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Calcium-sensing receptor signaling - how human disease informs biology

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Abstract

The calcium-sensing receptor (CaSR) is a class C GPCR that plays a fundamental role in extracellular calcium homeostasis by regulating parathyroid hormone (PTH) release. Although CaSR was identified over 25 years ago, new mechanistic details of how CaSR controls PTH secretion have recently been uncovered demonstrating heteromerization and phosphate binding affect CaSR-mediated suppression of PTH release. Additionally, understanding of how CaSR performs diverse functions in different cellular contexts is just beginning to be elucidated, with new evidence of tissue-specific regulation, and endosomal signaling. Insights into CaSR activation mechanisms and signaling bias have arisen from studies of CaSR mutations, which cause disorders of calcium homeostasis. Functional assessment of these mutations demonstrated the importance of the homodimer interface and transmembrane domain in biased signaling, and showed CaSR mutations can facilitate G-protein-independent signaling. Population genetics studies have allowed a greater understanding of the prevalence of calcemic disorders and revealed new pathophysiological roles.

Keywords (Maximum of 6)

Hypercalcemia; hypocalcemia; parathyroid hormone; signaling bias; sustained signaling

Introduction

The calcium-sensing receptor (CaSR), plays a critical role in extracellular calcium (Ca^{2+}_e) homeostasis by regulating parathyroid hormone (PTH) release and urinary calcium excretion (Figure 1). CaSR binds Ca^{2+} within its extracellular venus fly-trap (VFT) domain[1,2] to activate signaling pathways via: $G_{i/o}$, suppressing cAMP and activating mitogen-activated protein kinase (MAPK); and $G_{q/11}$-phospholipase-C (PLC), mobilising intracellular calcium (Ca^{2+}_i) release and activating MAPK[3,4]. CaSR mutations cause disorders of calcium homeostasis. Inactivating mutations cause familial hypocalciuric hypercalcemia type-1 (FHH1), characterized by lifelong elevated serum calcium, moderate-to-high PTH concentrations and low renal calcium excretion; and rarely cause neonatal severe hyperparathyroidism, which can be fatal if untreated[5]. Activating CaSR mutations cause autosomal dominant hypocalcemia type-1 (ADH1), characterized by mild-to-moderate hypocalcemia, with inappropriately low-to-normal serum PTH[5]. Additionally, inactivating mutations in the G-protein-α11 (Gα11), by which CaSR signals, and the adaptor protein-2 σ-subunit (AP2σ), which regulates endocytosis, cause FHH2 and FHH3, respectively; while activating Gα11 mutations cause ADH2[6-8]. This review focusses on studies from the last three years and begins with new insights into CaSR-mediated control of PTH secretion, before discussing how CaSR mutations have provided insights into receptor activation, internalization; and diverse physiological functions.

Regulation of PTH secretion

PTH acts on bone to enhance resorption, and at kidneys to activate calcium reabsorption and stimulate 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which mobilises intestinal calcium absorption (Figure 1). A number of new mechanisms by which CaSR regulates PTH secretion have recently been uncovered[9-12].

The transient receptor potential canonical channel-1 (TRPC1) is expressed at plasma membranes and mediates calcium entry in response to PLC-coupled receptor activation or calcium store depletion[13]. TRPC1 has recently been described to facilitate CaSR-mediated suppression of PTH secretion[10,13] (Figure 2A). *Trpc1*$^{-/-}$ mice have hypercalcemia, inappropriately high PTH, and reduced urinary calcium excretion; their parathyroid glands (PTG) have impaired PTH secretion; and CaSR-mediated signaling is reduced and PTH secretion enhanced in rat parathyroid-like cells (PTH-C1) depleted of *Trpc1*[10]. These effects were independent of Ca^{2+}_i store depletion, but were $G\alpha_{11}$-dependent, with co-immunoprecipitation indicating $G\alpha_{11}$ may interact with TRPC1[10]. Whether TRPC1, $G\alpha_{11}$ and CaSR form a complex together to potentiate the effects of CaSR on PTH secretion remains to be investigated[10].

Heteromer formation between CaSR and γ-aminobutyric acid-B_1 receptor ($GABA_{B1}R$) has recently been shown to regulate tonic PTH secretion[9] (Figure 2B). In mouse PTG, the $GABA_{B1}R$ agonist baclofen stimulates acute PTH secretion, particularly in low Ca^{2+}_e ranges, whilst a $GABA_{B1}R$ antagonist reduced PTH-max[9]. In contrast, mice with PTG-specific ablation of $GABA_{B1}R$ had reduced

serum calcium and PTH, and their PTG had reduced tonic PTH secretion[9]. In PTH-C1 cells, $GABA_{B1}R$ expression decreased the efficacy of high Ca^{2+}_e to activate G_i and G_q, while addition of baclofen further exacerbated the effects[9]. Treatment with baclofen alone did not modulate cAMP or IP_1, indicating heteromer formation is required for $GABA_{B1}R$ effects. CaSR-$GABA_{B1}R$ heteromers also contribute to pathophysiology: PTGs of patients with primary or secondary hyperparathyroidism (PHPT and SHPT, respectively) had increased heteromer expression; while in mice, deletion of $GABA_{B1}R$ alleviated serum PTH excess and hypercalcemia in mice lacking one CaSR allele, and rescued mice from early death in bialleic CaSR knockouts[9]. It is hypothesised that baclofen transmits a conformational change from $GABA_{B1}R$ to CaSR to reduce G_q and G_i activation[9]. Further studies are required to understand these activation mechanisms.

Phosphate is known to stimulate PTH secretion, but how cells detect phosphate has only recently been revealed[11]. Based on CaSR crystal structures, that harbour putative anion-binding sites, Centano *et al* hypothesised that CaSR may detect phosphate[1,11]. Using HEK293 overexpressing CaSR (HEK-CaSR), pathophysiological concentrations of phosphate, observed in chronic kidney disease (CKD), were shown to significantly reduce CaSR-mediated Ca^{2+}_i and pERK signaling[11]. In isolated human parathyroid cells and mouse PTG, PTH secretion was increased by high phosphate, and reduced when phosphate was restored to physiological levels. CaSR-null mice had no phosphate-mediated stimulation of PTH secretion[11]. An Arg62 residue is critical for phosphate binding: HEK293 expressing Arg62Ala lost phosphate-mediated inhibition of CaSR; and Arg62 forms a salt bridge in the CaSR active state between lobes of the VFT domains, which is broken in the presence of phosphate[11] (Figure 2). Thus, phosphate regulates the equilibrium of active-inactive CaSR. Elevated plasma phosphate shifts the equilibrium towards the inactive conformation, permitting elevated PTH secretion, which may contribute to SHPT and bone loss observed in CKD[1].

In addition to hyperphosphatemia, elevated FGF23 and klotho contribute to SHPT in CKD[14,15]. FGF23 is secreted by bone in response to elevated serum $1,25(OH)_2D$ and phosphate, and by PTH in vitamin D-independent mechanisms[14-17]. FGF23 binds to FGF-Receptor-1 (FGFR1), which with its co-receptor klotho, reduces renal expression of sodium-phosphate-cotransporters to increase phosphate excretion[18]. Elevated serum calcium and FGF23 suppress PTH production by negative-feedback mechanisms, which are disrupted in CKD, resulting in simultaneous increases in serum PTH and FGF23[12]. Why these regulatory mechanisms are disrupted is incompletely understood but recent studies using mice with PTG-specific deletions of CaSR (*PTHCre;*$CaSR^{fl/fl}$), klotho (*PTHCre;*$KL^{PTGfl/fl}$) or both CaSR and klotho (DKO) has revealed klotho regulates PTH secretion in CaSR-dependent and -independent mechanisms[12]. Both DKO and *PTHCre;*$CaSR^{fl/fl}$ mice had reduced body weights, shorter life expectancies, hypercalcemia and hypophosphatemia[12]. Serum PTH, FGF23 and $1,25(OH)_2D$ were elevated in *PTHCre;*$CaSR^{fl/fl}$ mice and significantly higher in DKO mice[12]. The PTG of *PTHCre;*$CaSR^{fl/fl}$ and DKO mice were enlarged and had increased proliferation, which was more severe in DKO mice[12]. These studies indicate klotho is a negative regulator of PTH synthesis in

the absence of CaSR[12]. Klotho expression was decreased in CaSR-deleted PTGs, and reciprocally, CaSR expression was reduced in *PTHCre;KL*$^{fl/fl}$; while co-immunoprecipitation experiments indicated klotho and CaSR may interact[12]. This is consistent with previous studies in which PTGs of patients with PHPT, SHPT, and end-stage renal failure have reduced CaSR and klotho expression[19,20]. Further studies are required to understand how CaSR and klotho function together to control PTH.

Tissue-specific bias

The CaSR is widely expressed and has diverse physiological functions indicating tissue- or ligand-specific CaSR signaling may exist. Two recent publications provide possible insights into this phenomenon.

CaSR activation in osteoblasts is essential for differentiation and bone remodeling[21]. Studies indicate CaSR in osteoblasts activates Akt pathways that phosphorylate β-catenin, facilitating nuclear translocation and promoting expression of genes involved in differentiation and growth[22]. Recent studies revealed a role for the scaffold protein Homer1 in CaSR-Akt pathways[23]. Silencing CaSR or Homer1 in human osteoblasts reduced Ca^{2+}-induced phosphorylation of Akt, GSK-3α/β, β-catenin and mTOR, and nuclear translocation of β-catenin[23]; and suppressed alkaline phosphatase activity[23]. In HEK-CaSR, Ca^{2+}_e did not phosphorylate Akt, but transfection of Homer1 restored Akt signaling[23], indicating this pathway may only be present in tissues expressing Homer1.

CaSR is inhibited by protein kinase-C (PKC)-mediated phosphorylation at Thr888. However, cells expressing the ADH1 mutant Thr888Met, which cannot be phosphorylated, had residual signaling, indicating other phosphorylation sites likely exist[24,25]. Ser875 was predicted as another PKC regulatory site, based on phosphorylation sites in related class C GPCRs[25]. Cells expressing Ser875Ala had increased pERK activity similar to that of Thr888Ala, while cells expressing a double mutant CaSR (S875A/T888A) had increased Ca^{2+}_e sensitivity, indicating an additive effect when both phosphorylation sites are mutated[25]. Distinct tissue- or ligand-specific phosphorylation patterns could be envisaged that activate different signaling pathways as demonstrated for other GPCRs[25-27] and remains to be further investigated.

Receptor activation and signaling bias

Studies of the >400 germline CaSR mutations has provided insights into CaSR activation mechanisms demonstrating the importance of the homodimer interface and TM3-TM6, similar to other GPCRs[5,28] (Figure 3). FHH1 and ADH1 extracellular domain mutations cluster in the homodimer interface, loop 1 and 2 (which span the interface to stabilise dimerization), and ligand-binding sites[1,2,5]. FHH1 transmembrane domain (TMD) mutations are present in the TM1-TM2-TM7 interface, consistent with studies showing loss-of-function GPCR mutations concentrate in these

regions[5,29]. However, there is a larger cluster of inactivating mutations at TM5. In other class C GPCRs TM5 is important in dimerization in the inactive state, which then evolves into a TM6-TM6 interface on receptor activation[30,31]. Therefore, these FHH1 mutations may prevent TMD transitions that are important for receptor activation. Consistent with this, ADH1 transmembrane domain (TMD) mutations cluster at the extracellular side of TM6-ECL3-TM7[1,2,5], indicating TM6 movement is likely important in receptor activation and that ADH1 mutations may favour formation of an active receptor. Residues in which both inactivating and activating mutations occur are also clustered at the dimer interface, TM3 and TM6[32], and are associated with signaling bias. These residues are hypothesised to act as molecular switches that undergo conformational changes on ligand binding, and their mutation facilitates receptor structures that preferentially signal via Ca^{2+}_i or pERK[32].

Studies of an ADH1-associated Arg680Gly mutation provides further details regarding CaSR activation. Arg680Gly biases signaling to enhance MAPK pathways via a G-protein-independent β-arrestin-mediated pathway[33]. Homology modelling and mutagenesis studies revealed Arg680 forms a critical salt-bridge with Glu767 in extracellular loop-2. Its disruption is predicted to allow lateral displacement of TM3 away from TM4 and TM5, facilitating β-arrestin binding[33,34] (Figure 3). The study of an autoantibody to CaSR, causing acquired hypocalciuric hypercalcemia, has revealed further insights into biased signaling. This autoantibody targets the VFT and acts as an allosteric modulator that favours activation of Ca^{2+}_i and impairs pERK signaling[35]. This autoantibody was hypothesised to act in a similar way to allosteric modulators of the GPCR taste receptors which target the VFT and facilitate conformational changes in which the TM5-TM6 interface evolves into closer interactions between TM6-TM6[35,36]. Detailed studies of other CaSR mutations, and those in its signaling protein $G\alpha_{11}$, are likely to provide further insights into receptor activation and G-protein coupling[5,28].

CaSR internalization

Unlike other GPCRs, CaSR is constantly exposed to its ligand, and the ability of the receptor to respond to Ca^{2+}_e fluctuations is aided by the existence of large intracellular reserves of mature CaSR that can rapidly mobilise to cell surfaces by agonist-driven insertional signaling (ADIS)[37]. A lack of consensus regarding CaSR internalization has existed, but was understood to be largely constitutive[38,39].

Two recent studies have investigated CaSR endocytosis in detail. Total internal reflection fluorescence microscopy (TIRFm) was used to measure ADIS and CaSR internalization simultaneously[40]. These studies showed internalization of CaSR by constitutive and agonist-driven mechanisms, which was recently confirmed using diffusion-enhanced resonance energy transfer (DERET) assays[41]. DERET studies also showed the negative allosteric modulator, NPS-2143, and positive allosteric modulator, NPS-R-568, reduce and enhance internalization, respectively[41]. Using G-protein inhibitors and CRISPR/Cas9-edited cells lacking $G_{q/11}$ or β-arrestin1/2, CaSR constitutive

internalization and agonist-driven internalization were shown to require β-arrestin1/2, but were largely G-protein-independent[41]. In contrast, TIRFm studies using the same knockout cells showed $G_{q/11}$ is required for internalization[40]. More detailed studies using both experimental systems are required to investigate the role of G-proteins in CaSR internalization.

TIRFm studies demonstrated AP2σ mutations reduce ADIS and prolong residency time in clathrin structures resulting in impaired CaSR internalization, with a net increase in CaSR surface expression[40]. However, AP2σ mutations reduce CaSR-mediated signaling[7,40]. To explain this paradox it was proposed that CaSR may continue signaling from within cells (sustained signaling)[40], which has previously been shown for other GPCRs[42-46] (Figure 4). A MAPK sustained signal was demonstrated in CaSR-expressing cells that was sensitive to the dynamin inhibitor Dyngo and dominant-negative Rab5, and was absent in AP2σ mutant cell-lines[40]. Furthermore, while plasma membrane signals required both $G_{q/11}$ and $G_{i/o}$, sustained signals were mediated by $G_{q/11}$ only, indicating spatially-directed G-protein selectivity by CaSR[40]. Detailed investigation of sustained signaling in different CaSR-expressing tissues is required to determine whether compartmental bias accounts for diverse CaSR functions.

Pathophysiology using large-scale population genetics

Recent studies of CaSR variants in the DiscovEHR cohort comprising 51,289 individuals showed 60% had at least one common or rare (mean allele frequency <0.01) CaSR variant[47]. Investigation of serum calcium levels of these individuals showed: nonsense/frameshift variants were associated with serum calcium concentrations outside the normal range; individuals with missense variants predicted to be benign were normocalcemic; while individuals with variants predicted to be pathogenic and shown to functionally impact CaSR expression and/or signaling, had changes in serum calcium[47]. This allowed prediction of prevalence estimates within the population of 74.1 per 100,000 for FHH1, and 3.9 per 100,000 for ADH1, far greater than previous analyses[47,48].

Three common CaSR variants (Ala986Ser, Arg990Gly, Glu1011Gln) have previously been inconsistently associated with pathologies including variations in urinary calcium excretion, serum calcium concentrations, nephrolithiasis and coronary artery disease[49-55]. The DiscovEHR cohort revealed: a positive association with serum calcium, hypercalcemia and hyperparathyroidism for the Ala986Ser variant; while the Arg990Gly variant was negatively associated with serum calcium (Smelser *et al*, bioRxiv doi: 10.1101/644559). A much larger study utilising data from the UK biobank and 25 cohorts from the UK, USA, Europe, and China, similarly identified associations between Ala986Ser and serum calcium changes. This was not associated with changes in bone mineral density or risk of fracture[56]. Therefore, common CaSR variants may be associated with lifelong elevated serum calcium levels.

Associations with other pathologies were also explored in the DiscovEHR cohort. Consistent with previous studies[54,55,57], individuals with the Ala986Ser variant had increased risk of

cardiovascular disease, while the Arg990Gly variant was associated with reduced risk of cardiovascular disease (Smelser *et al*, bioRxiv doi: 10.1101/644559). A PheWAS of rare CaSR variants also identified associations with cardiovascular disease. Other disease associations included: a significant increase in type-2 diabetes in individuals homozygous for Ala986Ser; a reduction in CKD in homozygous Arg990Gly individuals; and rare CaSR variants were associated with neurological diseases including dementia and depression, as well as fractures. Further studies in diverse tissues are required to determine whether the CaSR has direct effects or whether these disease associations are secondary to changes in serum calcium.

Summary

Recent studies have highlighted new potential targets for the treatment of calcium-sensing disorders. Dual therapies targeting both CaSR and other proteins involved in PTH secretion can be envisaged; while studies of mutant CaSR proteins have provided important insights into receptor activation that can aid drug design. Additionally, new understandings regarding tissue-specific and sustained signaling offer new avenues to explore for future therapies.

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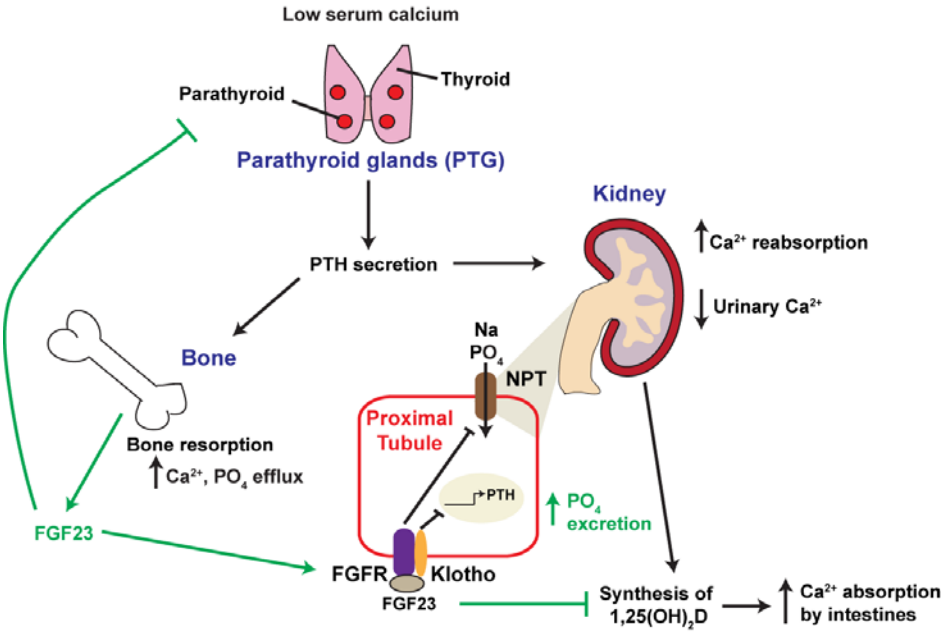
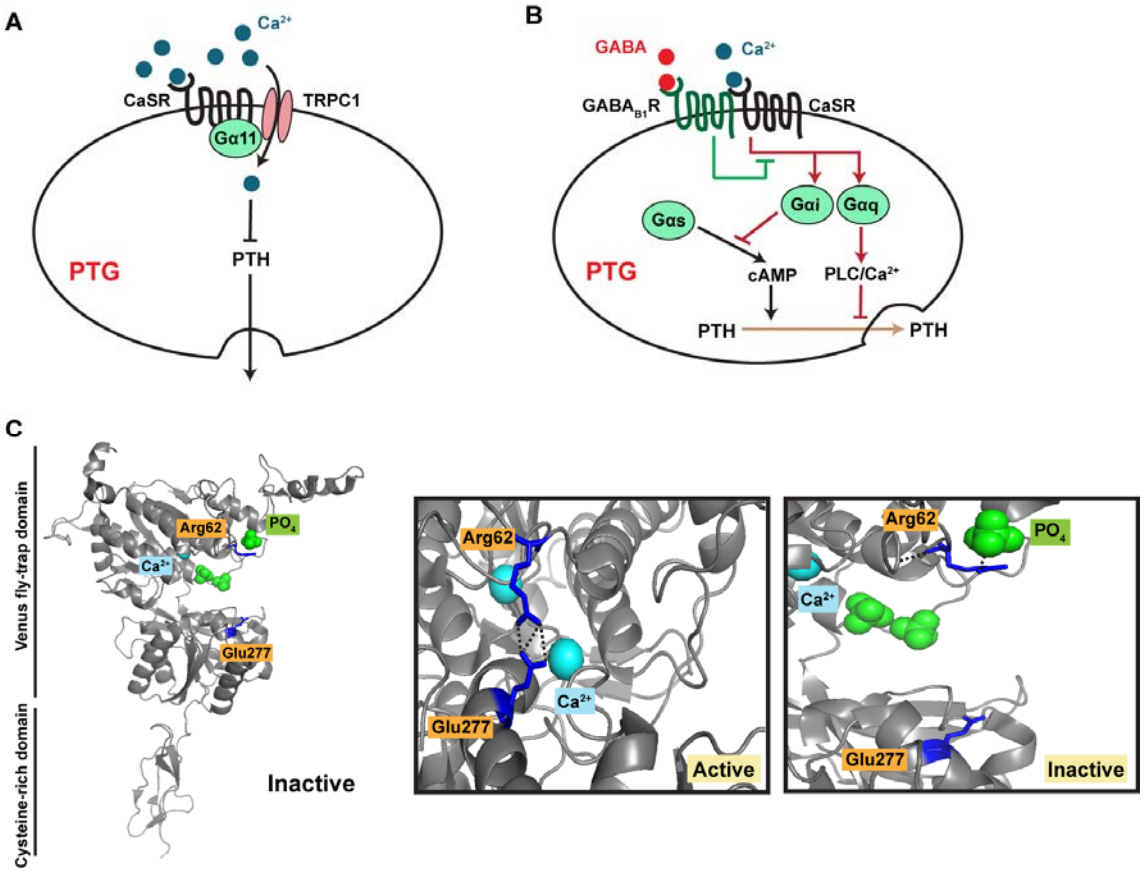
Figures and Tables

Figure 1 Relationship between PTH, 1,25(OH)$_2$D, FGF23 and klotho



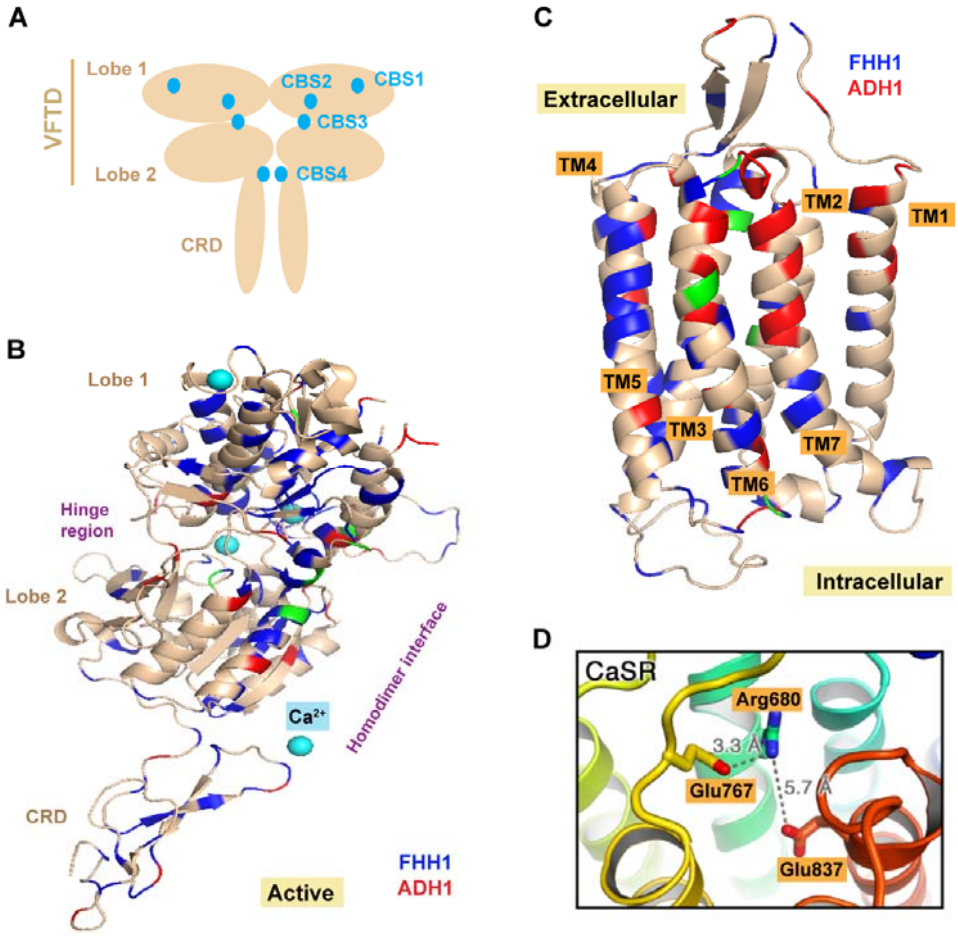
Reductions in serum calcium stimulate PTH secretion from the parathyroid glands. PTH acts at: bone to enhance resorption leading to increased efflux of calcium and phosphate; and kidney reducing calcium excretion and enhancing 1,25(OH)$_2$D synthesis, which stimulates calcium absorption by intestines. The net effect is to normalize serum calcium levels. Elevations outside the normal range activate CaSR on PTG leading to suppression of PTH. In the FGF23-klotho axis (effects shown in green), FGF23 is produced by bone and binds to FGFR-klotho, which reduces PTH secretion and plasma membrane expression of sodium-phosphate transporters (NPT) at the renal proximal tubule. Reduction of NPT at plasma membranes reduces phosphate uptake and increases urinary excretion. FGF23 also inhibits 1,25(OH)$_2$D production and may directly target the PTG to reduce PTH secretion. *Figure adapted from Quarles et al, 2008, JCI*[58].

Figure 2 **New insights into how CaSR regulates PTH secretion**



(A) Schematic showing the proposed role for TRPC1 in PTH secretion from a parathyroid gland (PTG) cell. Recent studies show that TRPC1 suppresses PTH secretion from PTG downstream of CaSR in response to high extracellular Ca^{2+} concentrations. Co-immunoprecipitations indicate that TRPC1 may directly interact with $G\alpha_{11}$. **(B)** Schematic showing the proposed role of CaSR-$GABA_{B1}R$ heteromers in PTG. CaSR homomers couple to G_q and G_i to activate phospholipase-C (PLC) and calcium mobilization, and reduce cAMP, respectively. Both signaling pathways reduce PTH secretion. In CaSR-$GABA_{B1}R$ heteromers (illustrated as a 1:1 stoichiometry, although these details are yet to be elucidated), $GABA_{B1}R$ impairs CaSR signaling by preventing G-protein coupling to CaSR. $GABA_{B1}R$ is important for tonic PTH secretion. **(C)** Left: Crystal structure showing how phosphate interacts with the Arg62 residue in the CaSR venus fly-trap domain to maintain the inactive state. Right: Close view of Arg62 and phosphate in the active and inactive states. In the active state Arg62 in the upper lobe forms a salt-bridge (black dotted line) with Glu227 on the lower lobe. This is broken in the presence of phosphate (inactive state). Images adapted from Centano *et al*, Chang *et al*, Onopiuk *et al*[9-11]. Structures use inactive and active structures from Geng *et al*[1] (PDB:5K5T, PDB:5K5S)[1].

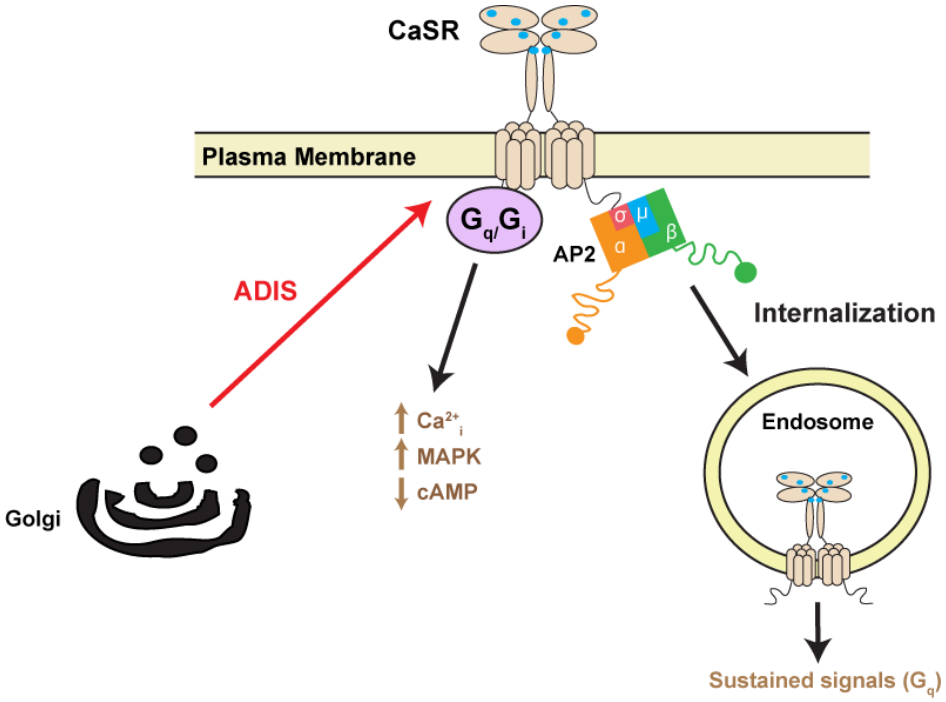
Figure 3 CaSR mutations provide insights into receptor activation



(A) Cartoon of the active CaSR homodimer showing locations of calcium-binding sites (CBS1–4) based on crystal structures (PDB:5K5S)[1]. The CaSR comprises a bi-lobed venus fly-trap domain (VFTD) and a cysteine-rich domain (CRD). **(B)** Structure of the CaSR protomer showing the location of all published FHH1 mutations (blue), ADH1 mutations (red) and sites of both FHH1 and ADH1 mutations (green). Mutations in the ECD are concentrated at the homodimer interface and close to calcium-binding sites. **(C)** Homology model of the CaSR transmembrane region based on the structure of class C GPCR metabotropic glutamate receptor 5 (mGluR5)[31]. FHH1 mutations are present in the TM1-TM2-TM7 interface as observed for other inactivating GPCR mutations. However, there is a cluster of mutations in TM5 indicating this region may be important in retaining the receptor in its inactive state. ADH1 mutations cluster in TM6-ECL3-TM7 indicating TM6 movement is likely important in CaSR activation as observed for other GPCRs. **(D)** Homology model of the CaSR TM3, TM7 and ECL2 region, reproduced from Gorvin, et al, 2018[33]. The homology model is based on the published structure of metabotropic glutamate receptor 1 (GluR1)[59]. The Arg680 residue is shown projecting from TM3 and is predicted to form a salt-bridge with Glu767 on

ECL2. Its disruption is predicted to allow lateral displacement of TM3 away from TM4 and TM5, facilitating β-arrestin binding[33].

Figure 4 **CaSR signals from the plasma membrane and endosomes**



The CaSR exists at plasma membranes as a homodimer. Mature CaSR is made at the Golgi and exists in large intracellular reserves that can be rapidly mobilized to the cell surface in response to receptor activation in a process known as agonist-driven insertional signaling (ADIS). CaSR signals from the plasma membrane predominantly via G_q and G_i. CaSR endocytosis is both constitutive and agonist-driven. AP2σ plays an important role in CaSR internalization and mutations in the protein cause FHH3. CaSR is also able to activate sustained signals from the endosome that use G_q. Mutations in AP2σ impair ADIS, CaSR internalization and sustained signaling.