Chapter 41
Sensing Extracellular Calcium – An Insight into the Structure and Function of the Calcium-Sensing Receptor (CaSR)

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Abstract The calcium-sensing receptor (CaSR) is a G protein-coupled receptor that plays a key role in calcium homeostasis, by sensing free calcium levels in blood and regulating parathyroid hormone secretion in response. The CaSR is highly expressed in parathyroid gland and kidney where its role is well characterised, but also in other tissues where its function remains to be determined. The CaSR can be activated by a variety of endogenous ligands, as well as by synthetic modulators such as Cinacalcet, used in the clinic to treat secondary hyperparathyroidism in patients with chronic kidney disease. The CaSR couples to multiple G proteins, in a tissue-specific manner, activating several signalling pathways and thus regulating diverse intracellular events. The multifaceted nature of this receptor makes it a valuable therapeutic target for calciotropic and non-calciotropic diseases. It is therefore essential to understand the complexity behind the pharmacology, trafficking, and signalling characteristics of this receptor. This review provides an overview of the latest knowledge about the CaSR and discusses future hot topics in this field.

Keywords Extracellular calcium · Parathyroid hormone · G protein-coupled receptor · G proteins · Biased signalling · Calcimimetics · Calcilytics · Allosteric modulators · Orthosteric ligands · Cellular trafficking

Abbreviations

1,25D3	1α,25-dihydroxyvitamin D3
AC	Adenylate cyclase
ADH	Autosomal dominant hypocalcaemia
AP2	Adaptor protein-2
cAMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium
CaSR	Calcium-sensing receptor
CR	Cysteine rich domain
$[Ca^{2+}]_o$	Extracellular calcium concentration
$Ca^{2+}{}_i$	Intracellular calcium
$[Ca^{2+}]_i$	Intracellular calcium concentration
CKD	Chronic kidney disease
DAG	Diacylglycerol
ECD	Extracellular domain
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FHH	Familial hypocalciuric hypercalcaemia
GABA	Gamma-aminobutyric acid
GAPs	GTPase-activating proteins
GEFs	Guanine nucleotide exchange factors
GDIs	Guanine nucleotide dissociation inhibitors
GPCR	G protein-coupled receptor

GRKs	G protein-coupled receptor kinases
GSK3	Glycogen synthase kinase-3
HEK	Human embryonic kidney
HEK-CaSR	HEK293 cells stably expressing the CaSR
ICD	Intracellular domain
IGF-1	Insulin-like growth factor 1
IP3	Inositol 1,4,5-trisphosphate
JNK	C-Jun amino-terminal kinases
mGlu	Metabotropic glutamate receptor
NAM	Negative allosteric modulator
LB	Lobe-shaped domain
MAPKs	Mitogen-activated protein kinases
NAM	Negative allosteric modulator
NSHPT	Neonatal severe hyperparathyroidism
NKCC2	Na-K-Cl cotransporter 2
PA	Phosphatidic acid
PAM	Positive allosteric modulator
PDEs	Phosphodiesterases
Pi	Inorganic phosphate
PI3Ks	Phosphoinositide 3-kinases
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PreProPTH	Prepro-parathyroid hormone
PT	Parathyroid
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PTx	Pertussis-toxin
RGS	Regulator of G protein signalling
RAMPs	Receptor activity-modifying proteins
TMD	Transmembrane domain
TAS1R	Taste 1 receptors
VFD	Venus flytrap domain

41.1 Introduction

Calcium (Ca^{2+}) is a macro element representing 1.5–2% of an adult's total body weight and is mostly found in bones and teeth. Only 1% of the body's Ca^{2+} is located in cells and tissues, where it regulates numerous critical cellular responses.

The extracellular calcium concentration $[Ca^{2+}]_o$ is much higher (20,000-fold) than in the cytosol and changes in this balance trigger various signalling pathways. This gradient allows Ca^{2+} to act as a second messenger in intracellular signalling [1]. Many tissues are equipped with a cell-surface sensor for Ca^{2+} that extends the signalling properties of Ca^{2+} to being an extracellular first messenger also. This receptor is known as the extracellular calcium-sensing receptor (CaSR) and is a G protein-coupled receptor (GPCR).

CaSR is a member of the class C GPCRs which also includes the metabotropic glutamate (mGlu) receptors, the gamma-aminobutyric acid (GABA) receptors, the taste 1 receptors (TAS1R) and 8 orphan receptors [2]. The class C orphan receptor GPRC6 shares the highest sequence similarity with the CaSR (Fig. 41.1).

The CaSR plays an essential role in calcium homeostasis and its existence was confirmed by cloning in 1993 [3]. CaSR senses changes in $[Ca^{2+}]_o$ and also interacts with other multivalent cations (Mg^{2+}, Gd^{3+}), organic cations (neomycin), polyamines (spermine, polyarginine and polylysine) and possibly even beta amyloid [4]. It is highly expressed in the parathyroid glands, pancreas, duodenum and kidney and less in the digestive system, stomach and respiratory system [5]. This review will give an overview of the (patho)physiological roles, structure, ligands, trafficking, signalling pathways and tissue specific functions of the CaSR.

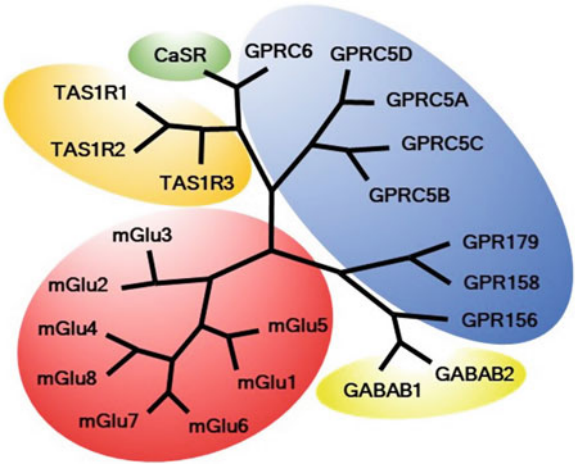

Fig. 41.1 Phylogenetic tree of the class C GPCRs generated with the neighbour-joining method of the full receptor sequences; The different colors represent the receptor families based on the endogenous ligand affiliation: CaSR family: calcium-sensing receptor (green), GPR family: class C orphan receptors with unknown endogenous ligands (blue), GABA family: gamma-aminobutyric acid receptors (yellow), mGlu family: metabotropic glutamate receptors (red), TAS1R family: taste 1 receptors (orange)

41.1.1 Physiological Role of the CaSR in Calcium Regulation

The blood $[Ca^{2+}]_o$ is influenced by parathyroid hormone (PTH), 1α,25-dihydroxyvitamin D_3 ($1,25D_3$) and calcitonin [4, 6]. PTH is expressed by and secreted from chief cells of the parathyroid gland and acts upon the kidneys, bones, and intestines. In the kidney, CaSR stimulates Ca^{2+} and Mg^{2+} reabsorption in the distal tubules whereas in the proximal tubules it promotes the excretion of hydrogen phosphate and dihydrogen phosphate. PTH induces the 25-hydroxyvitamin D_3 1α-hydroxylase to produce 1,25D3 which is essential for intestinal Ca^{2+} absorption [7]. Hypercalcaemia is prevented by CaSR, which inhibits PTH secretion and suppresses the transcription of PreProPTH and cell proliferation (Fig. 41.2) [8]. PTH stimulates bone remodelling. Calcitonin protects against hypercalcaemia and inhibits osteoclast activity and consequently the release of Ca^{2+} from bones. The transcription of calcitonin in thyroidal C-cells is inhibited by increasing 1,25D3 concentrations [6]. However, the impact of calcitonin in maintaining systemic

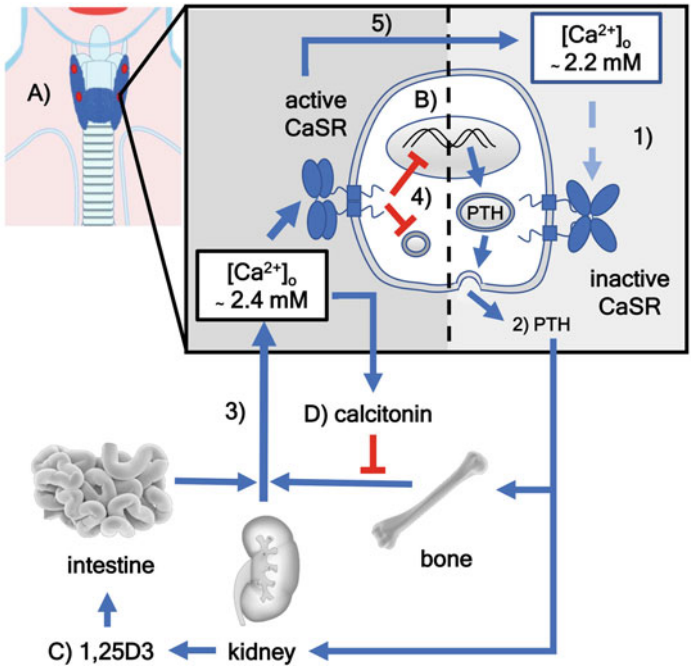

Fig. 41.2 Overview of the calcium homeostasis. (**a**) Location of the parathyroid glands (red dots); (**b**) Chief cell of the parathyroid gland, 1) CaSR is inactive at low $[Ca^{2+}]_o$ and PTH is secreted; 2) PTH stimulates Ca^{2+} release from bone, the reabsorption from the kidney and the $1,25D_3$ synthesis which induces Ca^{2+} uptake from the intestine (**c**); 3) The resulting increase in blood Ca^{2+} activates CaSR and at high concentrations calcitonin secretion; (**d**) Calcitonin inhibits the osteoclast activity and transiently the Ca^{2+} release from bone; 4) The active CaSR inhibits the PTH expression and secretion and consequently lowers the blood Ca^{2+} level; 5) Until the process starts again at 1)

blood Ca^{2+} is still contradictory because its absence or excess does not result in any significant metabolic abnormalities.

The physiological range of serum $[Ca^{2+}]_o$ is tightly regulated between 2.2 and 2.4 mM by the CaSR, facilitated by the high cooperativity of Ca^{2+} on the receptor. About half of the $[Ca^{2+}]_o$ is free, and the rest of it is bound mainly to albumin. PTH secretion is induced when the free $[Ca^{2+}]_o$ drops below 1.2 mM (to ~2.2 mM total Ca^{2+}) and it is effectively suppressed by CaSR activation when free $[Ca^{2+}]_o$ rises above 1.2 mM (towards 2.5 mM) [9]. High free $[Ca^{2+}]_o$ activates renal CaSR leading to inhibition of Ca^{2+} reabsorption resulting in elevated renal Ca^{2+} excretion [10, 11].

41.1.2 Pathophysiological Role of the CaSR

After the successful cloning of the bovine parathyroid CaSR [3], a number of diseases were identified which are caused by CaSR mutations.

Heterozygous loss-of-function mutations in CaSR are associated with familial hypocalciuric hypercalcaemia (FHH1) and homozygous CaSR mutations to neonatal severe hyperparathyroidism (NSHPT) [12]. FHH1 is characterised by disabled Ca^{2+} reabsorption causing hypocalciuria, moderate hypercalcaemia, hypermagnesaemia and a disabled inhibition of PTH secretion which leads to an elevated steady-state $[Ca^{2+}]_o$ level [4, 13]. Usually this disease remains asymptomatic over one's lifetime, but a few patients show signs of pancreatitis or chondrocalcinosis. FHH2 and FHH3 are the result of mutations in the G protein GNA11 and APS1 gene, respectively [13], these proteins acting downstream of CaSR signalling.

NSHPT is characterised by severe hypercalcaemia and very high PTH levels. The defective feedback regulation of the CaSR leads to bone demineralisation and to pathological fractures [4]. It is currently treated with bisphosphonates, dialysis, calcimimetics or by total parathyroidectomy [13, 14].

In contrast, autosomal dominant hypocalcaemia type 1 (ADH1) and type 2 (ADH2) are caused by gain-of-function mutations of the CaSR and the GNA11 gene, respectively. ADH1 results in a reduced steady-state of blood $[Ca^{2+}]_o$ and causes low PTH levels, hypercalciuria, hypomagnesaemia, and hyperphosphataemia. Symptoms of type 1 are paraesthesia, tetany, epilepsy, severe hypocalcaemia and basal ganglia calcification which are the same for ADH2 but without hypercalciuria and hypomagnesaemia [12]. Another gain-of-function disease is connected to a renal salt-wasting form called Bartter Syndrome type-5. It is the result of unrestrained CaSR activity which leads to dysfunctional Na-K-Cl cotransporter (NKCC2)-dependent NaCl reabsorption [4].

Autoimmune diseases of the CaSR have also been described in rare cases due to the presence of anti-CaSR antibodies. These antibodies can have CaSR-stimulating or CaSR-blocking effects causing a form of acquired autoimmune hypoparathyroidism or autoimmune hypocalciuric hypercalcaemia, respectively [13].

Mutations of the CaSR are also observed in a variety of non-calciotropic diseases, for example the R990G variant is associated with an elevated risk for hypercalciuria and nephrolithiasis [15]. Other diseases are connected to changed expression levels of the receptor. In colorectal and parathyroid cancer CaSR expression is decreased or lost, attenuating its tumour preventive effect. In breast and prostate tumours, CaSR is overexpressed which correlates with an increasing risk for metastases to the bone [16]. There is also evidence that changes in CaSR activity or expression are associated with alterations in cardiac function, insulin secretion, postprandial blood glucose regulation, lipolysis and inhibition of myocardial cell proliferation. In the digestive tract, CaSR shows anti-inflammatory, anti-secretory, pro-absorbent, and obstructive properties while in the respiratory tract CaSR activation is associated with inflammation and nonspecific hyperresponsiveness in asthma [13, 17].

41.2 Structure of the CaSR

The CaSR functions as a disulphide-tethered homodimer composed of three main domains: an extracellular domain (ECD), a heptahelical transmembrane domain (TMD) and an intracellular C-terminal domain (ICD) [18] (Fig. 41.3).

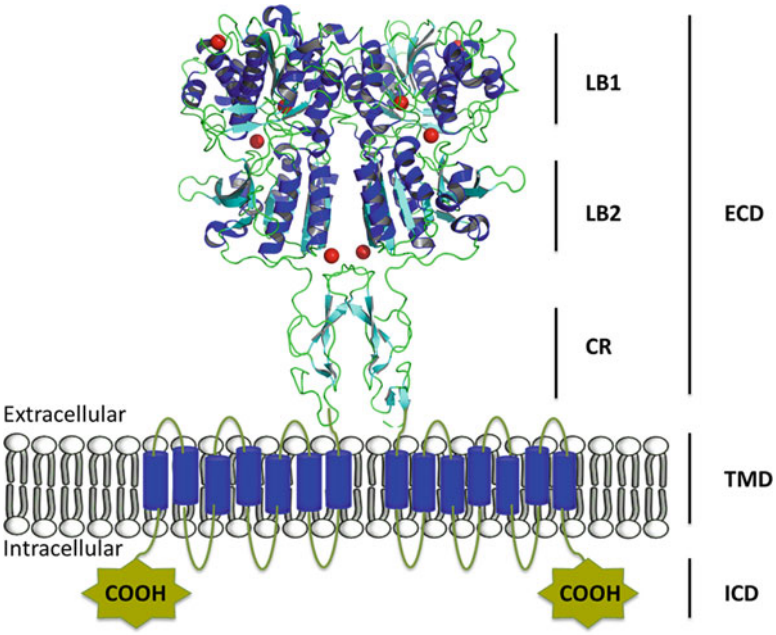

Fig. 41.3 Structure of the CaSR including the crystal structure of the ECD from the human CaSR, formed by LB1, LB2 and CR (PDB: 5k5s), and a schematic representation of the TMD formed by the seven transmembrane helixes followed by the ICD. Calcium ions are represented as red spheres in the ECD

The human CaSR ECD contains 612 amino acids and consists of two lobe-shaped domains (LB1 and LB2) that form the N-terminal Venus Fly Trap (VFT) domain, and the cysteine-rich (CR) domain [19]. The VFT is the ligand-binding region, reminiscent of gram-negative bacterial periplasmic binding proteins [20]. Both LB domains are formed by typical β-sheets and α-helices, where the central parallel β-sheets are sandwiched by α-helices [21]. The CR region, located between the ECD and the TMD contains nine-conserved cysteines. It transmits and amplifies signals from the VFT domain to the intracellular loops of the TMD [22]. The CR region is present in all class C GPCRs except in GABA B receptors and is required for receptor activation [21–23].

CaSR is expressed on the cell surface as a homodimer formed by direct interactions involving the ECD and the TMD. The ECDs of both monomers interact in a side-by-side fashion by a covalent disulphide bridge involving residues Cys-129 and Cys-131, whereas the TMDs establish hydrophobic interactions between them [24, 25]. However, there is also evidence suggesting heterodimerisation with other class C GPCRs. These heterodimers are considered new types of receptors that lead to changes in CaSR expression, signalling and sensitivity. For instance, CaSR may form dimers with mGlu1a or mGlu5, in hippocampal and cerebellar neurons, and with GABA B receptors [26, 27].

The CaSR ECD also includes 20–40 kDa of either high mannose or complex carbohydrates. These glycosylations are believed to be important for cell-surface localisation of the CaSR, intracellular trafficking, protein folding and secretion [25].

Recently, two different groups have simultaneously resolved the crystal structures of the human CaSR ECD in resting and active conformations [28, 29]. Zhang and partners crystallised the ECD in the active conformation and identified two Ca^{2+} binding sites plus an additional orthosteric binding site for L-Trp. The Ca^{2+} binding sites can also be occupied by other divalent metals such as Mg^{2+} whereas, the additional orthosteric binding site was occupied by a L-Trp derivate, L-1,2,3,4,-tetrahydronorharman-3-carboxylic acid and it was located in the hinge region between the two subdomains. The L-Trp binding site was described as crucial for receptor activation and stabilisation of the active conformation [29]. Meanwhile, Geng and colleagues crystallised the receptor in its active and inactive conformations. The active structure was obtained in the presence of 10 mM Ca^{2+} and 10 mM L-Trp, when the receptor is in its closed conformation (active state, closed-closed) (Fig. 41.4 left). They also identified the same orthosteric binding site described by Zhang and partners, located in the ligand-binding cleft of each protomer and also occupied by L-Trp. In this model, the authors defined four different Ca^{2+}-binding sites in the active structure, including one Ca^{2+}-binding site in each protomer that is common in the active and inactive structures suggesting an integral part of the receptor. On the other hand, the inactive CaSR ECD structure was obtained in the presence and absence of 2 mM Ca^{2+}, when the receptor is in open conformation (inactive state, open-open) and the interdomain cleft is empty (Fig. 41.4 right). In this model, they also defined three anion-binding sites. The authors proposed a CaSR activation model where L-Trp facilitates the CaSR-ECD closure by contacting LB1 and LB2 domains of the VFT module to bring the CR domains

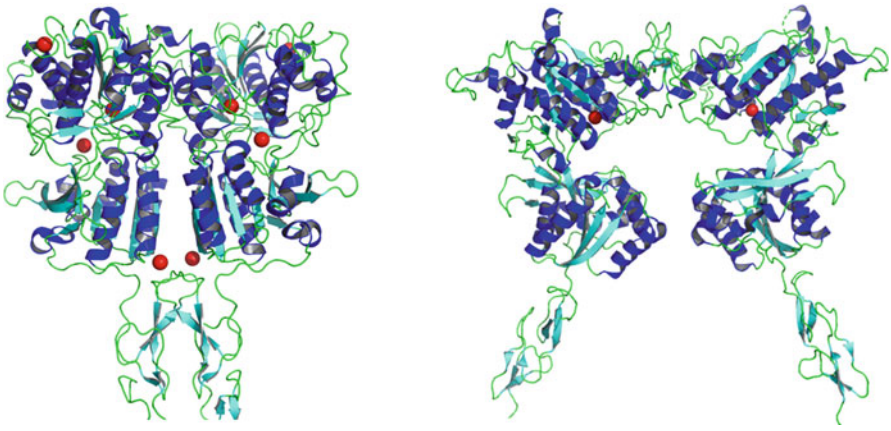

Fig. 41.4 Crystal structure of the human CaSR ECD in its active (left) and inactive (right) conformations. In the active conformation the VFT is closed and LB1 and LB2 interact to bring the CR domains closer together. In the inactive conformation the VFT is open and the interactions between LB1 and LB2 are minimal, therefore the CR domains do not interact. Calcium ions are shown as red spheres. PDB accession numbers: 5k5s and 5k5t [28]

closer together. These interactions form a large homodimer interface that is unique for the active state, reduce the distance between the C-terminal tails and might cause a rearrangement of the TMD [28] (Fig. 41.4).

Results from both groups suggest that the CaSR follows a universal activation mechanism similar for all class C GPCRs, despite the low sequence similarity (20–30%) [30]. This mechanism can be summarised in three steps. First, agonist binding causes the closure of the VFT. Second, membrane-proximal domains associate forming a homodimer interface between LB2 and CR domains. Third, agonist binding is accompanied by an approach between the C-terminal ends of ECDs of both protomers suggesting rearrangement of the TMD [31].

The ICD allows accurate receptor-specific control of diverse downstream signalling pathways. It represents the most diverse region of the class C GPCRs and determines selectivity of CaSR by coupling to different G proteins through the intracellular loops [32]. The ICD is exposed to the cytoplasm and begins with Lys-863 [33]. The amino acid sequence of the ICD is well conserved among species, although amino acids in the C-terminal tail are quite diverse [32]. Until now, two residues (Phe-706 and Leu-703) in intracellular loop two and eight residues (including Leu-797 and Phe-801) in intracellular loop three have been shown to be important for activating phospholipase C (PLC), the major pathway of the CaSR intracellular signalling [34]. Furthermore, there are several well-defined phosphorylation sites, especially Thr-888, in the ICD that are important for protein kinase C (PKC)-dependent inhibition of CaSR [22, 35, 36]. Also, this inhibitory effect may be counteracted by a protein phosphatase (most likely PP2A) that dephosphorylates Thr-888, restoring CaSR responsiveness [37].

41.3 CaSR Modulation

GPCRs can recognise diverse extracellular stimuli and are one of the most successful pharmaceutical target classes for different disorders. The ligands for GPCRs are typically polypeptides, amino acids and/or other small biological molecules that bind in well-defined pockets [38]. The CaSR, as a multifaceted receptor, is able to bind a broad range of molecules in addition to Ca^{2+}, its primary ligand. CaSR modulators can be divided into two groups: type I or orthosteric modulators, which bind to the active site, and type II or allosteric modulators, which bind elsewhere in the receptor.

41.3.1 Orthosteric Modulators of the CaSR

Orthosteric modulators are type I CaSR agonists and include all ligands that are thought to compete with Ca^{2+} for the same binding sites on the receptor. In addition, they are sufficient to activate the CaSR on their own, in the absence of Ca^{2+}.

Although Ca^{2+} is crucial for CaSR function, many other organic cations can activate CaSR in vitro for instance the divalent cations Mg^{2+} and Sr^{2+} and trivalent cations such as Gd^{3+}, as well as heavy metals such as Pb^{2+} and Co^{2+} which are more potent than Ca^{2+} [39]. In fact, the order of agonist potency for inositol metabolism in bovine parathyroid cells depends on two factors; the charge of the ion and the ionic radii. Thus, among ions with the same charge, those with greater radius have a greater potency and among ions of a similar size those with greater charge have a greater potency [40]. The order of potency for the main orthosteric modulators is as follows: $Gd^{3+} > La^{3+} > Ca^{2+} = Ba^{2+} > Sr^{2+} > Mg^{2+}$ [39]. Many organic polycations, such as the poly-amino acids poly-L-lysine or poly-arginine and aminoglycoside antibiotics such as neomycin, are also orthosteric modulators of the CaSR [41–43]. Polyamines produced in the gut and in the synaptic cleft in vivo, are also CaSR agonists. Spermine is the most potent polyamine followed by spermidine and putrescine. In this case, potency is linked to the number of amine groups in the ligand [44].

41.3.2 Allosteric Modulators of the CaSR

In addition to orthosteric agonists, the CaSR can also be activated by allosteric modulators, sometimes referred to as type II CaSR agonists, and these do not compete for the same binding sites as Ca^{2+}, instead they allosterically modify the endogenous affinity of the receptor for $Ca^{2+}{}_{o}$ [45]. The allosteric modulators affect the conformational equilibrium of the receptor and they can be divided into

two groups: activators or positive allosteric modulators (PAM) if they shift the equilibrium towards the active state, and inhibitors or negative allosteric modulators (NAM), if they stabilise the inactive state. Both types of modulators include compounds that can be found in the body under physiological conditions like L-aromatic amino acids, glutathione, ionic strength and alkalinisation [46], but also synthetic compounds like calcimimetic drugs [47].

L-aromatic amino acids were the first endogenous PAMs identified and these include L-Phe, L-Tyr, L-His and L-Trp, with the short aliphatic amino acids L-Thr and L-Ala also effective [48]. L-amino acids increase CaSR sensitivity in the presence of other agonists, such as Ca^{2+} or Gd^{3+}. This demonstrates that CaSR is able to sense a broad range of nutrients having special relevance in the gastrointestinal tract where the CaSR has been identified as an L-amino acid sensor for macronutrient-dependent hormone secretion [49, 50]. In addition, increased aromatic L-amino acid concentration suppresses PTH secretion stereoselectively by activating endogenous CaSR [51]. Therefore, L-amino acids may play an important role as physiological regulators of PTH secretion and calcium metabolism via CaSR modulation.

Interestingly, pH and ionic strength play a double role in modulating CaSR sensitivity. In the case of pH, CaSR sensitivity can be enhanced when pH is elevated (>7.5), but also reduced when pH is low (<7.3) [52]. Decreasing blood pH by only 0.2–0.4 units significantly increases PTH secretion, suggesting a functionally less active CaSR [53]. In contrast, moderate alkalinisation equivalent to that seen in metabolic alkalosis significantly inhibits PTH secretion independently of a change in $[Ca^{2+}]_o$, suggestive of a more sensitive CaSR [54]. This has been confirmed in vitro, whereby small pathophysiologic pH changes (0.2 units) significantly inhibit CaSR-induced intracellular calcium $Ca^{2+}{}_i$ mobilisation (and also extracellular signal-regulated kinase (ERK1/2) phosphorylation and actin polymerisation [55]) in CaSR-HEK cells and in bovine parathyroid cells [34]. Similarly, increasing the ionic strength of the surrounding buffer can also reduce CaSR sensitivity, whereas reducing the buffer's ionic strength enhances CaSR sensitivity [46]. This suggests that protons and Na^+ can both act as NAMs of the CaSR.

41.3.3 Synthetic Modulators of the CaSR

Over the last 20 years, scientists have been looking for drugs to alleviate pathological abnormalities in plasma PTH and Ca^{2+} levels. As the secretion of PTH is mainly regulated by CaSR, compounds that affect this receptor are good candidates to treat PTH disorders. Thus, new synthetic allosteric modulators with higher potency and specificity have been developed.

Nemeth and colleagues at NPS Pharmaceuticals Inc. successfully identified two small organic molecules that caused a leftward shift in the concentration-response

curve of the CaSR for $[Ca^{2+}]_o$. They named them calcimimetics. These compounds are able to potentiate the effects of $[Ca^{2+}]_o$ probably by stabilising the active conformation of the receptor by binding to the TMD [56–58]. Calcimimetics are considered type II CaSR agonists and most of them are phenylalkylamines and derivatives of Ca^{2+} channel blockers [57, 59]. Some Ca^{2+} channel blockers can also activate the CaSR, worsening the effects in pulmonary arterial hypertension [60].

Cinacalcet, a calcimimetic molecule more easily absorbed than the initially identified analogue NPS R-568, was the first PAM acting on a GPCR to receive FDA approval and enter the clinic. It represents a targeted therapy for the treatment of disorders linked to hyperparathyroidism, including chronic kidney disease (CKD), life-threatening NSHPT, and parathyroid carcinoma [61–63]. In patients with end-stage CKD, treatment with Cinacalcet lowers PTH levels after 2–4 h [64]. However, calcimimetics can evoke significant side effects including adverse gastrointestinal effects, due to the fact that the CaSR is expressed in many other tissues, where it activates different signalling pathways [64, 65]. Apart from nausea, the main side effect of Cinacalcet is hypocalcaemia [66]. Recently, a new peptide calcimimetic called Etelcalcetide (Parsabiv) has just received FDA approval for the treatment of secondary hyperparathyroidism in adult haemodialysis patients with CKD [66, 67]. Other calcimimetics in use either as research tools or as potential clinical agents include Calindol (AC265347) and Velcalcetide (AMG416) [68].

In contrast, synthetic CaSR NAMs called calcilytics have opposite effects to calcimimetics. Calcilytics include the substituted phenyl-O-alkylamine NPS 2143 and NPS 89636 [56]. Their binding site is located within the CaSR TMD and is partly overlapping with the calcimimetic binding site. Two other structural types of compounds, amino alcohols (e.g. Ronacaleret) and quinazolinones (e.g. ATF936), were identified by high-throughput screening and shown to reduce CaSR affinity for $[Ca^{2+}]_o$ [56, 69]. As calcilytics can increase endogenous PTH secretion by inhibiting CaSR they were initially developed to treat osteoporosis by delivering endogenous, anabolic pulses of PTH but they had insufficient efficacy [65]. Currently, calcilytics are being studied in different drug repurposing projects, including asthma and other lung-related diseases [70].

41.4 CaSR Trafficking

Receptor trafficking plays a critical role in GPCR activity through tight regulation of GPCR expression levels at the cellular surface. This regulation can be divided into two opposing routes: (1) trafficking of newly synthesised GPCRs to the cellular surface (i.e. exocytic trafficking) and (2) removal of GPCRs from the cell surface to intracellular compartments (i.e. endocytic trafficking) [71]. This section will focus on the processes and interacting partners involved in CaSR trafficking.

41.4.1 From Protein Synthesis to the Cellular Surface

To initiate downstream signalling, a GPCR is required to be present at the cellular surface where the agonist binding site is accessible to ligand stimulation and its intracellular part can interact with G proteins or other binding partners [71–73]. The outward motion of newly synthesised GPCRs to the cellular surface is driven by exocytic receptor trafficking. In this section, the term exocytic trafficking will be used in the broadest sense to refer to protein synthesis, protein maturation and the transport of newly synthesised GPCRs from the endoplasmic reticulum (ER) and Golgi system to the cellular surface [74].

To date, the processes and binding partners involved in exocytic CaSR trafficking are poorly understood. In humans, the gene that encodes for CaSR is located on chromosome 3q13.3-21 [75]. The CaSR protein is transcribed from six out of the eight mapped exons in this gene and transcription can be initiated from two different promoter sites (i.e. promoter P1 or P2) [76, 77]. In an investigation into the regulation of CaSR transcription, Canaff and Hendy have identified functional vitamin D and NF-κB response elements within both promoters of the CaSR gene [78, 79]. In agreement with these findings, vitamin D and several proinflammatory cytokines have been reported to upregulate rodent and human CaSR expression [79–82].

Correct protein folding and protein maturation through post-translational modifications are essential for cell-surface targeting of GPCRs. Protein folding into the GPCR's functional three-dimensional conformation is assisted by chaperones. To date, a large number of chaperones or GPCR-interacting proteins with chaperone function have been identified, but none of these proteins have been associated with CaSR folding [72, 83]. CaSR maturation involves extensive N-linked glycosylation in the ECD. A total of 11 potential N-linked glycosylation sites have been identified in the CaSR protein. Glycosylation of at least three sites have been found crucial for cell-surface expression. Moreover, western blot analyses of cell lysates containing CaSR demonstrate immunoreactive bands at approximately 140–160 kDa corresponding to immature monomeric CaSR and fully mature monomeric CaSR respectively [25, 84–86].

As mentioned earlier, the CaSR predominantly exists on the cell surface as a homodimer, but with evidence suggesting potential heterodimerisation with other class C GPCR members including mGlu and GABA B receptors [26, 87]. The CaSR homodimerisation process takes place in the ER and is directed by the formation of disulphide linkages and non-covalent interactions at the dimer interface, as confirmed by the recently resolved crystal structures of the CaSR ECD [28, 29].

Protein synthesis and maturation are strictly regulated by the cell to ensure that only correctly folded and fully matured GPCRs are targeted for trafficking towards the cellular surface. This quality control system is proposed to be regulated by GPCR-interacting proteins such as the previously mentioned chaperones as well as by recognition of conserved retention or export motifs [71, 83]. Bouschet et al. have investigated the involvement of receptor activity-modifying proteins (RAMPs)

in exocytic CaSR trafficking. According to Bouschet et al., CaSR interaction with RAMP subtype 1 or 3 facilitates delivery to the cellular surface [88]. This view is supported by Desai and co-workers who demonstrated direct interactions between CaSR and both RAMP subtypes at the cellular surface using FRET-based stoichiometry [89]. The CaSR-interacting protein dorfin mediates ER-associated degradation of the receptor, while filamin A, another interacting protein, protects CaSR from degradation [90–93]. Furthermore, an extended phosphorylation-regulated arginine-rich region was identified in the carboxyl terminus of CaSR which has been shown to be involved in intracellular retention through interaction with 14-3-3 proteins [94–97].

In general, the number of receptors expressed at the cellular surface influences the magnitude of downstream signalling responses. Multiple studies have demonstrated that differences in cell surface expression levels influence CaSR-mediated signalling. Cell surface expression levels of CaSR can be influenced by multiple factors [84, 98, 99]. First, CaSR expression is affected by numerous naturally occurring mutations and polymorphisms. Interestingly, cell surface expression levels of most CaSR mutants could be effectively rectified towards wild-type expression levels upon treatment with calcimimetics or calcilytics [90, 94, 100–102]. Second, phosphorylation at residue Ser-899, a protein kinase A (PKA) phosphorylation site located next to the extended arginine-rich region, has been reported to increase CaSR surface localisation by disruption of 14-3-3 protein binding [94, 95]. Third, a novel trafficking mechanism, referred to as agonist-driven insertional signalling, has been proposed to regulate cell surface expression in response to CaSR activation. According to this mechanism, agonist binding promotes an increase in the forward trafficking of newly synthesised CaSR to the cellular surface from a consistently present intracellular CaSR pool [95].

41.4.2 From the Cellular Surface to Protein Degradation

Endocytic receptor trafficking, also commonly referred to as receptor endocytosis or receptor internalisation, regulates the duration and magnitude of GPCR-activated G protein signalling responses by effective removal of GPCRs from the cellular surface. Besides its crucial role in the termination of GPCR activity, multiple studies have linked receptor endocytosis to the initiation of non-canonical G protein-independent signalling pathways [71, 103]. Endocytic trafficking of CaSR was described to play a role in parathyroid hormone-related protein (PTHrP) secretion and ERK1/2 activation [104, 105].

The molecular mechanism underlying endocytic CaSR trafficking is still poorly investigated. The reported experimental data is rather controversial, and there is no general agreement about the endocytic trafficking route of CaSR. One of the main findings related to CaSR endocytosis is the ability to initiate endocytic trafficking independently of ligand activation [95, 104, 106]. Pi and colleagues stated that phosphorylation preferentially by GRK4 promotes ß-arrestin binding

[107]. However, Lorenz et al. argue that phosphorylation by PKC rather than GRKs mediates ß-arrestin recruitment [108]. This disagreement could potentially be linked to receptor origin as Pi et al. measured desensitisation of rat CaSR while the studies of Lorenz et al. were conducted with human CaSR.

The internalised CaSR can be either recycled or degraded [95, 104, 106, 109]. Similarly as for the exocytic trafficking pathway, the endocytic trafficking pathway is strongly regulated by GPCR-interacting proteins and conserved motifs [103, 110, 111]. In 2012, a presumed internalisation motif linked to lysosomal degradation has been discovered at the CaSR carboxyl terminus [106]. Interestingly, this motif shows an overlap with the filamin A binding site indicating that filamin A might be involved in both exocytic and endocytic trafficking of the CaSR [91]. This hypothesis is supported by the finding that filamin A contributes to the localisation of CaSR to caveolae, a specialised cell membrane region known to be involved in clathrin-independent endocytosis [92, 112, 113]. Furthermore, the CaSR-interacting protein AMSH-1 (associated molecule with the SH3 domain of STAM) has been reported to promote ubiquitin-mediated degradation of internalised CaSR [104, 114].

The ability to rectify the expression of disease-related mutants by calcimimetics and calcilytics highlights the therapeutic potential of modulating endocytic CaSR trafficking in the treatment of CaSR-related diseases. However, further research is needed to fully understand the molecular mechanism underlying CaSR trafficking and its potential as therapeutic target.

41.5 Overview of Signalling Pathways Activated by the CaSR

This section will focus on the signalling pathways mediated by the CaSR, with special attention to the diversity of responses elicited upon activation of the receptor in different tissues. Figure 41.5 shows a simplified overview of what is known to date about CaSR signalling.

41.5.1 G Proteins Activated by the CaSR

G proteins, also called guanine nucleotide-binding proteins, can bind the guanine nucleotides GDP and GTP. GTP-bound, active G proteins have GTPase activity, hydrolysing GTP into GDP and inorganic phosphate (Pi), returning the G protein to its inactive GDP-bound form. The equilibrium of GDP- and GTP-bound forms of the G proteins is a result of the activities of three groups of molecules. Guanine nucleotide exchange factors (GEFs) activate the G proteins by exchanging GDP for GTP. GTPase-activating proteins (GAPs) accelerate the GTPase activity of the G protein and thus terminate its activity. Guanine nucleotide dissociation inhibitors (GDIs) bind GDP-bound G proteins and inhibit activation by the GEFs. G proteins

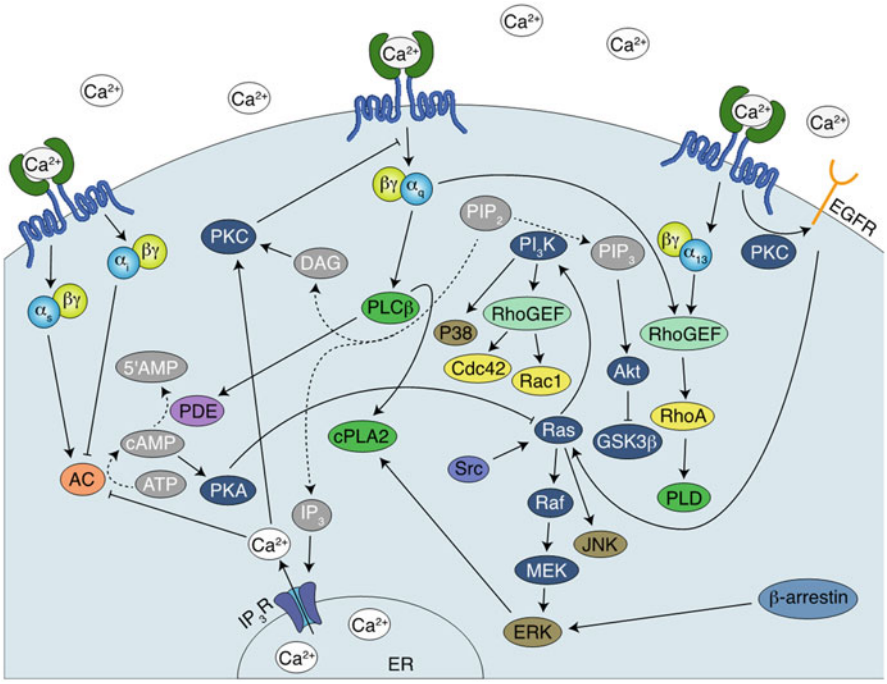

Fig. 41.5 General representation of the signalling pathways activated by CaSR. Arrows, bar-headed lines, and dashed arrows represent activation, inhibition, and a chemical reaction respectively. In green and blue, a CaSR homodimer; in light blue and yellow, the α and βγ subunits of the G proteins; in dark blue, kinases; in light green, phospholipases; in light brown, MAP kinases; in light yellow, Rho GTPases; in grey, second messengers and derivatives

can be either heterotrimeric or monomeric. In this section we will refer to them as "G proteins" and "small GTPases" respectively.

G proteins are heterotrimers composed of α, β, and γ subunits. To date, a total of 21 α, 6 β, and 12 different γ subunits have been identified in humans. G proteins are activated by GPCRs, such as the CaSR, which act as GEFs. The exchange of GDP for GTP, bound to the α subunit, results in the dissociation of the α subunit from the βγ dimer. Subsequently, both the GTP-bound α subunit and the βγ heterodimer activate signalling pathways until the GTP is hydrolysed into GDP and the heterotrimer is reassembled. GAPs accelerate the hydrolysis and are also known as regulators of G protein signalling (RGS). These events are illustrated in Fig. 41.6.

Among the well-accepted effects of the βγ dimer are the regulation of K^+ and voltage-dependent Ca^{2+} channels, adenylyl cyclases (ACs), phospholipases C (PLCs), and phosphoinositide 3-kinases (PI_3Ks) [115]. In addition, βγ heterodimers have been suggested to affect transcription, trafficking and signalling at different subcellular locations [116].

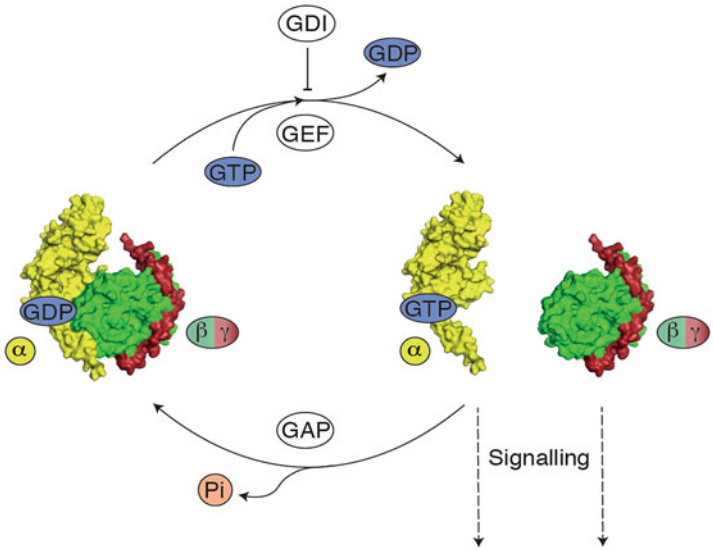

Fig. 41.6 Simplified representation of the cycles of activation and deactivation of G proteins

As for many GPCRs, the CaSR couples to more than one family of heterotrimeric G proteins, especially to $G_{q/11}$ and $G_{i/o}$. However several studies also suggest that the CaSR may couple to members of the $G_{12/13}$ family, and also to G_s in cancer-derived cell lines [18].

41.5.1.1 $G_{q/11}$

G_q and G_{11} share 90% sequence homology, are ubiquitously expressed, and have similar functions. For historic reasons, most studies focus on G_q and to a lesser extent on G_{11} [117]. The α subunits of $G_{q/11}$ activate PLCβ, which cleaves membrane-located phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 diffuses into the cytosol and binds the IP_3 receptors that reside in the ER, causing the release of $Ca^{2+}{}_i$. IP_3 is then metabolised to IP_2 and IP_1. Increased $[Ca^{2+}]_i$ together with DAG, localised in the plasma membrane, results in the recruitment and activation of multiple isoforms of PKC. PKC phosphorylates numerous other proteins [118], including CaSR at Thr-888 to regulate $Ca^{2+}{}_i$ oscillations [119]. CaSR-induced IP_3 generation and $Ca^{2+}{}_i$ mobilisation was first shown in *Xenopus laevis* oocytes expressing the bovine parathyroid CaSR in the original cloning paper [3] but is more commonly investigated in HEK293 cells stably expressing the CaSR (HEK-CaSR) [18] [120] thus confirming the central role of the $G_{q/11}$-PLCβ pathway in CaSR signalling.

In addition to the classic $G_{q/11}$-PLCβ pathway, recent studies have shown that $G_{q/11}$ can also activate other signalling pathways via RhoGEFs such as RhoA [121–123], although the relevance of this pathway for the CaSR has not yet been determined.

41.5.1.2 $G_{i/o}$

The members of the $G_{i/o}$ family are characterised by their sensitivity to pertussis toxin (PTx), which inhibits their interaction with the GPCR, the only exception being G_z [124]. G_{i1}, G_{i2}, G_{i3} and G_o subtypes share a high sequence homology and probably have overlapping functions, although G_o is localised predominantly in the central nervous system [125]. Due to the relatively high abundance of this family of G proteins compared to the others, and since the majority of signalling events activated by βγ are sensitive to PTx [126], the signalling by βγ dimers is often attributed to activation of $G_{i/o}$ [127].

Activation of G_i inhibits several types of ACs. ACs increase cytosolic levels of cAMP and therefore G_i activation lowers cAMP levels. Studies in HEK-CaSR cells show that increased $[Ca^{2+}]_o$ decreases forskolin-induced increase in cAMP, suggesting the activation of G_i [120]. In bovine parathyroid and HEK-CaSR cells, CaSR stimulates ERK1/2 phosphorylation via $G_{q/11}$ and G_i pathways [128]. A later study suggested that G_{i2} is the $G_{i/o}$ subtype responsible for ERK1/2 activation [105].

41.5.1.3 $G_{12/13}$

The activation of $G_{12/13}$ proteins recruit to the membrane and activate RhoGEFs that specifically activate RhoA, such as p115-RhoGEF, which also acts as a GAP through its RGS domain terminating the activity of $G_{12/13}$ [129]. Using Madin-Darby canine kidney cells stably overexpressing CaSR, Miller and collaborators found that $[Ca^{2+}]_o$ activated phospholipase D via RhoA, and that this was mediated by $G_{12/13}$ and independent of $G_{q/11}$ and G_i [130]. Another study, suggested a pathway specifically activated by L-Phe via $G_{12/13}$ in mouse embryonic fibroblasts that resulted in $Ca^{2+}{}_i$ oscillations [131].

41.5.1.4 G_s

A few studies have reported G_s coupling to the CaSR. Wysolmerski and collaborators first showed that CaSR couples to G_i in healthy mammary epithelial cells, but then switches to G_s in both MCF-7 human breast cancer cells, and in Comma-D immortalised murine mammary cells. Surprisingly, no IP_1 accumulation was observed, whereas the levels of active mitogen-activated protein kinases (MAPKs)

were increased upon stimulation with high $[Ca^{2+}]_o$. They also found that cAMP regulated the secretion of PTHrP via PKA [132], which was corroborated in a recent study [133]. In mouse pituitary gland tumour derived AtT-20 cells, the same group showed that CaSR activation stimulated PTHrP via the same mechanism, G_s-cAMP-PKA, independently of PLC or PKC [134]. In a previous study using the same cell line, increases in IP_1 concentrations were sensitive to PTx, showing simultaneous coupling both to G_i and G_s [135]. G protein switching has been observed also for the β2-adrenergic receptor, where PKA phosphorylates the receptor, increasing its affinity for G_i versus G_s. As a result, it switches signalling from cAMP/PKA to MAPK activation [136].

41.5.2 Rho GTPases

Rho GTPases belong to the Ras family of GTPases, which are the most known small monomeric GTPases. Among the Rho GTPases, the best characterised are RhoA, Rac1 and Cdc42. Rho GTPases play a central role in cell migration, cell polarity, and cell cycle progression, by regulating cell adhesion and actin cytoskeleton dynamics [137]. The activation of RhoA has been traditionally associated exclusively with $G_{12/13}$ signalling, however there is increasing evidence of activation by $G_{q/11}$ via RhoGEFs and independent of PLCβ [138]. It has been suggested that the CaSR activates PI_4-kinase via Rho [139]. CaSR activation produced actin stress fibre assembly in HEK-CaSR, in a Rho kinase-dependent mechanism. This phenomenon was PTx-insensitive and the PLCβ inhibitor U73122 showed no effect [140]. Since U73122 can activate ion channels at the concentrations used to inhibit PLCβ [141] the recently available potent and specific $G_{q/11}$ inhibitors FR900359 and YM-254890 may prove better reagents for the investigation of $G_{q/11}$ signalling [142].

A study in human keratinocytes showed that CaSR-dependent activation of RhoA plays a role in cell-cell adhesion [143], whereas experiments in human podocytes showed that CaSR activated RhoA via $Ca^{2+}{}_i$ mobilisation, in a mechanism dependent of the ion channel TRPC6 [144].

The activation of Rac and Cdc42 by G proteins is less clearly defined. In highly motile cells βγ-mediated activation of PI_3K and the GEF PRex resulted in Rac1 activity. Whether these signalling modules play a role in CaSR signal transduction remains to be demonstrated [145]. In primary human monocyte-derived macrophages CaSR activated Rac and/or Cdc42, but no RhoA, to regulate membrane ruffling via a mechanism dependent on PI_3K [146]. A study in a human T cell line found that CaSR can promote cell migration by activating Cdc42, also via a PI_3K-dependent mechanism [147]. A study in HEK-CaSR cells showed that membrane ruffling is $G_{q/11}$-dependent and $G_{12/13}$-independent, suggesting activation of Rho GTPases by $G_{q/11}$ [148].

41.5.3 β-Arrestins

In addition to their key role in terminating G protein signalling pathways activated by GPCRs, β-arrestins can also activate signalling events [149]. Specifically in HEK-CaSR cells, β-arrestin 1 is involved in CaSR-induced plasma membrane ruffling [150] while β-arrestins 1 and 2 are involved in CaSR-induced ERK1/2 activation [151].

41.5.4 CaSR-Induced Protein Kinase Activation

The CaSR activates a number of protein kinase families including glycogen synthase kinase-3 (GSK3), Akt, and the MAPKs, and these will be detailed in turn.

41.5.4.1 Akt and GSK-3β

Akt, or protein kinase B, is a protein kinase that regulates multiple functions such as growth, proliferation and transcription. The first step for Akt activation is binding to PIP_3 in the membrane. PIP_3-bound Akt is sequentially phosphorylated first at Thr-308 and then at Ser-473 for full activation [152]. GSK3 is involved in the phosphorylation of over a hundred substrates, and it interacts with multiple types of receptors. It exists in two isoforms, α and β, and it can be phosphorylated by PKA, PKC, and Akt, among others. Phosphorylation of GSK3-β at Ser-9 results in inhibition of the binding to certain substrates that require binding to a domain in the protein prior to phosphorylation [153].

Studies in fetal rat calvarial cells, murine osteoblast 2T3 cells, and human osteoblasts, show that CaSR activation results in phosphorylation of Akt at Thr-308 and Ser-473, and of GSK3-β at Ser-9 [154, 155]. Further, in proximal tubular opossum kidney cells, the CaSR ligands neomycin and gentamicin elicit phosphorylation of Akt and GSK3-β in a PI_3K-dependent fashion [42].

41.5.4.2 MAPKs

Several studies have recently explored the role played by CaSR in the phosphorylation of protein kinases, both in healthy tissue and in disease models. The MAPKs include ERK, c-Jun amino-terminal kinases (JNK), and P38. These proteins are activated by phosphorylation and thus we will refer to the active phosphorylated forms as p-ERK1/2, p-JNK and p-P38.

Activation of ERK1/2 can be Ras- or PKC-dependent. Ras-dependent activation involves PI_3K, Src family kinases, and receptor tyrosine kinases such as the epidermal growth factor receptor. A study in HEK-CaSR cells showed that ERK1/2

activation by CaSR was Ras-dependent, relied largely on PI_3K activity, and was independent of tyrosine kinase activity [156]. In contrast, another study in HEK-CaSR cells and in bovine parathyroid cells, showed that the cytoplasmic tyrosine kinase inhibitor, herbimycin, inhibited ERK1/2 phosphorylation [128]. A similar result was observed for ERK1 in Rat-1 fibroblasts [157]. In proximal tubular opossum-kidney cells, activation of CaSR by neomycin induced P38 activation via a PI_3K-mediated mechanism [41].

Across different tissues, increased CaSR expression and activation correlates positively with an increase in p-ERK1/2 levels [41, 154, 158–162], except for a study on hearts of a rat epilepsy model where p-ERK1/2 levels decreased [163]. A similar positive correlation was observed for p-JNK [161–164], whereas one study showed no effect [160]. As for p-P38, a similar number of studies show a positive correlation [41, 161, 163, 164] or no effect [158, 160, 162].

Overall, a prolonged exposure to CaSR agonists increases mRNA or protein expression levels of CaSR, and this phenomenon often correlates positively with an increase in active ERK1/2, JNK, and P38. Differences in phosphorylation are observed reflecting the different signalling profiles of CaSR in different tissues.

41.6 Ligand-Biased Signalling Through the CaSR

Ligand-biased signalling is a relatively new concept based on the idea that a receptor can exist in multiple active conformations, each stabilised by a specific ligand, with characteristic binding kinetics, and therefore with a particular signalling profile [165]. For GPCRs, this would translate into different coupling behaviours towards G proteins and β-arrestins [166]. For allosteric modulators, the concept extends to how these molecules affect positively or negatively each of the pathways activated by the orthosteric ligands. Exploiting this phenomenon offers great potential for the discovery and development of new drugs with increased efficacy and safety, which is of course of interest for the pharmaceutical industry [167].

Several studies have used the CaSR as a model to study ligand-biased signalling, given that it can activate multiple signalling pathways and it can be modulated by a wide range of different ligands [168]. This phenomenon has been studied using pharmacological assays, applied in a high-throughput manner, and using multiple agonists. The receptor readouts usually follow changes in $Ca^{2+}{}_i$ mobilisation, IP_1 accumulation, cAMP levels, phosphorylation of ERK1/2, and plasma membrane ruffling. The first two provide information on the $G_{q/11}$-PLCβ pathway; cAMP on G_i and G_s activity; p-ERK1/2 on G_q, G_i, and β-arrestins; and PM ruffling on Rho GTPases and β-arrestins.

These studies often rely on obtaining concentration-response curves for different ligands, and comparing calculated values such as EC_{50}, dissociation constant, maximum response, or cooperativity αβ for allosteric modulators. Additionally, receptor expression levels are often also determined, and in fact regulation of cell surface expression has been proposed as a mechanism of bias by allosteric

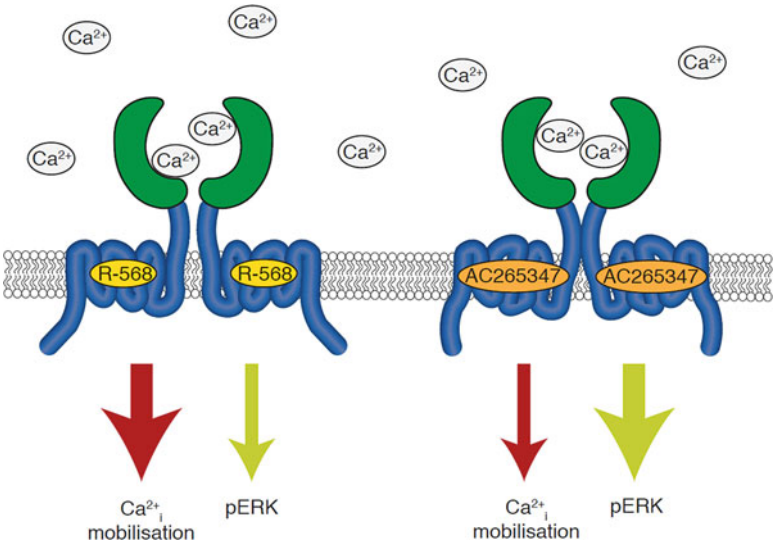

Fig. 41.7 Representation of bias by the positive allosteric modulators R-568 and AC265347 in $Ca^{2+}{}_i$ mobilisation and phosphorylation of ERK1/2 upon activation of the CaSR by $Ca^{2+}{}_o$

modulators [100, 169]. Multiple studies in HEK-CaSR cells have addressed ligand-bias by orthosteric ligands, as well as positive and negative allosteric modulators [151, 170–173], including one that explored the effect of naturally occurring CaSR mutations on ligand-bias [102].

These systematic in vitro studies provide valuable information to understand the differences in the effects of the ligands in vivo. For example, Leach and collaborators found that the calcimimetic AC265347 tunes the effect of $[Ca^{2+}]_o$ to favour phosphorylation of ERK1/2 and accumulation of IP_1, as compared to $Ca^{2+}{}_i$ mobilisation. Interestingly, AC265347 did not increase trafficking of loss-of-expression CaSR mutants, an effect observed by other calcimimetics, suggesting that it may act via a new mechanism [170]. Figure 41.7 shows an example of the signalling bias caused by two of the allosteric modulators used in this study.

41.7 Tissue-Specific Signalling of the CaSR

The pleiotropy of the CaSR arises as a result of its ability to couple to various G proteins and thus to mediate distinct signalling pathways. Consequently, the CaSR may fine-tune several physiological processes in a tissue-specific manner. The ability of GPCRs to mediate tissue-specific signalling is dictated by the cellular environment, as evidenced by recombinant systems where the same GPCR can have different pharmacological profiles in different cellular backgrounds. This

phenomenon is termed tissue-specific signalling or system bias. It arises when ligands favour the interaction of a receptor ensemble to auxiliary proteins, or when receptors form heterodimers with distinct pharmacological signatures [174]. The capacity of CaSR ligands to promote coupling to multiple G proteins and to differing extents was previously discussed in the context of biased signalling. Here, tissue-specific signalling is discussed in light of the evidence showing interaction of the CaSR with other proteins and the formation of heterodimers in different cellular environments.

The CaSR interacts with various proteins that influence its signalling signature. Several CaSR interacting proteins have been identified and these include inwardly-rectifying potassium channels [175] and the previously described filamin A [92, 93, 176] as well as the RAMPs [88]. In addition, the CaSR may form heterodimers with other class C GPCRs including mGlu1a, mGlu5 and GABA B receptors, as shown in endogenous and recombinant systems [87]. Such heterodimerisation could thus provide the CaSR another mechanism for tissue-specific signalling.

41.8 Future Topics and Concluding Remarks

The recently published crystallographic data of the CaSR ECD structure has shed some light on CaSR ligand recognition, receptor activation, allosteric modulation, as well as on the structural basis of dimerisation. The medicinal importance of CaSR modulation is clear and therefore obtaining the ECD structure will facilitate structure-based drug discovery and might open up further therapeutic approaches. These data also raise the question of whether L-aromatic amino acids and relevant anions should be added to the experimental buffers when studying CaSR function to preserve the receptor's native conformation. The full structure of the CaSR has yet to be determined and thus obtaining the crystal structures of the CaSR's TMD and ICD is a high priority in the field as this will help understanding effector interactions. In addition, the current structural data provides only a snapshot of the receptor in a fixed conformation, whereas in physiology the receptor is a dynamic molecule wobbling between multiple conformations. Thus, we need a new and more dynamic approach able to reveal a protein's structure in its transition states, ideally allowing us to see conformational changes upon agonist/antagonist binding at the receptor.

The CaSR field would also benefit from new reagents such as a CaSR-selective radioligand, as calcium itself is a too low-affinity ligand to be of use in binding studies. Such a radioligand would allow researchers to investigate whether CaSR biased signalling might be driven by ligand binding kinetics. Moreover, the radioligand could be used to study CaSR expression in native tissues and cells as CaSR expression analysis is currently hampered by nonspecific staining of commercially available CaSR antibodies. Next, the newly emerging FRET-based biosensors can be used to observe G protein activation of the CaSR directly, providing dynamic information on the first step in the signalling cascade [177]. Indeed by taking

advantage of the plethora of fluorescent biosensors available, given particular spectroscopic properties, it is possible to measure activation of multiple proteins simultaneously and in real-time [178]. This would be of particular value when studying biased signalling, as current protocols are susceptible to time- and assay-specific artefacts.

Finally, a recent publication suggested that internalised CaSR could have a role in sustained signalling [148]. This phenomenon has been proposed before for some class A GPCRs [179], and implies a new signalling mechanism by CaSR. The relevance of the observations for internalised CaSR signalling needs to be addressed in follow-up studies.

We can conclude therefore that CaSR activation results in a wide range of downstream signals and functions at different timescales and across a variety of tissues. To make sense of this complexity will require better understanding of a range of factors including differential ligand affinity and bias, receptor heterodimerisation, as well as downstream effector selection. The benefit of such information could be the rational development of novel drugs with improved efficacy and safety.

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