995)	203
E7-TM2	G670E	FHH/NSHPT	Kobayashi *et al.* (1997)	197
E7-ECL1	R680C	FHH	Pearce *et al.* (1995)	203
E7-ECL1	R680C	NSHPT	Waller *et al.* (2004)	365
E7-ECL1	R680C	NSHPT	Christie *et al.* (2007)	302
E7-ECL1	R680H	FHH/NSHPT	Arunchaiya *et al.* (1998)	294
E7-ECL1	R680H	FHH	Cole *et al.* (2009)	225
E7-TM3	V689M	FIHP	Warner *et al.* (2004)	269
E7-ICL2	W718X	FHH	Rus *et al.* (2008)	123
E7-TM4	V728I	FHH	Nissen *et al.* (2007)	344
E7-TM4	M734R	FHH	Rus *et al.* (2008)	123
E7-TM4	W742R	FHH	Nissen *et al.* (2007)	344
E7-ECL2	P747ins/fsX776	NSHPT	Pearce *et al.* (1995)	203
E7-ECL2	P748H	FHH	Cetani *et al.* (2008)	120
E7-ECL2	P748L	FHH	Mahto *et al.* (2006)	335
E7-ECL2	P748L	FHH	Christie *et al.* (2007)	302
E7-ECL2	P748R	FHH	Heath *et al.* (1996)	217
E7-ECL2	I761del	FHH	Cole *et al.* (2009)	225
E7-ECL2	C765W	FHH	Cetani *et al.* (2008)	120
E7-TM5	G778D	FHH	Ward *et al.* (2006)	368
E7-ICL3	R795W	FHH	Pollak *et al.* (1993)	25
E7-ICL3	R795del/fsX836	FHH	Nissen *et al.* (2007)	344

(*Continues*)

TABLE II (*Continued*)

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E7-ICL3	P798T	FHH	Lam *et al.* (2005)	327
E7-ICL3	A804D	FHH/NSHPT	Miyashiro *et al.* (2004)	339
E7-TM6	F809L	FHH	Timmers *et al.* (2006)	290
E7-TM6	V817I	FHH	Pearce *et al.* (1995)	203
E7-TM6	A826T	Poly	Cetani *et al.* (1999)	272
E7-ECL3	S834del/fsX836	FHH	Ma *et al.* (2008)	334
E7-TM7	L849P	FHH	Rus *et al.* (2008)	123
E7-TM7	A850ins/fsX981	FHH	D'Souza-Li *et al.* (2002)	218
E7-TM7	C851S	Poly	Baron *et al.* (1996)	295
E7-TM7	C851S	Poly	Vigouroux *et al.* (2000)	364
E7-ICD	T876 Alu ins	FHH/NSHPT	Janicic *et al.* (1995)	196
E7-ICD	F881L	FHH	Carling *et al.* (2000)	185
E7-ICD	R886P	FIHP	Simonds *et al.* (2002)	268
E7-ICD	R886W	FHH	Nissen *et al.* (2007)	344
E7-ICD	R896H	FHH	Felderbauer *et al.* (2006)	314
E7-ICD	Q926R	FHH	Rus *et al.* (2008)	123
E7-ICD	A986S	Poly	Heath *et al.* (1996)	217
E7-ICD	D1005N	FHH	Rus *et al.* (2008)	123
E7-ICD	Q1011E	Poly	Heath *et al.* (1996)	217

[a]E1–E7, exons 1–7; IVS, intervening sequence; 5′-UTR, 5′ untranslated region; SP, signal peptide; ECD, extracellular domain; TM1–7, transmembrane helices 1–7; ICL1–3, intracellular loops 1–3; ECL1–3, extracellular loops 1–3; ICD, intracellular domain.

[b]Mutation nomenclature according to Ref. 375.

[c]FHH, familial hypocalciuric hypercalcemia; NSHPT, neonatal severe hyperparathyroidism; FIHP, familial isolated hyperparathyroidism; TCP, tropical chronic pancreatitis; Poly, polymorphism.

mutation within TM7 is particularly active and has been found in patients with Bartter's syndrome subtype V. Further studies are needed to throw light on the mechanistic importance of this region.

Within the ICD, activating mutations are present in which almost all (S895-V1075del) or significant parts (K897ins/fsX979 and Q930del/fsX938) of the COOH-terminal tail are deleted. For the S895-V1075del mutant, increased cell-surface expression (relative to wild type) contributes to the activation. Missense mutations R898G, recurrent A988V, and A988G presented as

idiopathic epilepsy and H994Y as idiopathic hypercalciuria rather than the typical picture of ADH. Thus, portions of the ICD may have selective importance in the role of the CASR in neural transmission or function of the distal nephron (Table III).

XVI. Autoantibodies and the CASR

CASR mutations are found in two-thirds of FHH kindreds. Even taking into account that the disorder is heterogeneous with a gene other than *CASR* being responsible in a few other kindreds, the etiology of one-third of cases is not known. The disease may be due to mutations in parts of the *CASR* gene not currently examined, for example, the promoters and introns and large insertions/deletions.[225] Some patients with anti-CASR autoantibodies (of the inactivating type) associated with autoimmune disorders such as sprue or autoimmune thyroid disease present as an FHH phenocopy termed acquired hypocalciuric hypercalcemia (AHH).[226–228] The anti-CASR antibodies are directed against the ECD and interfere with elevated extracellular Ca^{2+}-mediated suppression of PTH release and perturb Ca^{2+}-sensing in the kidney, thereby closely mimicking FHH.[226] The autoantibodies studied from one patient with AHH potentiated the calcium-activated G_q pathway leading to phosphatidyl inositide (PI) turnover while inhibiting the G_i pathway that phosphorylates ERK1/2.[228] The calcimimetic NPS-R-568, in the presence of extracellular calcium, overcame the effects of the antibody and increased both PI turnover and ERK1/2 activation. It was proposed that the antibody acted in an allosteric manner to maintain the CASR in an active conformation with respect to one signaling pathway only.

Autoantibodies from a subset of patients with autoimmune hypoparathyroidism that inhibited PTH secretion were identified several years ago.[229,230] More recently, the CASR has been identified as a self-antigen in patients with autoimmune polyendocrine syndrome type 1 (APS1) or acquired hypoparathyroidism associated with autoimmune hypothyroidism or idiopathic hypoparathyroidism.[231–235] The activating antibodies are directed against epitopes in the ECD of the receptor and increase IP_3 accumulation and activate MAPK in HEK293 cells stably expressing the CASR, and inhibit PTH secretion from parathyroid cells.[232]

XVII. CASR Polymorphisms

Various polymorphisms (both exonic and intronic) at the *CASR* locus have been identified. Linkage disequilibrium analysis of a large Caucasian cohort using the Haploview program showed that the *CASR* locus is divided into

TABLE III
ACTIVATING MUTATIONS IN THE *CASR*

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E2-ECD	K29E	ADH	Hu *et al.* (2004)	323
E2-ECD	K47N	ADH	Okazaki *et al.* (1999)	347
E3-ECD	A116P	ADH	Christie *et al.* (2007)	302
E3-ECD	A116T	ADH	Baron *et al.* (1996)	295
E3-ECD	N118K	ADH	Pearce *et al.* (1996)	31
E3-ECD	N118K	ADH	De Luca *et al.* (1997)	306
E3-ECD	N118K	ADH	Cole *et al.* (2009)	225
E3-ECD	N124K	ADH	Hu *et al.* (2002)	322
E3-ECD	L125F	ADH	Cole *et al.* (2009)	225
E3-ECD	L125P	ADH	Sato *et al.* (2002)	354
E3-ECD	L125P	ADH	Vargas-Poussou *et al.* (2002)	211
E3-ECD	E127A	ADH	Pollak *et al.* (1994)	26
E3-ECD	E127G	ADH	Christie *et al.* (2007)	302
E3-ECD	E127K	ADH	Lienhardt *et al.* (2001)	32
E3-ECD	E127K	ADH	Christie *et al.* (2007)	302
E3-ECD	F128L	ADH	Pearce *et al.* (1996)	31
E3-ECD	C129F	ADH	Lienhardt *et al.* (2001)	32
E3-ECD	C129R	ADH	Cole *et al.* (2009)	225
E3-ECD	C129S	ADH	Hirai *et al.* (2001)	321
E3-ECD	C129Y	ADH	Burren *et al.* (2005)	296
E3-ECD	C129Y	ADH	Christie *et al.* (2007)	302
E3-ECD	C131W	ADH	Watanabe *et al.* (2002)	212
E3-ECD	C131F	ADH	Suzuki *et al.* (2005)	360
E3-ECD	C131Y	ADH	Christie *et al.* (2007)	302
E3-ECD	T151M	ADH	Lovlie *et al.* (1996)	333
E3-ECD	T151M	ADH	Pearce *et al.* (1996)	31
E3-ECD	T151M	ADH	Hoff *et al.* (1999)	224
E3-ECD	T151R	ADH	Haag *et al.* (2005)	317
E4-ECD	E191K	ADH	Pearce *et al.* (1996)	31
E4-ECD	P221L	ADH	Conley *et al.* (2000)	304
E4-ECD	P221L	ADH	Lienhardt *et al.* (2001)	32
E4-ECD	P221L	ADH	Poppe *et al.* (2002)	349

(*Continues*)

TABLE III (*Continued*)

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E4-ECD	P221L	ADH	Chikatsu *et al.* (2003)	299
E4-ECD	P221L	ADH	Haag *et al.* (2005)	317
E4-ECD	P221L	ADH	Christie *et al.* (2007)	302
E4-ECD	E228K	ADH	Cole *et al.* (2009)	225
E4-ECD	E228Q	ADH	Conley *et al.* (2000)	304
E4-ECD	E241K	ADH	Christie *et al.* (2007)	302
E4-ECD	Q245R	ADH	Conley *et al.* (2000)	304
E4-ECD	E297D	ADH	Silve *et al.* (2005)	93
E4-ECD	E354A	IE	Kapoor *et al.* (2008)	326
E4-ECD	N419S	ADH	Christie *et al.* (2007)	302
E6-ECD	E556K	ADH	Livadariu *et al.* (2008)	332
E6-ECD	C565G	ADH	Cole *et al.* (2009)	225
E7-ECD	F589L	ADH	Leinhardt *et al.* (2001)	329
E7-ECD	E604K	ADH	Tan *et al.* (2003)	361
E7-ECD	E604K	ADH	Alvarez-Hernandez *et al.* (2003)	293
E7-ECD	E604K	ADH	Cole *et al.* (2009)	225
E7-ECD	E610G	ADH	Christie *et al.* (2007)	302
E7-ECD	F612S	ADH	Pearce *et al.* (1996)	31
E7-ECD	F612S	ADH	Mancilla *et al.* (1997)	336
E7-TM1	L616V	ADH	Stock *et al.* (1999)	359
E7-ECL1	Q681H	ADH	Baron *et al.* (1996)	295
E7-TM3	I686V	IE	Kapoor *et al.* (2008)	326
E7-TM4	L727Q	ADH	Mittelman *et al.* (2006)	338
E7-ECL2	E767K	ADH	Uckun-Kitapci *et al.* (2005)	363
E7-TM5	L773R	ADH	De Luca *et al.* (1997)	306
E7-TM5	F788C	ADH	Watanabe *et al.* (1998)	370
E7-TM5	F788C	ADH	Lienhardt *et al.* (2001)	32
E7-TM5	F788C	ADH	Mora *et al.* (2006)	340
E7-TM5	F788L	ADH	Hendy *et al.* (2003)	319
E7-ICL3	E799K	ADH	Lienhardt *et al.* (2001)	32
E7-ICL3	N802I	ADH	Cole *et al.* (2009)	225
E7-TM6	F806S	ADH	Baron *et al.* (1996)	295
E7-TM6	M811V	ADH	Cole *et al.* (2005)	303
E7-TM6	S820F	ADH	Yamamoto *et al.* (2000)	372

(*Continues*)

TABLE III (*Continued*)

Region affected[a]	Mutation name[b]	Disease[c]	References	
			Author	No.
E7-TM6	F821L	ADH	Shiorara *et al.* (2004)	356
E7-TM6	A824S	ADH	Inoue *et al.* (1998)	324
E7-ECL3	T828N	ADH	Christie *et al.* (2007)	302
E7-ECL3	G830S	ADH	Cole *et al.* (2009)	225
E7-ECL3	F832L	ADH	Cole *et al.* (2009)	225
E7-ECL3	F832S	ADH	Dreimane *et al.* (2001)	309
E7-ECL3	A835T	ADH	D'Souza-Li *et al.* (2002)	218
E7-ECL3	V836L	ADH	Hu *et al.* (2005)	110
E7-TM7	A843E	ADH	Nakae *et al.* (1997)	342
E7-TM7	A843E	ADH	Zhao *et al.* (1999)	374
E7-TM7	A843E	ADH	Lienhardt *et al.* (2001)	32
E7-TM7	A843E	ADH	Watanabe *et al.* (2002)	212
E7-TM7	A843E	ADH	Sato *et al.* (2002)	354
E7-TM7	A843E	ADH	Sanda *et al.* (2008)	352
E7-TM7	A844T	ADH	Haag *et al.* (2005)	317
E7-TM7	S845N	ADH	Christie *et al.* (2007)	302
E7-ICD	S895_V1075del	ADH	Lienhardt *et al.* (2000)	330
E7-ICD	K897ins/fsX979	ADH	Lienhardt *et al.* (2001)	331
E7-ICD	R898Q	IE	Kapoor *et al.* (2008)	326
E7-ICD	Q930del/fsX938	ADH	Christie *et al.* (2007)	302
E7-ICD	E942K	ADH	Ekhzaimy *et al.* (2006)	312
E7-ICD	A988V	IE	Kapoor *et al.* (2008, Case 1)	326
E7-ICD	A988V	IE	Kapoor *et al.* (2008, Case 2)	326
E7-ICD	A988G	IE	Kapoor *et al.* (2008)	326
E7-ICD	R990G	Poly	Heath *et al.* (1996)	217
E7-ICD	H994Y	IH	Christie *et al.* (2002)	301

[a]E1–E7, exons 1–7; ECD, extracellular domain; TM1–7, transmembrane helices 1–7; ICL1–3, intracellular loops 1–3; ECL1–3, extracellular loops 1–3; ICD, intracellular domain.

[b]Mutation nomenclature according to Ref. 375.

[c]ADH, autosomal dominant hypocalcemia; IE, idiopathic epilepsy; IH, idiopathic hypercalciuria; Poly, polymorphism.

(at least) three haplotype blocks, coincident with 5′ regulatory, coding, and 3′ domains.[35] Three single nucleotide polymorphisms (SNPs) in exon 7 encode nonconservative amino acid changes in the carboxy-terminal tail of the CASR

protein.[217] The most common missense SNP in Caucasians, c.2956G > T, results in a serine substitution for alanine-986. The other nearby SNPs, c.2968A > G and c.3031C > G, encode a glycine substitution at arginine-990 and a glutamate substitution at glutamine-1011, respectively. Their role in disease pathogenesis is the subject of ongoing investigation. There are large ethnic differences in their frequencies[35] and linkage disequilibrium exists between them, making it difficult to isolate the effects of a single SNP. In some association studies, trilocus haplotypes may be better predictors than any single SNP genotype alone.[236,237]

Functionally activating and inactivating CASR mutations exert their most obvious clinical effects on serum and urine calcium, and it is these quantitative traits that have been scrutinized most with respect to *CASR* SNPs. Initial surveys suggested that both total and ionized serum calcium concentrations were increased (within the normal range) in young women carrying the 986S allele.[238–240] With phase ascertainment across the three missense SNPs, the trilocus haplotype was also predictive of blood ionized calcium in a cohort of healthy Italian men and women.[236] Similar significant differences[241,242] or (nonsignificant) trends[243–246] have been reported in healthy adult controls. On the other hand, several negative studies with apparently adequate design and sufficient power[247–249] suggest that ascertainment issues, such as founder effects or age of population or environmental confounders (e.g., vitamin D status) make replication difficult. Although a few studies have found associations between *CASR* polymorphisms and bone mineral density (BMD),[240,250,251] the majority have found no evidence for this.[246,249,252–255]

Some studies indicate a small but significant overall association between the PHPT phenotype and both A986S and R990G loci.[237,243,244] The minor 986S allele appears to act as a mild inactivating variant promoting increases in serum calcium and relative decreases in calcium excretion. A986S is not a strong predictor of hypercalciuria.[256] While 986S is more common in PHPT patients overall,[237] in those patients having renal stones it is less common with 990G being more frequent.[237,257] The 990G variant has been implicated in increased calcium excretion in idiopathic hypercalciuria consistent with it having an activating function in the kidney.[258,259]

In keeping with the widespread expression of the *CASR*, there is evidence that the three variants (A986S, R990G, or Q1011E) and other SNPs at the *CASR* locus may be associated with a more diverse set of phenotypes than just those associated with bone and mineral disorders. Association studies that have examined either single *CASR* variants or haplotypes as predictors for a variety of common disorders, including hypertension,[260,261] coronary artery disease,[241] cancer,[262–264] chronic pancreatitis,[265] and Alzheimer's disease[266] have provided evidence of a role of the *CASR* gene in these disorders.

XVIII. Altered Expression of CASR and Disease

Altered expression of the *CASR* may play a part in disease pathogenesis or progression (hyperparathyroidism, cancers), a resetting of the calciostat during inflammatory responses (septic shock, burn injury) or hyperresponsiveness of the renal CASR to vitamin D (in ADH patients).

A. Hyperparathyroidism

Hyperparathyroidism is usually not part of the FHH syndrome and histologically the parathyroid glands removed from FHH patients are only occasionally hyperplastic.[178,179] This contrasts with the marked hypercellularity of the glands removed from NSHPT patients and the *Casr* knockout mouse model.[149] However, some FHH kindreds (and FIHP kindreds with *CASR* mutations) have affected family members with elevated PTH levels and parathyroid tumors.[185,267–269] Thus, it may be that some mutant CASRs, even in the heterozygous state, have deleterious effects with respect to parathyroid cell proliferation. The link between parathyroid calcium sensing and proliferative pathways suggested that somatic alterations in the *CASR* gene could be tumorigenic. However, somatic mutation of the *CASR* gene rarely if ever contributes to the pathogenesis of sporadic parathyroid tumors.[270–273] On the other hand, the majority of the parathyroid glands of patients with primary and severe uremic secondary hyperparathyroidism have reduced *CASR* expression.[38,273,274] Thus, mutations in other genes regulating the calcium-sensing pathway may play a significant role in the initiation or the progression of parathyroid tumorigenesis.

Vitamin D insufficiency is common among patients with PHPT and vitamin D deficiency is associated with more severe and progressive disease. The active metabolite, $1,25(OH)_2D$, regulates parathyroid cell proliferation[275] and expression of the *PTH* and *CASR* genes.[39] Humans or mice in which there is homozygous inactivation of the VDR or its ligand[152] manifest marked parathyroid hyperplasia and elevated circulating PTH levels. Like the *CASR* gene, somatic mutation of the *VDR* gene does not contribute to parathyroid tumorigenesis, but VDR expression is reduced in both primary and secondary hyperparathyroid patients. Thus the reduced CASR expression may, in part, be secondary to decreased VDR expression. In FHH-affected individuals, the clinical severity can be exacerbated by vitamin D deficiency promoting overt hyperparathyroidism and vitamin D supplementation can restore the clinical picture to that of modestly elevated serum calcium with PTH levels within the normal range.[81]

B. Hypercalciuria

In the CTAL of the distal nephron, the activated CASR signals to inhibit the K^+ channel that drives the paracellular uptake of cations (see earlier section). Hence, the activated CASR promotes hypercalciuria. Patients with ADH provide a special management problem relative to other forms of hypoparathyroidism. The normal treatment with vitamin D metabolites carries the risk of excessively stimulating renal calcium excretion (while failing to bring serum calcium to the normal range) leading to nephrocalcinosis, nephrolithiasis, and renal damage. Upregulation by 1α-hydroxylated vitamin D metabolites exacerbates the responsiveness to cations by the already oversensitive CASR.[31,32]

Altered regulation of CASR expression by vitamin D metabolites may be critical in genetic hypercalciuria contributing to stone formation. A genetic hypercalciuric stone-forming rat model demonstrates features of human hypercalciuric nephrolithiasis. The rat model exhibits elevated levels of the VDR in the nephron and concomitantly increased CASR expression and defective renal calcium reabsorption.[276,277]

C. CASR and Cancer

The CASR is involved in normal cell proliferation and differentiation control in several tissues (not only the parathyroid gland). Alterations of CASR expression have been implicated in parathyroid neoplasia and breast, prostate and colon cancers.[278,279]

The CASR is expressed in human colon epithelium and regulates cell proliferation and differentiation. Cells of the colon crypt acquire CASR expression as they differentiate and migrate toward the apex of the crypt. CASR expression is weak or absent in colon carcinomas and is inversely correlated with differentiation status. Extracellular calcium and $1,25(OH)_2D$ upregulate CASR and cyclin-dependent kinase inhibitor, p21 and p27, expression in the colon and the chemopreventive activities of calcium and $1,25(OH)_2D$ in colon cancer may be mediated, in part, through the CASR.[280]

The CASR is expressed in both normal and malignant breast tissue and elevated levels are found in highly metastatic primary breast cancer cells and cell lines. In normal breast cells, activation of the CASR inhibits the release of the growth factor and promoter of bone metastases, parathyroid hormone-related peptide (PTHrP). In malignant breast cells, the CASR acts as an oncogene and stimulates the production of PTHrP.[48] The resulting hypercalcemia provides a potent stimulus to further activation of the breast CASR and release of PTHrP generating a so-called vicious cycle.[281] In a similar fashion, enhanced CASR expression and altered proliferation occur in prostate cancer cells.[282,283]

D. Proinflammatory Cytokines

Critically ill patients, with sepsis or burn injury, commonly have hypocalcemia.[284,285] The degree of hypocalcemia is inversely correlated with survival rate. The levels of circulating proinflammatory cytokines, interleukin-1β (IL-1β) and interleukin-6 (IL-6) are elevated and transactivate the *CASR* gene in parathyroid gland, thyroid C cells, and kidney.[40,42] PTH and 1,25 $(OH)_2D$ levels are reduced and those of calcitonin increased contributing to the hypocalcemia. The mechanism may represent a critical counter-regulatory system that attempts to minimize the effects of calcium and cytokines in promoting intravascular coagulation and artherosclerosis during the inflammatory response.

XIX. CASR Allosteric Modifiers in the Clinic

Calcimimetics have been approved for use in patients with chronic kidney disease on dialysis and those with parathyroid carcinoma, and by their direct action on the parathyroid gland CASR they provide an effective medical means of lowering PTH secretion.[286,287] Cinacalcet HCl is marketed as Sensipar in North America and Australia and Mimpara in the European Union. Ongoing clinical trials in patients with mild PHPT have shown that calcimimetics reduce serum calcium and PTH levels and increase serum phosphate levels but do not significantly affect bone turnover or BMD.[288] While calcimimetics provide an important addition to the armamentarium of drugs to treat the secondary hyperparathyroidism of chronic kidney disease, their more widespread use in the medical management of PHPT is uncertain at present.

CASR antagonists, calcilytics, are also being evaluated in clinical trials as a treatment of osteoporosis. As intermittent exogenous administration of PTH produces increases in BMD, it is proposed that once-daily administration of a short-acting calcilytic could achieve a similar result by producing a pulse of endogenous PTH secretion (see Ref. 289 for review).

Other potential areas where the use of CASR allosteric modifiers are being or could be explored include (but are not restricted to) individual cases of FHH[290,291] or ADH, breast, prostate and other cancers, and the exuberant hypocalcemic response of critically ill patients.

XX. Summary

The central role of the parathyroid and kidney CASR as the calciostat controlling systemic mineral ion homeostasis is exemplified by the identification of inactivating and activating mutations in the gene as well as inactivating

and activating autoantibodies. The widespread expression of the CASR predicts functions other than those involved in calcium homeostasis; and the calcimimetics and calilytics either in current or projected clinical use in mineral ion disorders may ultimately find application in a much broader array of diseases.

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