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# Insights into calcium-sensing receptor trafficking and biased signalling by studies of calcium homeostasis

Caroline M. Gorvin[a,b]

**Author affiliations**

[a]Institute of Metabolism and Systems Research (IMSR), University of Birmingham, Birmingham, B15 2TT.

[b]Centre for Endocrinology, Diabetes and Metabolism (CEDAM), Birmingham Health Partners, Birmingham, B15 2TH, UK

Email: C.Gorvin@bham.ac.uk

Telephone: +44 (0)121 414 6857

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## ABSTRACT

The calcium-sensing receptor (CaSR) is a class C G-protein coupled receptor (GPCR) that detects extracellular calcium concentrations, and modulates parathyroid hormone secretion and urinary calcium excretion to maintain calcium homeostasis. The CaSR utilises multiple heterotrimeric G-proteins to mediate signalling effects including: activation of intracellular calcium release; mitogen-activated protein kinase (MAPK) pathways; membrane ruffling; and inhibition of cAMP production. By studying germline mutations in the CaSR and proteins within its signalling pathway that cause hyper- and hypocalcaemic disorders, novel mechanisms governing GPCR signalling and trafficking have been elucidated. This review focusses on two recently described pathways that provide novel insights into CaSR signalling and trafficking mechanisms. The first, identified by studying a CaSR gain-of-function mutation that causes autosomal dominant hypocalcaemia (ADH), demonstrated a structural motif located between the third transmembrane domain and the second extracellular loop of the CaSR that mediates biased signalling by activating a novel β-arrestin-mediated G-protein-independent pathway. The second, in which the mechanism by which adaptor protein-2 σ-subunit (AP2σ) mutations cause familial hypocalciuric hypercalcaemia (FHH) was investigated, demonstrated that AP2σ mutations impair CaSR internalisation and reduce multiple CaSR-mediated signalling pathways. Furthermore, these studies showed that the CaSR can signal from the cell surface using multiple G-protein pathways, whilst sustained signalling is mediated only by the $G_{q/11}$ pathway. Thus, studies of FHH and ADH associated mutations have revealed novel steps by which CaSR mediates signalling and compartmental bias, and these pathways could provide new targets for therapies for patients with calcaemic disorders.

## 1 Introduction to the CaSR

Extracellular calcium ($Ca^{2+}_e$) is required for diverse biological functions ranging from blood coagulation, mineralisation of bone matrix, muscle contraction, and hormone secretion (Brown 1991). Thus, calcium concentrations within the blood are tightly regulated. The parathyroid gland plays an essential role in calcium homeostasis by detecting $Ca^{2+}_e$ in the blood, and in response to hypocalcaemia, secretes the parathyroid hormone (PTH) to normalize serum calcium concentrations. PTH achieves this by: enhancing bone resorption; activating calcium reabsorption at the kidneys; and stimulating the synthesis of 1,25-dihydroxyvitamin $D_3$, which mobilises intestinal calcium absorption (Fig. 1A) (Riccardi and Brown 2010). The net effect of these three pathways is to increase $Ca^{2+}_e$, which provides feedback inhibition to the parathyroid gland, to suppress PTH secretion (Conigrave and Ward 2013). The parathyroid is able to detect $Ca^{2+}_e$ concentrations in the blood using the cell-surface expressed calcium-sensing receptor (CaSR), a class C G-protein coupled receptor (GPCR), for which $Ca^{2+}$ is the major ligand (Conigrave and Ward 2013; Riccardi and Brown 2010).

The CaSR is a 1078 amino acid protein that exists at cell surfaces as a disulphide-linked homodimer (Ward, et al. 1998), although it is capable of forming heterodimers (Chang, et al. 2007; Gama, et al. 2001). The CaSR has a large extracellular domain, which was recently crystallized by two independent labs, and consists of a bilobed venus fly-trap ligand binding domain (VFTD), and a cysteine-rich domain (CRD) (Geng, et al. 2016; Zhang, et al. 2016) (Fig. 1B). $Ca^{2+}_e$ binds between the two lobes (lobe 1 and lobe 2) of the VFTD, which initiates a conformational change, facilitating lobe 2-lobe 2 dimerisation and allowing the cysteine-rich domains to interact (Geng et al. 2016; Zhang et al. 2016). These conformational changes are predicted to re-orientate the seven transmembrane (TM) domain (Geng et al. 2016), and consequently activate the associated G-proteins and initiate signal transduction (Standfuss, et al. 2011). The CaSR has been reported to couple to multiple G-protein subtypes, but predominantly signals by: the $G_{i/o}$ pathway, to suppress cAMP and activate mitogen-activated protein kinase (MAPK) cascades (Kifor, et al. 2001; Thomsen, et al. 2012); the $G_{q/11}$-phospholipase C (PLC)-mediated pathway, to generate inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol, that activate intracellular calcium ($Ca^{2+}_i$) mobilisations (Hofer and Brown 2003) and

MAPK pathways, respectively. The CaSR can also couple to a G-protein-independent mechanism involving β-arrestin proteins to also activate MAPK signalling (Thomsen et al. 2012) (Fig. 2).

## 2 Human disorders of the CaSR provide insights into receptor signalling mechanisms

The importance of the CaSR in the regulation of $Ca^{2+}_e$ is highlighted in patients with germline mutations of the receptor that have disorders of calcium homeostasis. Heterozygous loss-of-function mutations in the CaSR lead to the autosomal dominant condition familial hypocalciuric hypercalcaemia (FHH) (Pollak, et al. 1993), which is characterised by lifelong mild-to-moderate hypercalcaemia, normal or mildly raised serum PTH levels and low urinary calcium excretion (Firek, et al. 1991; Marx 2015). FHH is considered to be a benign disorder as most patients are asymptomatic, but it can be associated with chondrocalcinosis and pancreatitis in some cases (Hannan and Thakker 2013; Pearce, et al. 1996a; Volpe, et al. 2009). Homozygous and compound heterozygous loss-of-function mutations of the CaSR are the common cause of neonatal severe hyperparathyroidism (NSHPT), which is characterised by marked elevations in serum calcium and PTH, failure to thrive and hyperparathyroid bone disease (Chattopadhyay and Brown 2006; Hannan and Thakker 2013; Pollak et al. 1993). On occasion, heterozygous CaSR mutations may lead to an NSHPT-like phenotype, and this is largely due to the dominant-negative nature of the mutant receptor on the wild-type CaSR in these cases (Obermannova, et al. 2009; Pearce, et al. 1995).

Gain-of-function mutations of the CaSR cause autosomal dominant hypocalcaemia (ADH) characterised by mild-to-moderate hypocalcaemia and inappropriately low or normal PTH concentrations (Hannan and Thakker 2013; Pearce, et al. 1996b). Up to 50% of patients present with hypocalcaemic symptoms of paraesthesia, carpopedal spasms, seizures, and ectopic calcification of the kidneys and basal ganglia (Hannan and Thakker 2013; Pearce et al. 1996b). Some patients with gain-of-function mutations in CaSR may have Bartter syndrome type 5, which is characterised by renal salt wasting, hypokalaemia, hyperreninaemia and hyperaldosteronaemia (Vargas-Poussou, et al. 2002; Watanabe, et al. 2002).

## 2.1 Biased signalling of the CaSR

Functional studies in HEK293 cells have demonstrated that disease-causing mutations may influence CaSR signalling responses in a biased manner (Leach, et al. 2012). Despite the capability of the CaSR to signal via multiple signalling pathways, *in vitro* studies in HEK293 cells have shown that it preferentially couples to the $Ca^{2+}_i$ signalling pathway (Leach et al. 2012). In contrast, disease-causing CaSR mutants have been shown to switch this preferential signalling, with some FHH1-causing mutations signalling equally via the $Ca^{2+}_i$ and MAPK pathways, or predominantly via MAPK pathways; while many ADH1-associated mutants couple more strongly to $Ca^{2+}_i$ (Leach et al. 2012). Furthermore, studies of positive and negative allosteric CaSR modulating compounds, revealed they too can mediate a biased signalling response, with both classes of drugs influencing $Ca^{2+}_i$ to a greater extent than ERK1/2 phosphorylation (Leach, et al. 2013). Thus, these findings established that agonist-induced CaSR signalling may occur in a biased manner, although the GPCR structural motifs mediating ligand-dependent bias were not described.

## 2.2 Novel insights into mechanisms by which CaSR can mediate signalling bias

Recently, a novel ADH1-associated CaSR mutation, Arg680Gly, has provided some insights into the molecular mechanisms mediating signalling bias (Gorvin, et al. 2018a). *In vitro* analyses showed that $Ca^{2+}_i$ signalling in cells expressing the Arg680Gly mutation was not different to that observed in wild-type cells, in contrast to previously reported ADH1-associated CaSR mutants (Gorvin et al. 2018a). However, the Arg680Gly mutation did enhance MAPK signalling (Gorvin et al. 2018a). Furthermore, this type of signalling bias, in which an ADH1 mutation enhances MAPK signalling, but does not affect $Ca^{2+}_i$ has not previously been described and provided an opportunity to explore the role of the Arg680 residue in CaSR structure-function. As MAPK acts as a convergence pathway for multiple CaSR signalling pathways (Fig. 2) each was investigated using a single reporter assay measuring luciferase upstream of a serum-response element (SRE), which acts as a measure for MAPK-mediated transcription (Gorvin, et al. 2017b). By applying compounds that specifically block either the $G_{q/11}$

((YM-245890 and UBO-QIC), the $G_{i/o}$ (pertussis toxin) or the β-arrestin (β-arrestin-1 and β-arrestin-2 targeting siRNA) pathways, it was shown that the Arg680Gly mutation enhanced MAPK signalling by a β-arrestin1/2-mediated pathway (Gorvin et al. 2018a; Schrage, et al. 2015; Takasaki, et al. 2004).

The structural location of the mutant residue within the CaSR transmembrane domain (TMD) region provides some insights into the likely mechanism mediating this bias. Homology modelling of the CaSR TMD, based upon the structure of the closely related human metabotropic glutamate receptor 1 (mGluR1) (Gorvin et al. 2018a; Hu, et al. 2005), predicts that the Arg680 residue lies at the extracellular side of TM3, and that the residue forms salt bridge connections with the side chain of adjacent residues in extracellular loop 2 (ECL2) (Glu767) or TM7 (Glu837) (Fig. 3). Such connections between residues within TMDs or with ECLs of GPCRs are known to be important in receptor activity. For example, TM3 and TM6 of the β2AR forms ionic interactions which lock the receptor in an inactive state, and conformational changes within these helices governs G-protein coupling at the cytoplasmic face of the receptor (Ballesteros, et al. 2001; Rasmussen, et al. 2007). Furthermore, disruption of a salt bridge in β2-AR allows lateral displacement of TM3 away from TM4 and TM5, facilitating β-arrestin binding (Shukla, et al. 2014), and it was therefore hypothesised that the CaSR Arg680Gly mutation may break a salt-bridge between TM3 and ECL2 or TM7 allowing β-arrestin to bind more readily at the CaSR cytoplasmic face (Gorvin et al. 2018a). The importance of the Arg680, Glu767 and Glu837 residues in the CaSR has previously been recognised. Mutation of Glu767 and Glu837 has been shown to increase signaling by the CaSR (Hu, et al. 2006; Hu et al. 2005; Hu, et al. 2002; Uckun-Kitapci, et al. 2005), and previous structural homology models that investigated the binding of allosteric modulator drugs with the CaSR demonstrated that the three residues form critical contacts for drug binding (Miedlich, et al. 2004; Petrel, et al. 2004), indicating their importance in CaSR structure-function. To investigate the hypothesis that a salt-bridge is disrupted by mutation of Arg680, a series of mutations were engineered at the Arg680, Glu767 and Glu837 residues. First, Glu767 and Glu837 were mutated to Arg residues, to introduce unfavourable electrostatic interactions. Under these conditions, the Glu767Arg engineered mutant was shown to

enhance MAPK activity in a similar way to the Arg680Gly ADH1 mutant, a response that was also β-arrestin sensitive (Gorvin et al. 2018a). In contrast, responses in cells expressing the CaSR Glu837Arg engineered mutant were similar to those in cells expressing WT CaSR (Gorvin et al. 2018a). To confirm that a salt-bridge is required between residues 680 and 767 in the CaSR, a double mutant was generated by mutating the Glu767Arg mutant receptor with an additional missense mutation of Arg680 to Glu680. This should allow the salt-bridge between the two residues to reform. Indeed, in this double mutant (Glu680-Arg767) CaSR, MAPK signalling was restored to levels similar to those seen in wild-type cells (Gorvin et al. 2018a).

Thus, these studies have revealed some of the important structural motifs of CaSR that mediate signalling bias, and discovery of this novel β-arrestin-specific pathway may help facilitate the development of targeted therapeutics for CaSR. Furthermore, it demonstrates the importance of investigating multiple signalling outputs downstream of CaSR, to ensure that potentially disease-causing mutations are not classified as benign polymorphisms (Gorvin et al. 2018a).

## 3 Insights from human genetic mutations in components of the CaSR signalling pathway

Both FHH and ADH are genetically heterozygous conditions with mutations in the CaSR accounting for approximately 65% and 70% of cases, respectively (Hannan, et al. 2012; Nesbit, et al. 2013a). Two further genetically distinct forms of FHH, and one further distinct form of ADH, have been described (Mannstadt, et al. 2013; Nesbit et al. 2013a; Nesbit, et al. 2013b). Heterozygous loss- and gain-of-function germline mutations in the α-subunit of the G-protein 11 ($G\alpha_{11}$), a component of the CaSR signalling pathway, give rise to FHH type-2 (FHH2) and ADH type-2 (ADH2), respectively (Mannstadt et al. 2013; Nesbit et al. 2013a). Only four FHH2 and six ADH2 mutations have been described to date, and therefore these mutations account for only a small number of cases of these disorders (Gorvin, et al. 2016; Gorvin, et al. 2017a; Li, et al. 2014; Mannstadt et al. 2013; Nesbit et al. 2013a; Piret, et al. 2016). FHH type-3 (FHH3) is due to mutations in the sigma subunit of the adaptor

protein-2 (AP2σ), which plays a fundamental role in clathrin-mediated endocytosis of transmembrane proteins, such as GPCRs (Nesbit et al. 2013b). The AP2σ protein is ubiquitously expressed and clathrin-mediated endocytosis is a critical cellular process; however, FHH is a largely benign condition, and the phenotypes observed in FHH3 patients are largely CaSR-specific. Thus, by studying the AP2σ mutations identified in FHH3, which have been described in a single residue (Hannan, et al. 2015; Nesbit et al. 2013b), novel insights into the trafficking and signalling mechanisms of CaSR have been elucidated, and indicate an important interplay between these two processes (Gorvin, et al. 2018b).

### 3.1 Trafficking of the CaSR

The cell surface expression of the CaSR is important for detecting extracellular ligand and signalling by the receptor, and therefore the plasma membrane expression of CaSR is carefully regulated. This involves multiple pathways including: receptor synthesis and secretion, trafficking to the plasma membrane, and removal of the CaSR from the cell surface by endocytosis.

#### 3.1.1 Regulation of cell surface expression by functional desensitisation

In contrast to most GPCRs, which undergo agonist-dependent desensitisation by phosphorylation and/or by β-arrestin proteins, functional desensitisation appears to have only a minimal impact on CaSR expression, and there are inconsistencies between findings in these studies (Bouschet, et al. 2005; Lorenz, et al. 2007; Pi, et al. 2005; Thomsen et al. 2012). Initial studies of the CaSR showed that several proteins desensitise the receptor. These include: PKC, which phosphorylates the receptor following activation of signalling pathways and recruits β-arrestin; G-protein regulatory kinase (GRK) 2, which binds to $G\alpha_q$ and inhibits its signalling; and, GRK4 which phosphorylates CaSR to facilitate desensitisation (Lorenz et al. 2007; Pi et al. 2005). Furthermore, GRKs and β-arrestin have independent functions as combined treatment of cells with these proteins enhances desensitisation (Lorenz et al. 2007). The traditional view of GPCR desensitisation and internalisation was one in

which the receptor is phosphorylated and β-arrestin recruited, followed by internalisation of the receptor by clathrin-mediated endocytosis, which is facilitated by interactions between β-arrestin and AP2 (Shukla, et al. 2011). While studies of CaSR showed that β-arrestin can be recruited, this did not enhance receptor internalisation, which only occurred when cells were treated with high concentrations of $Ca^{2+}_e$ (10mM) (Lorenz et al. 2007). β-arrestin is also now recognised to have another function, as a scaffold protein that facilitates signalling either at the plasma membrane, or for some GPCRs, at the endosome (Shukla et al. 2014). This function of β-arrestin has been recognised for CaSR in some studies, providing seemingly contradictory information to that in studies of functional desensitisation. Thus, treatment of cells with dominant-negative forms of β-arrestin1 or β-arrestin2, or with siRNA targeting β-arrestin1 or β-arrestin2, reduces the pERK and membrane ruffling signals downstream of CaSR (Bouschet et al. 2005; Gorvin et al. 2018b; Thomsen et al. 2012). Further studies are required to determine whether the discrepancies within these data sets are due to experimental differences, differences in cell type, or if both desensitisation and enhanced signalling occur downstream of CaSR, but at different spatial or temporal points.

### 3.1.2 Regulation of cell surface expression by the secretory pathway and agonist-driven insertional signalling (ADIS)

The cell surface expression of CaSR, and mechanisms by which the receptor is synthesised and exported to the plasma membrane, are generally well understood. The CaSR is synthesised at the ER where it forms homodimers (Pidasheva, et al. 2006) and undergoes quality control and immature glycosylation steps prior to progression to the Golgi (Fan, et al. 1997). At the Golgi, the CaSR undergoes further maturation. This forward trafficking through the secretory pathway involves a number of regulatory proteins that have been well described in previous reviews (Breitwieser 2013; Huang, et al. 2011). The core-glycosylated CaSR is then retained within pre-plasma membrane compartments (Fan et al. 1997). Therefore CaSR is retained intracellularly within two large intracellular reserves: one at the ER and one at pre-plasma membrane compartments (Breitwieser 2012).

The large pool of fully mature CaSR plays a unique role in regulation of $Ca^{2+}_e$. Unlike many GPCRs, the CaSR is chronically exposed to its ligand at baseline concentrations, and thus the receptor undergoes very little functional desensitisation (Gama and Breitwieser 1998). Furthermore, CaSR is able to elicit signalling responses for as long as elevated $Ca^{2+}_e$ is available (Grant, et al. 2011; Lorenz et al. 2007; Pi et al. 2005). Experimental studies have sought to explain this apparent paradox and using a combination of total internal reflection fluorescence microscopy (TIRFm) and a construct that allows the simultaneous measurement of CaSR insertion within plasma membranes and endocytosis (known as BSEP-CaSR), a new model for CaSR cell surface expression has emerged (Fig. 4). This model proposes that in the basal state, CaSR is only weakly expressed at the plasma membrane, but on exposure to increases in $Ca^{2+}_e$ there is an increase in anterograde trafficking through the secretory pathway (Grant et al. 2011) (Fig. 4). This increase in the secretory pathway involves both mature CaSR located in pre-plasma membrane regions, and newly synthesised CaSR from the ER (Breitwieser 2013; Grant et al. 2011), and at present the mechanisms that trigger the ADIS events are not fully elucidated. However, studies in which ADIS and CaSR signalling were measured simultaneously revealed that elevated $Ca^{2+}_i$ and the $G\alpha_{q/11}$ pathway is likely involved (Gorvin et al. 2018b; Grant, et al. 2012). Furthermore, the 14-3-3 proteins, which bind CaSR at an Arg-rich site within the C-terminus, limits the release of CaSR from the endoplasmic reticulum, and therefore regulates CaSR cell surface expression by reducing the ADIS-mobilisable pool of the receptor (Grant, et al. 2015). The presence of the ADIS system and constitutive endocytosis may also explain some of the inconsistencies between studies of CaSR trafficking, as endocytosis of the receptor is balanced by the continual insertion of new CaSR from the secretory pathway (Breitwieser 2013). Those studies in which a tagged construct and cell surface labelling (e.g. FLAG) was used in isolation, without also measuring insertion of new CaSR within the plasma membrane, may not reflect the physiological state. Similarly, measurement of total cell surface expression of receptor cannot be used as a surrogate for determining the rate of internalisation.

These studies have demonstrated that trafficking of the CaSR plays a critical role in receptor signalling and calcium homeostasis. More recent studies, building upon these initial findings, have identified further insights into CaSR cell surface expression and endocytosis, and recognised further connections between the trafficking and signalling of this receptor.

### 3.1.3 Regulation of cell surface expression by endocytosis

The CaSR has been described to have two types of internalisation: a constitutive pathway (Grant et al. 2011), and an agonist-driven pathway (Gorvin et al. 2018b; Lorenz et al. 2007). The CaSR was shown to internalise using clathrin-mediated endocytosis in early studies (Holstein, et al. 2004). In addition, CaSR has been described to associate with several proteins that facilitate clustering at the plasma membrane and therefore increase the efficiency of internalisation, or act as scaffolds to enable signalling to occur. One such protein is caveolin-1, with which CaSR has been shown to coimmunoprecipitate (Kifor, et al. 1998). CaSR has been described to be enriched in caveolae structures in parathyroid chief cells and osteosarcoma cells (Jung, et al. 2005; Kifor et al. 1998; Sun and Murphy 2010). CaSR-mediated signalling is impaired in cells treated with caveolin-1 targeting siRNA, and it is likely that this signalling function is facilitated by caveolae acting as signalling hubs allowing the CaSR, G-proteins and PKC to cluster together (Jung et al. 2005; Kifor et al. 1998; Sun and Murphy 2010). However, it is currently unknown whether caveolin facilitates CaSR clustering within caveolae structures to enhance signalling, or whether the signalling itself drives this clustering to encourage endocytosis (Breitwieser 2013). Filamin is an actin binding cytoskeleton protein that is important for protein scaffolding (Hjalm, et al. 2001) and may also facilitate clustering that aids in receptor endocytosis. Filamin binds to the CaSR C-terminus and increases total cellular content of CaSR by preventing its proteosomal degradation (Hjalm et al. 2001), with some studies showing CaSR expression is reduced in cells treated with siRNA targeted against filamin. However, other reports show no such changes in CaSR expression, and this likely requires further investigation (Huang, et al. 2006; Mingione, et al. 2017). The net result of Filamin A binding to CaSR is increased MAPK signalling by the receptor (Hjalm et al. 2001; Pi, et al. 2002).

283

284 **3.1.4 Effect of AP2σ mutations on CaSR signalling and trafficking**

285 Mutations in AP2σ have been demonstrated to cause FHH3, and are associated with impaired CaSR-
286 mediated $Ca^{2+}_i$ signalling (Nesbit et al. 2013b). The AP2 complex is a ubiquitously-expressed
287 heterotetrameric protein which plays a fundamental role in the clathrin-mediated endocytosis of
288 transmembrane proteins, such as GPCRs. The two larger subunits, α and β, have appendages that bind
289 to the clathrin coat proteins, plasma membrane phospholipids, and endocytic accessory proteins (e.g.
290 β-arrestin); while the two smaller subunits, μ and σ, bind to endocytic motifs of cargo proteins
291 (Collins, et al. 2002; Jackson, et al. 2010; Kelly, et al. 2008; Kirchhausen, et al. 2014) (Fig. 4). The
292 AP2μ subunit recognises tyrosine-based motifs and the AP2σ subunit recognises dileucine-based
293 motifs (Haucke and De Camilli 1999; Kelly et al. 2008) (Fig. 4). FHH3-associated mutations in the
294 AP2σ protein have been reported to affect the Arg15 residue, and structural modelling studies using a
295 published structure of the AP2 complex, have shown these missense mutations (to Cys15, His15 and
296 Leu15) likely disrupt interactions with a putative dileucine motif in the CaSR C-terminus (Nesbit et
297 al. 2013b), and thus affect CaSR endocytosis.

298

299 Initially this hypothesis was tested using an ELISA assay of total CaSR at cell surfaces in HEK293
300 cells stably overexpressing CaSR (HEK-CaSR), and transiently transfected with AP2σ-mutant
301 proteins. This showed that AP2σ-mutant expressing cells had increased CaSR cell surface expression
302 when compared to AP2σ-WT cells following stimulation with 5mM $Ca^{2+}_e$ (Nesbit et al. 2013b). Thus,
303 it was concluded that CaSR endocytosis is impaired in these cells resulting in increased total CaSR
304 cell surface expression (Nesbit et al. 2013b). More detailed studies characterising the ADIS and
305 endocytosis components of CaSR regulation in AP2σ mutant cells using TIRFm and the BSEP-CaSR
306 construct, showed both ADIS and CaSR endocytosis were impaired resulting in the net effect of an
307 increased total CaSR cell surface expression in cells expressing AP2σ mutant protein compared to
308 WT cells (Gorvin et al. 2018b). Additional TIRFm studies focussing on CaSR and clathrin

309 demonstrated that CaSR and clathrin colocalise at plasma membranes, and that the duration of this
310 colocalisation is prolonged in AP2σ-mutant expressing cells (Gorvin et al. 2018b). Furthermore, the
311 vesicles containing both CaSR and clathrin in AP2σ-mutant cells, when compared to WT cells, were
312 less motile, which is an indication that the vesicles are less likely to result in viable endocytic events
313 (Gorvin et al. 2018b; Rappoport and Simon 2003). Thus, cells expressing AP2σ-mutant proteins have
314 delayed recruitment of clathrin, and colocalisation with CaSR is prolonged, resulting in impaired
315 CaSR endocytosis (Gorvin et al. 2018b).

316

317 The effect of the FHH3-associated AP2σ-mutant proteins on multiple CaSR-mediated signalling
318 pathways has been characterised in a number of cellular assays in HEK293 cells stable overexpressing
319 AP2σ WT and mutant proteins and lymphoblastoid cell-lines derived from blood samples from FHH3
320 patients with the AP2σ-Cys15 mutation (Gorvin et al. 2018b; Nesbit et al. 2013b). These studies
321 confirmed that AP2σ mutations of the Arg15 residue impair $Ca^{2+}_i$ mobilisation, phosphorylated
322 ERK1/2 (pERK1/2) MAPK signalling, membrane ruffling and suppression of cAMP, and that all of
323 these pathways occur downstream of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ (Gorvin et al. 2018b; Nesbit et al. 2013b).
324 Therefore, these studies have shown that AP2σ mutations reduce endocytosis resulting in increased
325 CaSR cell surface expression, but paradoxically decrease CaSR-mediated signalling (Gorvin et al.
326 2018b). To explain this paradox, we hypothesised that CaSR may be able to continue signalling from
327 within the cell (i.e. sustained signalling) (Gorvin et al. 2018b). Such sustained signalling has been
328 previously reported for some class A (e.g. β2-adrenergic receptor (β2AR), dopamine receptor D1
329 (DRD1), thyroid-stimulating hormone receptor (TSHR), vasopressin receptor 2 (V2R) and luteinizing
330 hormone receptor (LHR)), and class B (e.g. parathyroid hormone 1 receptor (PTH1R)) GPCRs
331 (Calebiro, et al. 2009; Feinstein, et al. 2013; Ferrandon, et al. 2009; Irannejad, et al. 2013; Jean-
332 Alphonse, et al. 2014; Kotowski, et al. 2011). Thus, in cells with the AP2σ mutation, in which there is
333 impaired endocytosis, the availability of internalised receptors from which sustained signals could
334 emanate would be reduced and thus the net effect would be impaired overall CaSR-mediated
335 signalling. To test this hypothesis, a combination of imaging and biochemical analyses, along with

chemical inhibitors were used in HEK-CaSR, HEK-AP2σ and CRISPR-Cas generated β-arrestin knockout cells to assess CaSR-mediated MAPK signalling (Gorvin et al. 2018b). To assess sustained signalling two primary assays were used: 1) assessment of pERK1/2 over 60 minutes following treatment of cells with a 5 min pulse of 5mM $Ca^{2+}_e$; and 2) analysis of SRE luciferase reporter responses over 12 hours, following a 5 min pulse of 5mM $Ca^{2+}_e$. Using these methods, MAPK sustained signals were demonstrated in HEK-CaSR cells, and evidence for an internal, likely endosomal, source was shown in three ways (Gorvin et al. 2018b) (Fig. 5). First, addition of Dyngo, a chemical inhibitor of dynamin, which is required for vesicle scission during clathrin-mediated endocytosis, abolishes sustained signals, whilst rapid plasma membrane mediated signals remain intact. Loss of this sustained response in Dyngo-treated cells was not due to increased apoptosis or decreased proliferation, and was unaffected by inhibition of CaSR protein synthesis as the sustained rise in pERK1/2 was not blocked by tunicamycin, an inhibitor of glycosylation of newly synthesised CaSR. Second, sustained signals were reduced in cells expressing a dominant-negative Rab5 protein, which delays maturation of early endosomes and therefore slows clathrin-mediated endocytosis. Third, reduction or loss of these sustained signals was observed in cells expressing AP2σ mutant proteins (Fig. 5).

The G-proteins involved in these sustained signals were also explored as MAPK signalling is a convergence pathway for multiple CaSR-mediated signalling pathways (Fig. 2). The ability of the $G\alpha_{q/11}$ and $G\alpha_{i/o}$ pathways to activate CaSR sustained signals was investigated using G-protein specific inhibitors (UBO-QIC and PTx) and the SRE luciferase reporter assay, which showed that inhibition of both $G\alpha_{q/11}$ and $G\alpha_{i/o}$ impaired the early, plasma membrane mediated CaSR signal, while only $G\alpha_{q/11}$ was important for the later sustained signal. Furthermore, confocal microscopy confirmed that $G\alpha_q$ and phosphatidylinositol 4,5-bisphosphate ($PIP_2$), the lipid hydrolysed by PLC, colocalise with CaSR at plasma membranes and a subpopulation of endosomes (Gorvin et al. 2018b). In addition, inhibitors of the PLC-DAG-$IP_3$ pathway (U73122, GF-109203X and 2-aminoethoxydiphenyl borate (2-APB), which inhibit PLC, PKC and the $IP_3$-receptor, respectively)

were shown to reduce sustained pERK1/2 signals, indicating that $G\alpha_{q/11}$ and its signalling pathway are important for CaSR-mediated signalling (Gorvin et al. 2018b). Finally, the effects of the β-arrestin scaffold proteins, which are important for the sustained endosomal signalling of some GPCRs such as V2R and PTH1R (Feinstein et al. 2013; Wehbi, et al. 2013), were assessed in HEK-CaSR cells with deletion of the β-arrestin1 and β-arrestin2 genes. In these cells both pERK and SRE reporter responses were unaffected by deletion of the β-arrestin proteins (Gorvin et al. 2018b). Thus, the CaSR mediates some MAPK signals from endosomes using $G\alpha_q$ and PLC, but does not require β-arrestin for this pathway.

**Conclusions and important lessons for the future (or unanswered questions)**

Since the cloning of the CaSR gene 25 years ago (Brown, et al. 1993), many insights have been gained into the role of the CaSR in calcium homeostasis, and by studying patients with mutations within this gene we have learnt much about CaSR signalling and trafficking mechanisms. The discovery that CaSR can signal from within the cell, most likely from an endosomal source, and that disruption of a specific structural motif can mediate G-protein independent signalling bias opens up many new avenues of investigation. How do mutations in the CaSR affect these endosomal pathways? Can drugs that target the CaSR affect these pathways? How are trafficking and signalling of the CaSR so intimately linked? Future studies investigating these questions will undoubtedly reveal further complexities into the regulation of CaSR, and could provide mechanisms relevant to other GPCRs.

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## Figures

### Figure 1 Regulation of extracellular calcium at the parathyroid glands

(**A**) Schematic illustrating how the parathyroid glands respond to changes in extracellular calcium concentrations [$Ca^{2+}_e$] in the blood. In the presence of low [$Ca^{2+}_e$] in the blood, the parathyroid glands secrete parathyroid hormone (PTH). PTH binds to PTH receptors at the kidney, resulting in calcium reabsorption, and activation of 1,25-dihydroxyvitamin $D_3$; which in turn acts upon bone to mediate calcium release, and the intestine to activate calcium resorption. PTH also acts directly on bone to activate calcium release. The tissue-specific effects on calcium are shown in the hatched box with an arrow to indicate that these are increased by low [$Ca^{2+}$] in the blood. (**B**) Schematic of the homodimeric calcium-sensing receptor (CaSR) showing the two protomers (CaSR1 and CaSR2) within the plasma membrane (PM). The calcium-sensing receptor is expressed highly at the parathyroid gland and its major function is to detect [$Ca^{2+}_e$] and regulate PTH secretion accordingly. The CaSR has a large extracellular domain (ECD) comprising the venus flytrap domain (VFTD) that contains two lobes (lobe 1 and lobe 2), and a cysteine-rich domain (CRD). The CRD connects the ECD to the 7 transmembrane domains (TMD), and the CaSR has a long cytoplasmic intracellular domain (ICD). Ionised calcium binds between the two lobes of the VFTD. In the presence of high calcium the CaSR is activated, leading to changes in its signal transduction and reduced PTH secretion.

### Figure 2 Major signalling pathways of the calcium-sensing receptor (CaSR)

Schematic diagram of the calcium-bound homodimeric CaSR and its major signalling pathways. The CaSR is expressed at the plasma membrane (PM) where its function is to detect [$Ca^{2+}_e$] in the blood. The CaSR activates two major signalling pathways: $G_{q/11}$ and $G_{i/o}$. Activation of the $G_{q/11}$ pathway leads to stimulation of its major effector protein phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to the second messengers diacylglycerol (DAG) and inositol trisphosphate ($IP_3$). DAG activates protein kinase C (PKC), and the mitogen-activated protein kinase (MAPK) signalling pathway. $IP_3$ binds to $IP_3$ receptors at the endoplasmic reticulum (ER),

which mobilise intracellular calcium ($Ca^{2+}_i$) release into the cytoplasm. This increase in $Ca^{2+}_i$ can further activate MAPK signalling. The CaSR also activates $G_{i/o}$ signalling pathways, which inhibits adenylate cyclase (AC), resulting in reductions in cAMP and protein kinase A (PKA) activity. This reduction in PKA relieves the inhibition on MAPK signalling and therefore provides another activation pathway for MAPK signalling. The net effect of all these signalling pathways is a change in gene transcription.

**Figure 3 Disruption of an Arg680-Glu767 salt-bridge within CaSR mediates signalling bias by activation of a G-protein independent, β-arrestin pathway**

(**A**) Schematic diagram of a CaSR monomer at the plasma membrane (PM) showing the seven transmembrane domains (TM1-7) with extracellular loops 1–3 (ECL1–3) and intracellular loops 1–3 (ICL1–3). Arg680 is located at the extracellular end of TM3 and is predicted to form a salt-bridge with either a Glu767 residue on ECL2 or a Glu837 residue on TM7. (**B**) Homology model of the CaSR TM3, TM7 and ECL2 region, reproduced with permission from Gorvin *et al*, 2018, Science Signaling (Gorvin et al. 2018a). The homology model is based on the published structure of mGluR1 (Wu, et al. 2014). The Arg680 residue is shown projecting from TM3 and is predicted to form a salt bridge with Glu767 on ECL2. The Glu837 residue on TM7 lies at a 5.7Å distance from the Arg680 residue, and therefore formation of a salt bridge between Arg680 and Glu837 is less likely, but was tested as the homology model may not reflect the true state of CaSR.

**Figure 4 Mechanisms by which calcium-sensing receptor (CaSR) expression is regulated at plasma membranes**

Schematic showing trafficking pathways regulating the expression of CaSR at the plasma membrane. The CaSR is continuously synthesised at the endoplasmic reticulum (ER) and undergoes post-translational modifications at the ER and Golgi, before export to the plasma membrane surface. In addition to this constitutive anterograde trafficking of the CaSR, an additional export pathway for the CaSR has been described. This pathway is activated by high $[Ca^{2+}]_e$ and has been named the agonist-

driven insertional signalling, or ADIS, pathway. Removal of the CaSR from the cell surface is regulated by clathrin-mediated endocytosis that requires the heterotetrameric adaptor protein-2 (AP2). AP2 binds directly to transmembrane proteins using its μ-subunit or σ-subunit. The CaSR has a putative dileucine motif within its C-terminus with which it is predicted to bind to AP2σ. The α-subunit and β-subunit have large appendages that are important for binding to clathrin coat proteins, plasma membrane phospholipids, and endocytic accessory proteins. Once internalised within clathrin-coated vesicles, CaSR is targeted to the endosomal-lysosomal system. Experimental evidence suggests CaSR is degraded at the lysosome, and very little, if any, recycling of the receptor occurs.

**Figure 5 The calcium-sensing receptor (CaSR) can activate plasma membrane and endosomal signalling pathways**

Schematic diagram showing the signalling pathways that occur downstream of the CaSR. As described in Figure 2, CaSR predominantly signals via: the $G_{q/11}$ pathway, leading to activation of the intracellular calcium ($Ca^{2+}_i$) and mitogen-activated protein kinase (MAPK) pathways; and the $G_{i/o}$ pathway leading to activation of MAPK and reductions in the cAMP pathways. These pathways are activated rapidly (within 2-5 minutes), and originate at the plasma membrane. The CaSR can also signal from an internal location using a $G_{q/11}$ pathway. This sustained signal that occurs later than the plasma membrane signal (from 30 minutes), is sensitive to: global clathrin-mediated endocytosis blockade (shown experimentally using dyngo); inhibition of CaSR-mediated internalisation (demonstrated in cells expressing FHH3-associated AP2σ mutants); and maturation of internalised vesicles to the early endosome (demonstrated experimentally using a dominant-negative (DN) form of Rab5). Thus, this sustained signal is likely to arise within endosomes.

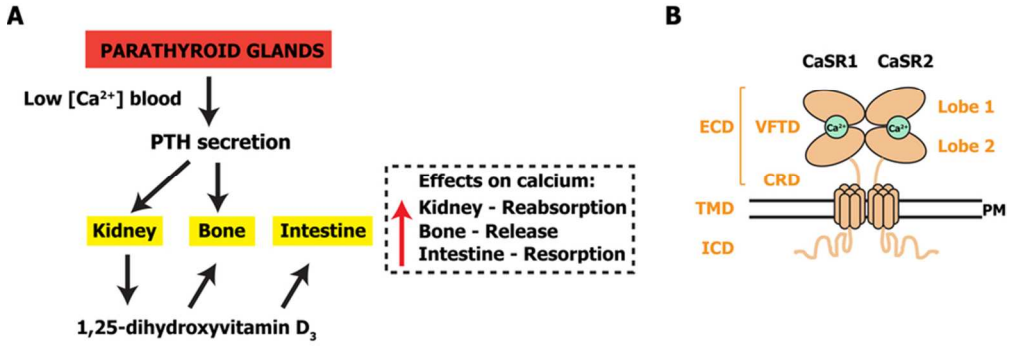


Figure 1

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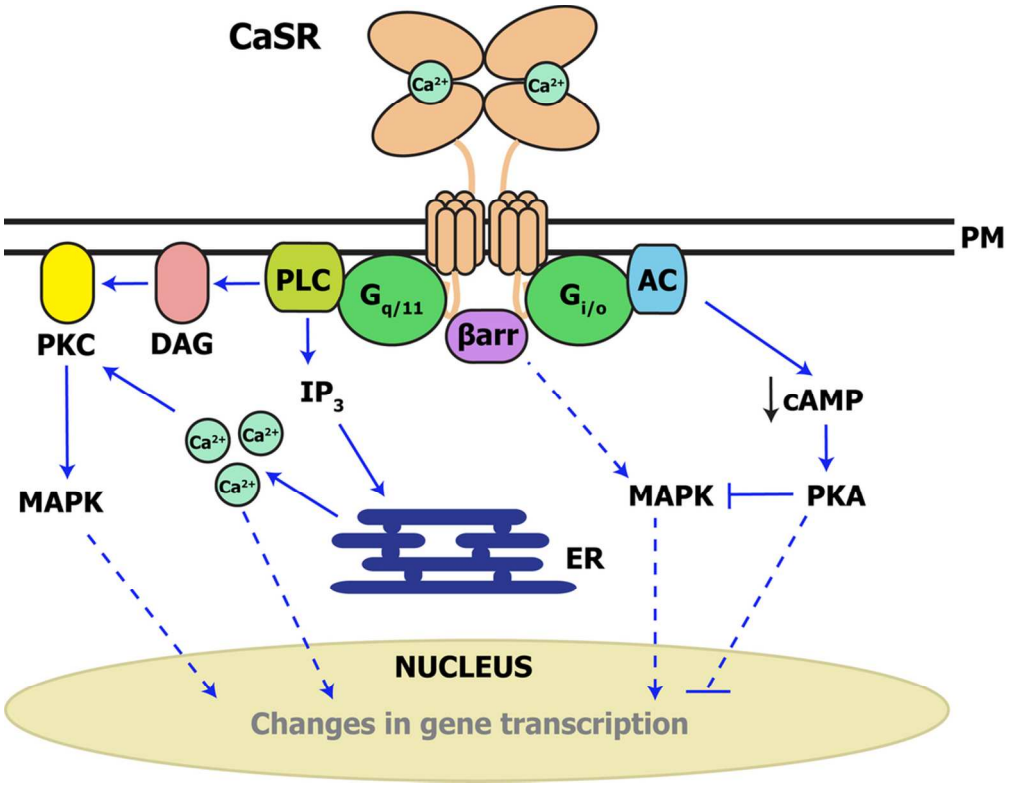


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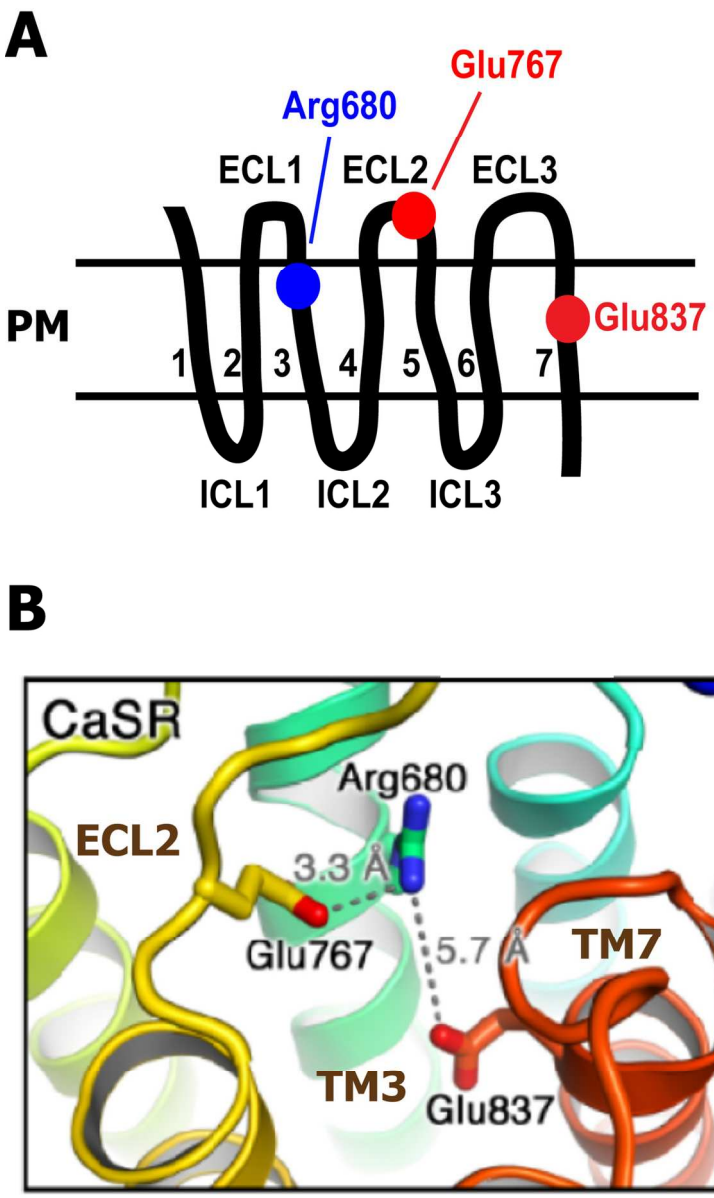


Figure 3

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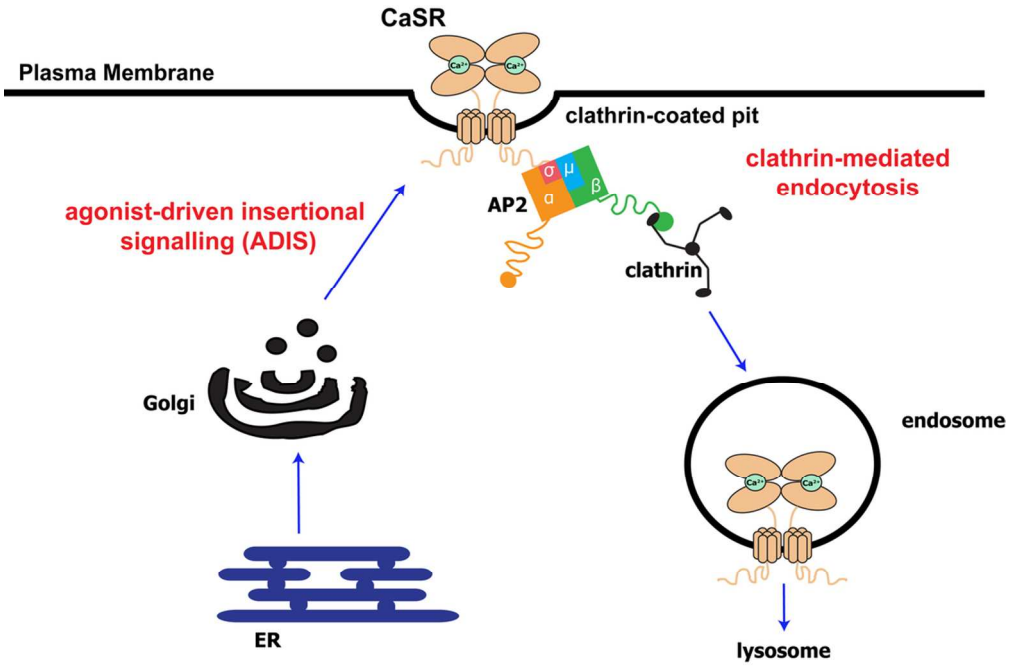


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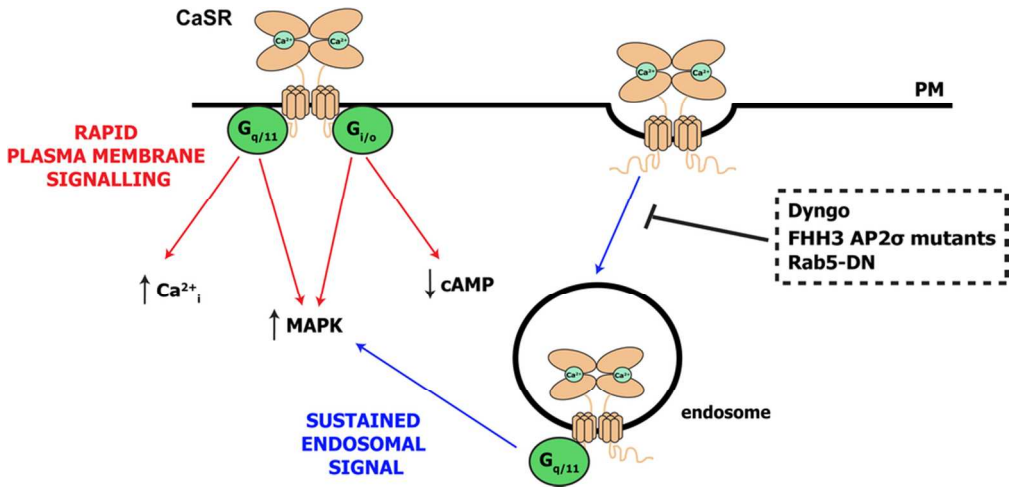


87x42mm (300 x 300 DPI)