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THEMED SECTION: MOLECULAR PHARMACOLOGY OF G PROTEIN-COUPLED RECEPTORS

REVIEW

Mechanisms of multimodal sensing by extracellular Ca^{2+}-sensing receptors: a domain-based survey of requirements for binding and signalling

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In this article we consider the molecular basis of sensing and signalling by the extracellular calcium-sensing receptor. We consider the nature of its ligands and sensing modalities, the identities of its major protein domains and their roles in sensing, signalling and trafficking as well as the significance of receptor homo- and hetero-dimerization. Finally, we consider the current, incomplete, state of knowledge regarding the requirements for ligand-specific signalling.
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Keywords: calcium-sensing receptor; class C GPCR; Venus Fly Trap domain; cysteine-rich domain; dimerization; heptahelical domain

Abbreviations: ADH, Autosomal Dominant Hypocalcemia; AMSH, associated molecule with the SH3 domain of STAM; CaR, calcium-sensing receptor; ERK, extracellular regulated kinase; FHH, familial hypocalciuric hypercalcemia; GPCR, G-protein-coupled receptor; GPRC6A, G-protein-coupled receptor family C (class C) member 6A; mGlu, metabotropic glutamate receptor; VFT domain, Venus Fly Trap domain

Introduction

Class C GPCRs

The extracellular calcium sensing receptor (CaR) belongs to class C of the G-protein-coupled receptor superfamily. Class C G-protein-coupled receptor (GPCRs) also include metabotropic glutamate receptors (mGlu-s) of which eight isoforms are recognized, GABA(B) receptors, T1R taste receptors (T1R1-3), the GPRC6A cationic amino acid receptor and various orphans including putative pheromone receptors (Pin *et al.*, 2004; Bräuner-Osborne *et al.*, 2007). These proteins are characterized by large N-terminal extracellular regions composed of 400–500 residue bilobed Venus Fly Trap (VFT) domains for nutrient binding tethered to GPCR heptahelical (HH) signalling domains. In most, but not all cases – the GABA(B) receptor is a notable exception (Hu *et al.*, 2000) – 60–70 residue Cysteine-rich domains act as necessary signal transmission units interposed between the VFT and HH domains. Class C receptors also exhibit large cytoplasmic C-terminal domains which act as scaffolds for the assembly of intracellular signalling units and for the binding of proteins that direct trafficking to specific compartments (Huang and Miller, 2007).

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The calcium-sensing receptor: physiological roles

The calcium-sensing receptor (CaR) is widely, almost ubiquitously, expressed (Brown and MacLeod, 2001). In addition to being expressed in endocrine glands such as the parathyroid and various tubular segments of the kidney, it is also expressed in the gastrointestinal tract, mesenchymal tissues including cartilage and bone, and even the brain in which it is expressed in neurons, glial cells and their precursors, apparently acting to modulate synaptic transmission and/or facilitate development (Hofer and Brown, 2003; Yano *et al.*, 2004). High level expression in the subfornical organ, which has a

recognized role in ionic strength sensing (Rogers *et al.*, 1997), indicates that CaR-mediated inputs may also modulate whole body salt and water homeostasis.

Expression cloning of the CaR (Brown *et al.*, 1993) revealed the molecular basis of extracellular Ca^{2+} (Ca^{2+}_o) – dependent feedback control of parathyroid hormone (PTH) secretion and other key aspects of whole body calcium homeostasis (reviews: Brown *et al.*, 1995; 1998). Parathyroid chief cells synthesize and secrete PTH, which acts on the renal proximal tubule to suppress inorganic phosphate reabsorption and stimulate the synthesis of calcitriol as well as the thick ascending limb and distal tubule to promote Ca^{2+} reabsorption (review: Houillier *et al.*, 2003). These effects restrain renal calcium losses and boost intestinal calcium absorption, thereby elevating the serum Ca^{2+} concentration. The attendant fall in the serum inorganic phosphate concentration limits the risk of calcium-phosphate precipitation.

As Ca^{2+}_o rises towards the lower limit of its normal range (around 1.0–1.1 mM) CaRs in the parathyroid trip a switch that turns off continued PTH release to close the feedback loop (review: Brown and MacLeod, 2001). As Ca^{2+}_o rises still further, CaRs in the renal tubules further adjust the Ca^{2+}_o and inorganic phosphate levels by directly suppressing Ca^{2+} reabsorption in the cortical thick ascending limb (reviews: Ba and Friedman, 2004; Gamba and Friedman, 2009) and antagonizing the effect of PTH to restore inorganic phosphate reabsorption in the proximal tubule (Ba *et al.*, 2003). Downregulation of the CaR and inorganic phosphate transporter Na^+/P_i-2 in the proximal tubule in response to a chronically elevated phosphate diet provides protection from hyperphosphatemia (Riccardi *et al.*, 2000).

Calcium-sensing receptor agonists, modulators and regulatory modalities

In addition to what is generally considered to be its primary physiological agonist Ca^{2+}, the CaR is sensitive to various other biochemical species and modalities such as ionic strength and pH (Quinn *et al.*, 1998; 2004), and even temperature (Breitwieser *et al.*, 2004) – pointing to roles beyond, but perhaps complementary, to its well-recognized role in the regulation of calcium metabolism.

Other activators that largely mimic the effect of Ca^{2+}, and are therefore considered to be agonists, include the inorganic divalent cation Mg^{2+}, which is a less potent activator than Ca^{2+}, various other divalent and tervalent inorganic cations including Gd^{3+}, the polyamines spermine and spermidine, antibiotics of the aminoglycoside class and cationic peptides including polyArg and amyloid peptides (reviews: Brown and MacLeod, 2001; Ward and Riccardi, 2002). The molecular basis for this extraordinary promiscuity is, thus far, unexplained.

L-amino acids including, in particular, aromatics, short aliphatics and small polar amino acids are physiological modulators of CaR function (Conigrave *et al.*, 2000a,b). These amino acid activators markedly sensitize the receptor to Ca^{2+}_o–induced activation of intracellular Ca^{2+} mobilization but have lesser impacts on Ca^{2+}_o-stimulated phosphatidylinositol-specific phospholipase-C (PI-PLC) (Rey *et al.*, 2005) and ERK1/2 (Lee *et al.*, 2007) activities. Pharmacologically significant modulators include calcimimetics of the phenylalkylamine (Shoback *et al.*, 2003; Nemeth *et al.*, 2004) and other classes (Dauban *et al.*, 2000; Kessler *et al.*, 2004a), which are positive allosteric modulators (Hebert, 2006; Nemeth, 2006), and calcilytics, which are negative allosteric modulators that stabilize the receptor in one or more inactive conformations (Nemeth *et al.*, 2001; Kessler *et al.*, 2004b; 2006; Nemeth, 2004; Balan *et al.*, 2009).

Variations in pH and ionic strength modulate the receptor's extracellular Ca^{2+} sensitivity. Ca^{2+}_o sensitivity is enhanced by increased pH and reduced by decreased pH (Quinn *et al.*, 2004) suggesting that the receptor may have pH-sensing properties in compartments in which dynamic variations in pH arise including, for example, the medullary collecting tubule, in which the receptor is expressed on the apical membrane (Sands *et al.*, 1997) and the stomach in which the CaR is expressed on the basolateral membranes of gastric mucosal cells (Cheng *et al.*, 1999; Rutten *et al.*, 1999). In addition, Ca^{2+}_o sensitivity is enhanced by decreased ionic strength (e.g. arising from reduced serum Na^+ concentration) and reduced by increased ionic strength (Quinn *et al.*, 1998) providing a possible explanation for the high level of CaR expression in the subfornical organ (Rogers *et al.*, 1997).

Calcium-sensing receptors: signalling mechanisms

In response to the binding of activators, the CaR's HH transmembrane domains couple to various heterotrimeric G-proteins, including $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ to initiate intracellular signalling pathways upstream of PI-PLC, MAP kinases including Erk1/2, p38 and JNK, PI-3 kinase, monomeric G-proteins such as Rho that regulate interactions with the cytoskeleton, and inhibition of adenylyl cyclase (reviews: Brown and MacLeod, 2001; Hofer and Brown, 2003; Ward, 2004; Brennan and Conigrave, 2009). This functional plasticity requires the assembly of complex signalling networks involving interactions between docked heterotrimeric G-proteins and the receptor's C-terminal domain. As a consequence of ligand diversity and functional plasticity, the CaR is particularly susceptible to the impact of mutations throughout its extracellular and intracellular domains (reviews: Brown, 1999; Thakker, 2004) and it may be possible in future to tailor pharmacotherapy to restore receptor function in individuals with CaR mutations that significantly impair receptor function (Hu and Spiegel, 2007).

Problems impeding a complete molecular pharmacological description

How do extracellular calcium-sensing receptors achieve their nutrient and multi-modal sensing tasks? How do they provide high fidelity signalling, differentiating between signals and integrating information from diverse inputs to operate effectively in diverse tissue contexts to control cell fate and function? The solution of these complex issues requires careful analysis on a tissue-by-tissue basis, taking account of the physiologically relevant nutrients and sensing modalities for the compartment and tissue, and taking advantage of modern molecular techniques. In the following sections we review

what is currently known of the CaR's ligand binding, signalling and trafficking functions focusing on the structures and functions of its key protein domains.

Major protein domains

Venus FlyTrap domain: major site of nutrient sensing

The CaR's N-terminal Venus FlyTrap (VFT) domain extends from residues 20 to 536. Like the corresponding N-terminus of metabotropic glutamate receptors, the CaR's N-terminus is homologous to nutrient-binding bacterial periplasmic binding proteins (O'Hara *et al.*, 1993; Brauner-Osborne *et al.*, 1999) and, based on the solved crystal structures of homologous mGlus including mGlu-1 (Kunishima *et al.*, 2000) as well as mGlu-3 and mGlu-7 (Muto *et al.*, 2007), takes the form of a bilobed structure in which ligand binding sites are located within the bilobed cleft and at the interprotomeric interface of homodimers (Figure 1). By analogy with the mGlu-1 VFT domain (Kunishima *et al.*, 2000; Tsuchiya *et al.*, 2002), the CaR's dimeric VFT domains are likely to adopt four major conformations: open-open, open-closed, closed-open and closed-closed, in which the closed forms favour receptor activation and are stabilized upon ligand binding.

In addition to its role in nutrient sensing, the VFT domain is an important site of dimerization (Ray *et al.*, 1999) and appears to be the primary location of Ca^{2+} ion binding (Huang *et al.*, 2007b; 2009) although additional cation binding site(s) have been identified in the HH transmembrane domains (Ray and Northup, 2002). The nature of the CaR VFT domain's interaction with the immediately contiguous Cysteine-rich domain is currently uncertain (see below) but turning moments induced by closure of the VFT domains are believed to control the conformation of the HH domains and thus the probability of G-protein docking and activation.

The VFT domain exhibits tight conservation of residues required for binding the α-amino and α-carboxylate groups of L-amino acid ligands in metabotropic glutamate receptors and other amino acid binding class C GPCRs (Table 1) and, indeed, on the basis of analyses of chimeric receptors (Mun *et al.*, 2004) and mutations (Zhang *et al.*, 2002b; Mun *et al.*, 2005) is the site of broad-spectrum L-amino binding in the CaR.

Cysteine-rich domain: signal transmission

The CaR's VFT domain connects with its HH domain via a 62 residue Cys-rich domain (Figure 2; Hu *et al.*, 2000) and a 14 residue linker (Ray *et al.*, 2007). Although the otherwise conserved Cys-rich domain is absent in GABA(B) receptors (Hu *et al.*, 2000), molecular analyses indicate that the CR domain plays a critical role in transmitting nutrient-derived signals from the VFT domain to the HH domains in other class C GPCRs. Thus, deletion of the entire CR domain eliminated high Ca^{2+}_o concentration-induced activation of PI-PLC with no significant effect on surface expression (Hu *et al.*, 2000) and mutational analyses indicate that all nine Cys residues are required for normal receptor function (Fan *et al.*, 1998; Ray *et al.*, 1999; Hu *et al.*, 2000).

Although no structures are available for any of the CaR's major domains, the recent solution of a crystal structure for the entire extracellular domain of the rat Group II metabotropic glutamate receptor, mGlu-3 (Muto *et al.*, 2007), provides new insights into the structural relationships between the CR domains and contiguous VFT and HH domains. In particular, the structure defines roles for the entire complement of nine conserved Cys residues in the CR domains. In the case of four pairs of CR domain Cysteines (mGlu-3 residues C509 and C528; C513 and C531; C534 and C546; and C549 and C562) a network of intra-domain disulfides stabilizes a rigid rod-like structure composed of several anti-parallel sheets to provide the VFT domain with caliper-like control over the HH domain

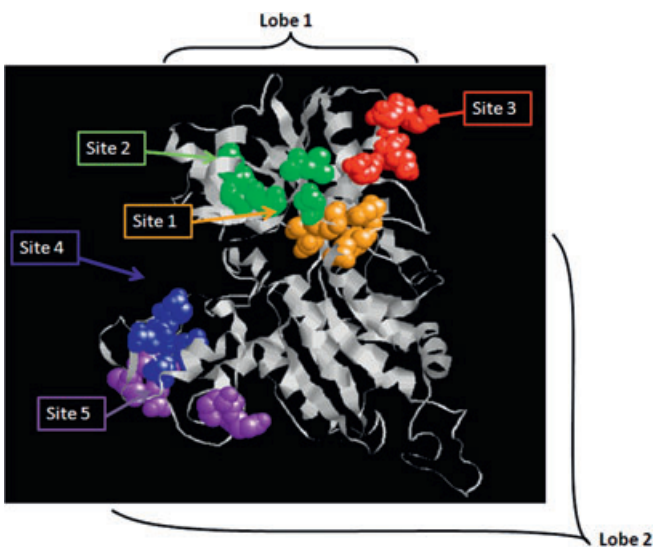

Figure 1 Molecular model of a CaR protomeric VFT domain. A model of a single subunit based on the mGlu-1 crystal structure 1EWK (Kunishima *et al.*, 2000). Putative Ca^{2+}_o binding sites (1–5) were identified by aromatized terbium luminescence analysis of globular sub-domains (Huang *et al.*, 2009). Site '1' also corresponds to the conserved L-amino acid-binding site of class C GPCRs raising the possibility that Ca^{2+} and amino acid binding are closely associated. CaR, calcium-sensing receptor; mGlu, metabotropic glutamate receptor; GPCR, G-protein-coupled receptor; VFT domain, Venus Fly Trap domain.

Table 1 Aligned α-carboxylate and α-amino VFT domain binding residues from a multiple sequence alignment of class C GPCRs

rmGlu-1	S165	T188	D208	Y236	D318
rmGlu-3	S151	T174	D194	Y222	D301
hCaR	S147	S170	D190	Y218	E297
mT1R1	T150	S173	D193	Y221	E302
mT1R3	S147	S170	D190	Y218	E301
mGPRC6A	S149	T172	D192	Y220	D303

Known α-amino and α-carboxylate binding residues from the crystal structures of the VFT domains of rat mGlu-1 (Kunishima *et al.*, 2000) and rat mGlu-3 (Muto *et al.*, 2007) are presented together with aligned residues for various L-amino acid binding members of GPCR class C including the human isoform of the CaR and mouse isoforms of T1R2, T1R3 and GPRC6A. The table has been modified (Conigrave and Hampson, 2006). The Protein Database accession numbers used in the analysis were as follows: NP_058707.1 (rmGlu-1), NP_001099182.1 (rmGlu-3), NP_000379.2 (hCaR), NP_114073.1 (mT1R1), NP_114078.1 (mT1R3), NP_694711.1 (mGPRC6A).
CaR, calcium-sensing receptor; GPCR, G-protein-coupled receptor; GPRC6A, G-protein-coupled receptor family C (class C) member 6A; mGlu, metabotropic glutamate receptor; VFT domain, Venus Fly Trap domain.

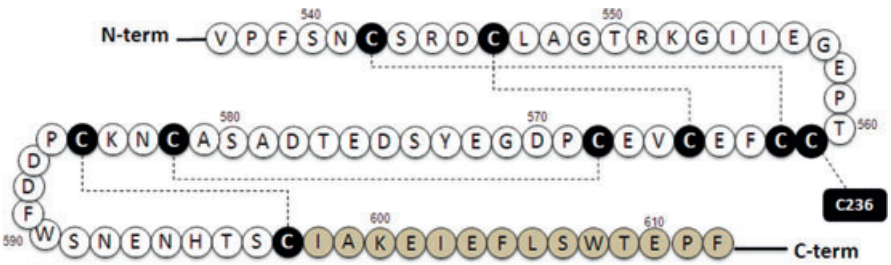

Figure 2 Schematic representation of the CaR's Cysteine rich domain. The Cys-rich (CR) domain has nine conserved cysteine residues (black circles), all of which are predicted to participate in di-sulfide bonds (broken lines). In total there are four predicted intra-domain di-sulfides and one disulfide between CaR residues 561 in the CR domain and 236 in lobe 2 of the VFT domain. A 14 amino acid linker (grey circles) supports signal transmission from the VFT domain to the heptahelical domain. CaR, calcium-sensing receptor; VFT domain, Venus Fly Trap domain.

(Muto *et al.*, 2007). In the case of the remaining mGlu-3 CR domain Cys residue, C527 (which aligns to CaR residue 561), an interdomain disulfide forms with C240 (aligning to CaR residue 236) in lobe-2 of the VFT domain. This disulfide appears to adjust the angle at which turning moments induced in the VFT domains are transmitted to the HH domains.

An inter-domain disulfide between the homologous Cys residues in mGlu-2 was predicted previously (Rondard *et al.*, 2006) but, despite conservation of both Cys residues, was excluded for the CaR based on analyses of proteolytic fragments released from an engineered tobacco etch virus cleavage site between the VFT and CR domains (Hu *et al.*, 2001). The discrepancy between the crystal structure-based findings of Muto *et al.* for mGlu-3 (Muto *et al.*, 2007) and mutational analysis of Rondard *et al.* for mGlu-2 (Rondard *et al.*, 2006), on the one hand, and Hu *et al.* for the CaR (Hu *et al.*, 2001) on the other, is currently unexplained. It suggests, however, that interdomain di-sulfide bridge formation in the CaR may be relatively unstable (review: Hu and Spiegel, 2007) and perhaps sensitive to ligand binding.

The HH domain: interactions with G-proteins

Members of the GPCR superfamily exhibit a characteristic HH domain consisting of seven helices connected by alternating intracellular loops (iL-s) and extracellular loops (eL-s) (Pierce *et al.*, 2002; Karnik *et al.*, 2003; Rosenbaum *et al.*, 2009). The N-terminus of the HH module is continuous with the N-terminal extracellular domain of the receptor and the C-terminus of the module extends into the cytoplasm as the C-terminal domain. The HH domain is considered critical for the docking and activation of hetero-trimeric G-proteins. Crystal structures of class A GPCRs including bovine rhodopsin (Palczewski *et al.*, 2000) and more recently β2-adrenergic (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007) and β1-adrenergic (Warne *et al.*, 2008) receptors, have greatly facilitated molecular modelling efforts aimed at understanding the mechanism(s) of GPCR activation. According to the current view of class A receptor action, ligand binding in a pocket formed by a cylindrical arrangement of HH helices releases inhibitory constraints on the docking and binding of G-proteins on the interior surface of the receptor (Kobilka and Schertler, 2008).

Although models based on the solved class A GPCR structures have been applied with some success to class C GPCRs including the CaR (Miedlich *et al.*, 2004; Petrel *et al.*, 2004), the nature of the interfaces between oligomeric HH domains, together with the selectivities and stoichiometries of receptor: G-protein binding are undefined. Nevertheless, significant progress has been made on the activation mechanism of the homologous mGlu-5 receptors, which exhibit subunit interchange between dimeric HH domains (Brock *et al.*, 2007). In addition, mutational analysis has demonstrated that CaR-dependent activation of PI-PLC, which is $G_{q/11}$-dependent, is dependent on residues in both intracellular loops 2 and 3 (Figure 3; Chang *et al.*, 2000).

Unlike, the mGlus which tend to be specialized for either the activation of $G_{q/11}$, in the case of Group I receptors, or $G_{i/o}$ in the case of the Group II and Group III receptors, the CaR is unusually pleiotropic, coupling to both $G_{q/11}$ and $G_{i/o}$, as well as $G_{12/13}$ (reviews: Hofer and Brown, 2003; Ward, 2004; Brennan and Conigrave, 2009). It may even couple to G_s under some circumstances (see below). Although the signalling potential of these G-protein partners is clear, the broader physiological significance of these interactions is, at present, largely unknown. A notable exception is the role of $G_{q/11}$ in CaR-mediated control of PTH secretion as parathyroid-specific ablation of both G_q and G_{11} in mice induced severe neonatal primary hyperparathyroidism (Wettschureck *et al.*, 2007).

G_q and G_{11} appear to interact with the CaR via residues in intracellular loop-2 (iL-2) and intracellular loop-3 (iL-3) as alanine scanning mutagenesis in both iL-2 and iL-3 impaired PI-PLC activation (Chang *et al.*, 2000). In the case of iL-2 (residues 700 to 727 in the bovine isoform), L704 and F707 were critical for high Ca^{2+}_o-mediated coupling to PI-PLC. In the case of iL-3 (residues 794–807), two closely associated patches were identified between residues R796 and P799 and between residues N801 and F807 (Chang *et al.*, 2000). The naturally occurring FHH-related mutation of the human CaR, R795W (R796 in the bovine isoform), which exhibits dominant negative activity, is located nearby.

The analysis described above for CaR coupling to $G_{q/11}$ has not yet been extended to other heterotrimeric G-proteins to which the CaR couples such as $G_{i/o}$ or $G_{12/13}$. In addition, the CaR's G-protein preference switches from $G_{i/o}$ in normal mammary epithelial cells to G_s in two breast cancer cell lines, thus reversing the polarity of its control over cAMP synthesis (Mamillapalli *et al.*, 2008) with potential significance for cancer cell growth and/or metastasis. The mechanism that underlies this effect is currently unknown.

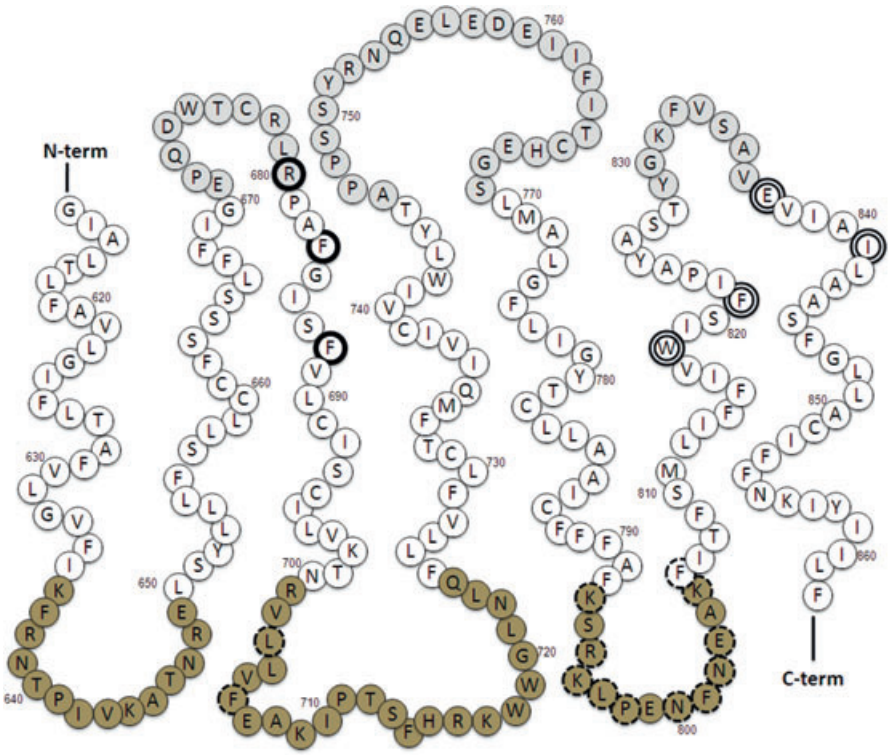

Figure 3 Schematic representation of the CaR's heptahelical domain. The seven transmembrane helices are shown together with the alternating intracellular loops (residues in dark grey) and extracellular loops (residues in light grey). Residues that interact with both calcimimetics and calcilytics are enclosed in double-lined circles. Residues that interact with calcilytics alone are enclosed in single, bold circles. Residues in iL-2 and iL-3 that support $G_{q/11}$-dependent activation of PI-PLC are highlighted with broken-lines. CaR, calcium-sensing receptor; PI-PLC, phosphatidylinositol-specific phospholipase-C.

C-terminal domain: Dynamic regulation of signalling and expression

The human CaR's C-terminal domain is composed of residues 863–1078 (Figure 4; Garrett *et al.*, 1995) and plays key roles in signalling, expression, trafficking, cooperativity and desensitization (reviews: Bai, 2004; Ward, 2004; Breitwieser, 2006; Hu and Spiegel, 2007). Based on analyses of truncation mutants and alanine scanning, the immediate membrane proximal loop (residues 863–874) promotes receptor expression and is required for PI-PLC activation (Ray *et al.*, 1997). In addition, several residues between 874 and 888, although not required for receptor expression, are necessary for PI-PLC activation. Residues beyond 887 although not required for PI-PLC activation (Ray *et al.*, 1997) are implicated in alternative downstream signalling pathways. In addition, residues 868–886 appear to contribute to cooperativity and protect the receptor from desensitization (Gama and Breitwieser, 1998); see Figure 4.

Various potential PK-C phosphorylation sites have been identified in the intracellular loops and C-terminal domain. Of these, T888 in the proximal C-terminal domain is considered to be the primary site and phosphorylation of this residue modulates receptor function, acting to uncouple PI-PLC and thus reduce sensitivity to elevated Ca^{2+}_o (Bai *et al.*, 1998; Jiang *et al.*, 2002). Interestingly, recent work employing an antibody that binds selectively to a CaR-tail phosphopeptide centred on T888 demonstrates that the activated receptor exhibits repetitive T888 phosphorylation and dephosphorylation (Davies *et al.*, 2007). Although the full significance of this result is uncertain, it suggests a mechanism by which activator-induced low frequency oscillations in Ca^{2+}_i concentration might arise (Davies *et al.*, 2007). In addition, T888 phosphorylation appears to promote G-protein receptor kinase-2 mediated desensitization via sequestration of alpha-q subunits and/or beta-arrestin binding to the HH domain (Pi *et al.*, 2005; Lorenz *et al.*, 2007). The tendency for T888 to undergo repetitive phosphorylation and dephosphorylation upon receptor activation may also contribute to the CaR's well-recognized resistance to desensitization (Brown and MacLeod, 2001).

In addition to its role in PI-PLC signalling, the C-terminal domain interacts either directly or indirectly with intracellular proteins that modulate or mediate receptor trafficking, subcellular localization and downstream signalling pathways (Huang and Miller, 2007). Interactions with inwardly rectifying K^+ channels, Kir4.1 and Kir4.2, for example, provide a mechanism by which alterations in Ca^{2+}_o modulate renal salt and water transport (Huang *et al.*, 2007a). Binding to filamin-A, on the other hand, establishes a link to the actin cytoskeleton to direct receptors to specific subcellular compartments for the creation of signalling scaffolds (Awata *et al.*, 2001; Hjalm *et al.*, 2001; Zhang and Breitwieser, 2005). Filamin may mediate, for example, the CaR's interactions with caveolin, thereby targeting the receptor to plasma membrane caveolae (Kifor *et al.*, 1998). In addition, the association between the CaR and filamin is required for coupling

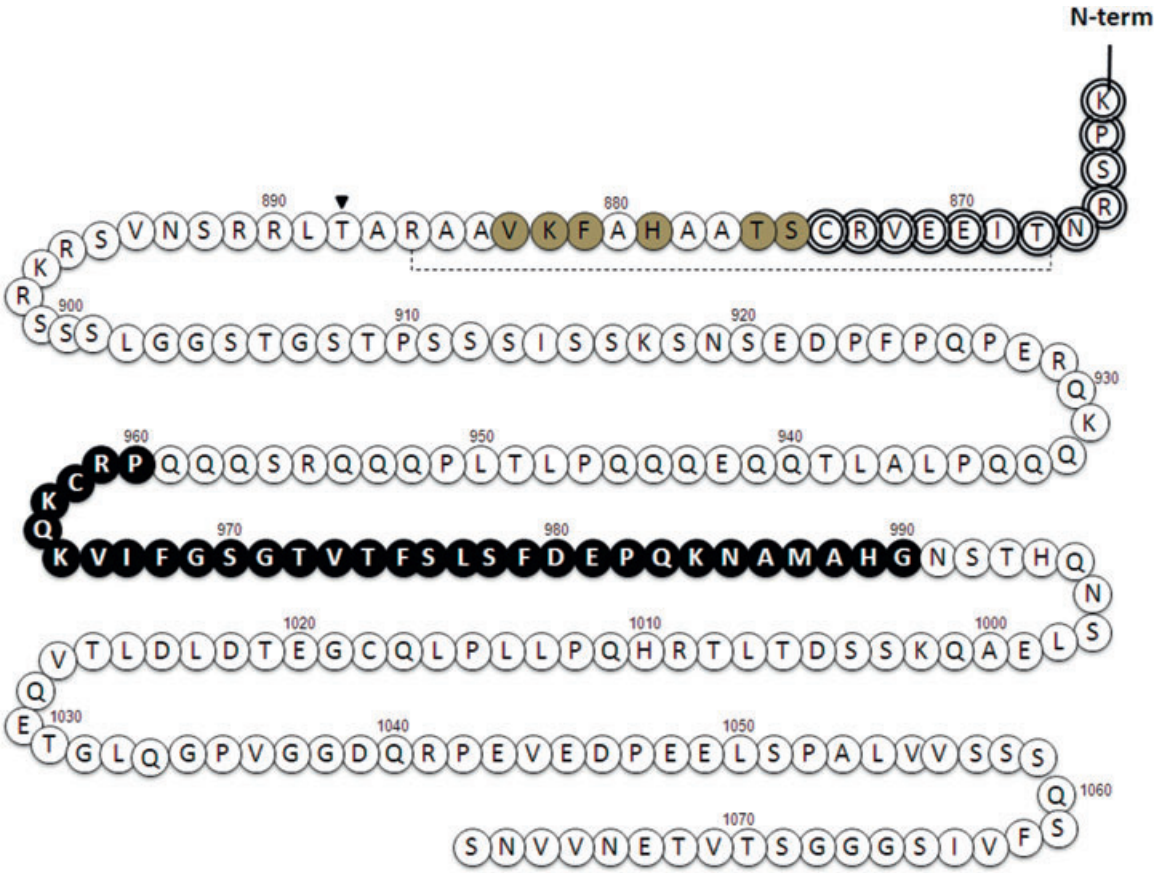

Figure 4 Schematic representation of the CaR's intracellular C-terminal domain. The C-terminal domain supports receptor expression and activation of PI-PLC (residues 863–874; double circles), activation of PI-PLC alone (highlighted in grey), as well as cooperativity and resistance to desensitization (residues 868–886 indicated by broken line). Residues 960–990 (labelled in black) provide a high-affinity binding site for filamin-A. The major PK-C phosphorylation site at T-888 is labelled '▼'. CaR, calcium-sensing receptor; PI-PLC, phosphatidylinositol-specific phospholipase-C.

between the receptor and ERK 1/2 (Awata *et al.*, 2001; Hjalm *et al.*, 2001) and may permit $G_{12/13}$ control of small G-proteins including Rho, upstream of PI-4 kinase and phospholipase D (Pi *et al.*, 2002; Rey *et al.*, 2005). Two filamin-A binding sites have been reported: a high affinity site located in the approximate region 960–990 (Awata *et al.*, 2001; Hjalm *et al.*, 2001; Zhang and Breitwieser, 2005) and a lower affinity, membrane proximal binding site that appears to contribute to Ca^{2+}_o-induced ERK1/2 activation (Zhang and Breitwieser, 2005).

Other CaR binding partners including the E3 ubiquitin ligase, dorfin (Huang *et al.*, 2006) and AMSH (Herrera-Vigenor *et al.*, 2006; Reyes-Ibarra *et al.*, 2007) promote intracellular trafficking to proteasomes for protein degradation. Surprisingly, recent studies employing co-immunoprecipitation and RNAi techniques indicate that trafficking of the CaR to the plasma membrane in COS-7 and HEK293 cells requires receptor activity modifying proteins (RAMPs) including RAMP-1 and RAMP-3 (Bouschet *et al.*, 2005; Bouschet *et al.*, 2008). These findings were unexpected as RAMPs have been considered specific partners of Class B GPCRs such as the calcitonin gene-related peptide receptor for which they modulate receptor expression, ligand selection and signalling properties (Hay *et al.*, 2006; Sexton *et al.*, 2006).

Roles of dimers in tissue-specific receptor function

Homodimers

Whether expressed heterologously in HEK293 cells or constitutively in cells with a primary role in extracellular Ca^{2+}-sensing, the CaR functions primarily as homodimers and both covalent and non-covalent interactions support dimerization at the interface between neighbouring VFT domains (reviews: Bai, 2004; Hu and Spiegel, 2007). Two asymmetric intermolecular disulfide bonds form between residues C129 and C131 of dimeric receptors (Ray *et al.*, 1999). In addition, non-covalent interactions involving L112 and L156 promote dimer stability (Jiang *et al.*, 2004). Dimerization is independent of agonist or modulator binding, and appears to arise at the time of insertion of the newly translated subunits into the endoplasmic reticulum (ER) membrane (Pidasheva *et al.*, 2006).

Dimerization promotes trafficking to the plasma membrane, perhaps by the mutual masking of peptides that act as ER retention signals (Chang *et al.*, 2007). If this is correct, CaR homodimers might operate in a manner analogous to GABA(B) heterodimers in which GABA(B2) residues R714-P820 mask an ER retention signal located in the GABA(B1) C-terminal domain (Pagano *et al.*, 2001). Dimerization also

appears to be required for normal receptor function. Firstly, co-expression of receptor mutants disabled in either their VFT domains or HH domains restored extracellular Ca^{2+}-stimulated PI-PLC (Bai *et al.*, 1999). Secondly, the CaR homolog, mGlu-5 exchanges loops of its HH domains upon receptor activation (Brock *et al.*, 2007) suggesting that the dimeric VFT-CR domain apparatus induces a functionally important interchange between associated HH domains.

Heterodimerization with other class C GPCRs: a mechanism for tissue-specific nutrient and multi-modal sensing?

In addition to forming functional homodimers, the CaR appears to form physiologically important heterodimers with other class C GPCRs dependent on their patterns of expression. Consistent with this idea, the CaR and mGlu-1α were co-immunoprecipitated from bovine brain (Gama *et al.*, 2001). Furthermore, the CaR formed disulfide-linked dimers with mGlu-1α or mGlu-5 when co-expressed with these receptors in HEK293 cells, acquiring glutamate-induced receptor internalization and exhibiting enhanced cell surface expression in the presence of the mGlu-1/5 binding partner Homer 1c (Gama *et al.*, 2001).

The CaR also forms heterodimers with co-expressed GABA(B) receptor subunits as revealed by co-immunoprecipitation analyses of growth plate chondrocytes (Cheng *et al.*, 2007) as well as whole brain and hippocampal neurons (Chang *et al.*, 2007). Interestingly, CaR expression was differentially modulated by co-expressed GABA(B1) or GABA(B2) receptors in HEK293 cells (Chang *et al.*, 2007). Whereas GABA(B2) markedly enhanced CaR surface expression, possibly by shielding a putative ER retention signal in the CaR tail, GABA(B1) suppressed CaR total and surface expression and promoted internalization (Chang *et al.*, 2007). On the other hand, the CaR promoted the expression of both GABA(B) subunits suggesting that it may play a role as a chaperone for the expression of some class C GPCRs. Taken together, the results suggest that the CaR preferentially forms heterodimers with other class C GPCRs but the mechanisms by which specific heterodimers are favoured are currently undefined.

A domain-based survey of CaR-ligand interactions

As noted above, the CaR mediates multi-modal, multi-metabolic sensing not only of nutrients including Ca^{2+} and L-amino acids but also ionic strength, pH and even temperature (Conigrave *et al.*, 2000a; Breitwieser *et al.*, 2004). Although the molecular requirements for the sensing of Ca^{2+}_o and amino acids as well as *calcimimetics* and *calcilytics* have been the subject of considerable scrutiny, the mechanisms that underlie the sensing of other modalities are largely unknown. Thus, the following discussion concentrates on Ca^{2+} and other multivalent cations, L-amino acids and calcimimetics and calcilytics.

Ca^{2+} and other multivalent cations

Although the CaR binds Ca^{2+} with apparently low affinity, it exhibits pronounced positive cooperativity in its control of proximal signalling pathways including PI-PLC and intracellular Ca^{2+} mobilization as well as its inhibitory control of PTH secretion (review: Brown and MacLeod, 2001). In particular, Hill coefficients for the Ca^{2+}_o-dependent activation of proximal signalling events are typically around 3–5 and approach 8–12 for the control of PTH secretion, indicating the existence of multiple interacting binding sites in receptor dimers or even oligomeric arrays. As a consequence, the concentration-response relationships are very steep at the points of inflection; for PTH secretion from normal human parathyroid cells this occurs at a Ca^{2+}_o concentration of around 1.1 mM (Conigrave *et al.*, 2004; Mun *et al.*, 2009).

The location(s) of the Ca^{2+} binding sites have been controversial. Chimeric receptors composed of the VFT domain of the CaR fused to the CR domain, HH domain and intracellular C-terminal domain of mGlu-1 exhibit normal or near-normal Ca^{2+}_o sensitivity in assays of PI-PLC activity (Hauache *et al.*, 2000) and intracellular Ca^{2+} mobilization (Mun *et al.*, 2004), consistent with the idea that the CaR's Ca^{2+} binding sites are located in the VFT domain. The interpretation of these experiments is compromised, however, by reports that mGlu-1 and some other mGlu-s are also Ca^{2+}_o-sensitive (Kubo *et al.*, 1998; Tabata *et al.*, 2002); for a review see: Tabata and Kano, 2004. In addition, studies of CaRs in which the entire extracellular domain has been replaced by the signal peptide of bovine rhodopsin (so-called 'headless' receptors), demonstrate that Ca^{2+}_o sensing can arise from the HH domain in the complete absence of the VFT domain (Ray and Northup, 2002; Mun *et al.*, 2004) and indicate that there is at least one Ca^{2+} binding site outside the VFT domain. Alanine scanning mutagenesis in the junction between transmembrane helices VI and VII, thus involving the third extracellular loop, identified a peptide (residues 819–837) required for Ca^{2+}_o-induced receptor activation but its significance for cation binding is unknown (Hu *et al.*, 2005).

Mutational analysis has also been employed in attempts to locate Ca^{2+}_o binding sites but is of limited value unless impaired receptor function and impaired cation binding can be causally associated. Based on molecular modelling, Silve *et al.* (Silve *et al.*, 2005) reported the presence of a Ca^{2+} binding site in the region of a conserved glutamate residue E297 in the VFT domain. They noted that E297K is an inactivating mutation associated with FHH whereas E297D is an activating mutation associated with ADH. The activating effect of the conservative mutation E297D was postulated to arise from the creation of an enlarged divalent cation binding pocket (Silve *et al.*, 2005).

More recent analysis using aromatized terbium luminescence to probe for Ca^{2+}_o binding sites, together with molecular modelling and mutational analysis, suggests the existence of several functionally significant Ca^{2+}_o binding sites in the VFT domain (Huang *et al.*, 2007b; Huang *et al.*, 2009). In particular, the independent expression of three globular subdomains corresponding to the hinge region of the VFT domain and distinct lobe 1 and lobe 2 proteins permitted analyses of Ca^{2+}_o binding and associated conformational changes (Huang *et al.*, 2009). Ca^{2+} binding 'site 1' in subdomain-1 was defined by residues S147, S170, D190, Y218 and E297, which are all highly conserved in class C GPCRs (Table 1) and known to contribute to the core amino acid binding site in mGlu-1

(Kunishima *et al.*, 2000; Tsuchiya *et al.*, 2002) as well as mGlu-3 and mGlu-7 (Muto *et al.*, 2007). Based on its apparent Ca^{2+}-binding affinity (K_d around 0.5 mM) it seems likely that this site is normally occupied at physiologically relevant Ca^{2+}_o concentrations and, therefore, may not contribute directly to the changes in receptor structure that arise from physiologically relevant changes in Ca^{2+}_o concentration (between around 1.1–1.3 mM). Instead 'site 1' may stabilize a receptor configuration necessary for Ca^{2+} binding to coupled lower affinity sites or a closely associated L-amino acid binding site.

The interpretation of the physiological significance of any of a large number of low affinity Ca^{2+}-binding sites is complicated by the demonstration that various multivalent cations including the inorganic polyvalent cations, Gd^{3+} and Mg^{2+} (review: Brown and MacLeod, 2001), as well as polyamines such as spermine (Quinn *et al.*, 1997), aminoglycoside antibiotics (Ward *et al.*, 2002; Gibbons *et al.*, 2008) and cationic peptides including PolyArg (Brown *et al.*, 1991) and amyloid peptides (Ye *et al.*, 1997) are all CaR activators. This extraordinary promiscuity is not clearly explained by currently reported binding analyses and the negatively charged surface(s) that mediate these effects are undefined.

L-Amino acids

L-Amino acids act as positive allosteric modulators promoting Ca^{2+}_o-induced intracellular Ca^{2+} mobilization in CaR-expressing HEK293 and CHOK1 cells (Conigrave *et al.*, 2000a,b) as well as normal human parathyroid cells (Conigrave *et al.*, 2004). In single cells, L-amino acids induce low frequency oscillations in the presence of submaximal Ca^{2+}_o concentrations (Young and Rozengurt, 2002) that arise from the activation of a signalling pathway coordinated by filamin binding to the C-terminal domain (Rey *et al.*, 2005). In addition, L-amino acids suppress PTH secretion (Conigrave *et al.*, 2004), an effect that is impaired in primary hyperparathyroidism (Mun *et al.*, 2009). The apparent requirement for a threshold extracellular Ca^{2+} level (typically around 0.5–1.0 mM in CaR-expressing HEK293 cells and parathyroid cells) indicates that the amino acid and Ca^{2+} binding sites interact. Furthermore, the L-amino acid and phenylalkylamine binding sites also exhibit positive cooperativity (Zhang *et al.*, 2002a).

Analysis of CaR/mGlu-1 chimeric receptors and a headless CaR construct expressed in HEK293 cells localized the L-amino acid binding site to the VFT domain (Mun *et al.*, 2004) indicating that the L-glutamate binding site in mGlu-1 is conserved in the CaR as a broad-spectrum L-amino acid binding site (Conigrave and Hampson, 2006). Consistent with this idea, the double mutant T145A/S170T markedly impaired L-Phe sensitivity but had little or no effect on Ca^{2+}_o-sensing in CaR-expressing HEK293 cells (Mun *et al.*, 2005). As noted above, this site may be closely associated with a moderately high affinity Ca^{2+} binding site located in the hinge region (Huang *et al.*, 2009) providing a potential explanation for the positive interactions between Ca^{2+} and L-amino acids (Conigrave *et al.*, 2007).

Calcimimetics and calcilytics

Calcimimetics are organic compounds that act as agonists or positive allosteric modulators, mimicking the effects of elevated Ca^{2+}_o on downstream signalling pathways. *Calcilytics*, on the other hand, are CaR inhibitors. In general, unlike amino acids, these compounds bind in the HH domains. The first classes of calcimimetics and calcilytics to be identified were phenylalkylamines including the calcimimetics NPS R467 and R568 (Nemeth *et al.*, 1998; Nemeth, 2006) and the calcilytics NPS 2143 and NPS 89636 (Nemeth *et al.*, 2001); for a review see: Nemeth (2002). NPS R467, R568 and their orally active analog cinacalcet are all stereoselective, positive allosteric modulators.

Unlike L-amino acids, which exhibit selectivity for specific signalling pathways including activation of low frequency intracellular Ca^{2+} oscillations (Rey *et al.*, 2005) and modestly enhance the Ca^{2+}_o sensitivity of ERK 1/2 activation (Lee *et al.*, 2007), phenylalkylamine calcimimetics exhibit broad specificity for CaR-linked signalling pathways (review: Brennan and Conigrave, 2009). Consistent with these effects cinacalcet is a potent activator of the CaR *in vivo* and has been successfully introduced for the medical therapy of secondary hyperparathyroidism in the context of chronic renal failure (Nagano, 2006; Wüthrich *et al.*, 2007). Its place in the treatment of primary hyperparathyroidism is less certain due to the effectiveness of modern surgical techniques (Bilezikian *et al.*, 2009; Khan *et al.*, 2009) but it is effective in long-term biochemical control for 1 year or more (Shoback *et al.*, 2003; Peacock *et al.*, 2005). Calcilytics, on the other hand, are being evaluated in the treatment of osteoporosis (Nemeth, 2004) although a reported inhibitory effect on osteoblast differentiation and function may limit their utility in this disorder (Dvorak *et al.*, 2004).

Based on studies with chimeric and 'headless' receptors, as well as molecular modelling and mutational analyses, calcimimetics and calcilytics bind in the HH domain (Hauache *et al.*, 2000; Ray and Northup, 2002; Miedlich *et al.*, 2004; Mun *et al.*, 2004; Petrel *et al.*, 2004) acting either positively or negatively to modulate the transmission of molecular signals to the G-protein docking site. Consistent with the idea that the binding sites of positive and negative allosteric modulators at least partially overlap, mutations of residues in transmembrane helix VI (Y818, F821) and VII (E837, I841) impaired the effects of the calcimimetics R-568 and calindol as well as calcilytics including NPS 2143 and Calhex 231 (Miedlich *et al.*, 2004; Petrel *et al.*, 2004). Mutations of residues in helix III (R680, F684, F688), however, selectively impaired the effects of calcilytics (Miedlich *et al.*, 2004; Petrel *et al.*, 2004); see Figure 3. Of the key HH domain residues identified in these studies, E837 between eL-3 and helix VII appears to be of particular interest as mutations of this residue can switch the efficacy of allosteric modulators between positive and negative (Miedlich *et al.*, 2004; Hu *et al.*, 2006).

Conclusions and future directions

Class C GPCRs exhibit dazzling promiscuity for ligands together with pluripotent activation of signalling pathways. The CaR's ability to control $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ appears to be an extreme example. Despite this craziness it works! In addition, molecular analysis is starting to locate ligand binding sites and unravel the mechanisms of receptor activation

including the internal transmission of signals, coordination of G-protein docking and formation of signalling scaffolds. Despite the impressive progress to date, the recognition that different ligands/signals induce distinct receptor conformations linked to the selective recruitment of signalling pathways indicates that important tasks in molecular analysis still lie ahead.

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