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Title Page

Agonists and allosteric modulators of the Calcium Sensing Receptor and their therapeutic applications

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Running title page

Running title: Agonists and allosteric modulators of the CaR

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Abbreviations:

ADH: autosomal dominant hypocalcemia; AGAs: aminoglycoside antibiotics; $Ca^{2+}{}_i$: intracellular Ca^{2+}; $Ca^{2+}{}_o$: extracellular Ca^{2+}; CaR: Calcium sensing Receptor; ECD: extracellular N terminal domain; FHH: Familial hypocalciuric hypercalcemia; GPCR: G protein-coupled receptor; HPT: hyperparathyroidism; IP_3: inositol 1,4,5-triphosphate; $Mg^{2+}{}_o$: extracellular Mg^{2+}; MAPK: mitogen-activated protein kinase; NSHPT: neonatal severe hyperparathyroidism; pH_o: extracellular pH; PLCβ: phospholipase Cβ; PTH: parathyroid hormone; RANK: Receptor Activator for NF-κB; TM: transmembrane; TRPC: transient receptor potential cation channel; VFTM: Venus Flytrap Domain Motif

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Abstract

The Calcium sensing Receptor (CaR) belongs to the G protein-coupled receptor superfamily, with a characteristic structure consisting of seven transmembrane helices, an intracellular C terminal and an extracellular N terminal domain. The primary physiological function of the CaR is the maintenance of constant blood Ca^{2+} levels, due to its ability to sense very small changes in extracellular Ca^{2+} ($Ca^{2+}{}_o$) concentrations. Nevertheless, in addition to being expressed in tissues involved in $Ca^{2+}{}_o$ homeostasis, the CaR is also expressed in tissues not involved in mineral homeostasis, suggestive of additional physiological functions. Numerous agonists and modulators of the CaR are now known in addition to $Ca^{2+}{}_o$, including various divalent and trivalent cations, aromatic L-amino acids, polyamines, and aminoglycoside antibiotics. The signaling of the CaR is also regulated by extracellular pH and ionic strength. The activated CaR couples mainly to the phospholipase Cβ and ERK1/2 signaling pathways, and it decreases intracellular cAMP levels, leading to various physiological effects. The recent identification of synthetic allosteric modulators of the CaR has opened up a new field of research possibilities. Calcimimetics and calcilytics, which increase and decrease agonist signaling via the CaR, respectively, may facilitate the manipulation of the CaR, and thus aid in further investigations of its precise signaling. These allosteric modulators, as well as strontium, have been demonstrated to have therapeutic potential for the treatment of disorders involving the CaR. This review discusses the various agonists and modulators of the CaR, differences in their binding and signaling, and their roles as therapeutics in various diseases.

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1. Introduction

a. Structure and signaling of the CaR

The Ca^{2+}-sensing receptor (CaR), initially cloned from the bovine parathyroid, belongs to family 3 (or C) of the G protein-coupled receptor (GPCR) superfamily (Brown et al., 1993). It has a structure characteristic of GPCRs, consisting of seven transmembrane (TM) helices, an intracellular C terminal and a large extracellular N terminal domain (ECD), typical of family 3 GPCRs. The ECD is structurally similar to the Venus Flytrap Domain Motif (VFTM) of bacterial periplasmic binding proteins (Brown and MacLeod, 2001; Pin et al., 2003). It has been demonstrated that when expressed at the cell surface the CaR exists mainly in the form of a dimer (Bai et al., 1998; Zhang et al., 2001), in which the monomers are covalently linked by disulfide bridges involving two cysteine residues (Cys129 and Cys131) within the VFTMs (Ray et al., 1999) (Figure 1.). As is the case for other GPCRs, the activated CaR is capable of activating multiple types of G proteins from different G protein subfamilies, primarily $G\alpha_{q/11}$ and $G\alpha_i$. This leads to a range of cellular responses, including stimulation of phospholipase Cβ (PLCβ), production of inositol 1,4,5-triphosphate (IP_3), release of intracellular Ca^{2+} ($Ca^{2+}{}_i$), stimulation of mitogen-activated protein kinases (MAPKs), and an inhibition of adenylate cyclase, causing a decrease in cAMP levels (Brown and MacLeod, 2001; Maiti et al., 2008) (Figure 1).

b. Physiological functions of the CaR

The primary physiological role of the CaR is the maintenance of constant blood Ca^{2+} levels (1.1-1.3 mM) through continuous adjustments of parathyroid hormone (PTH) release from the parathyroid chief cells, which are highly sensitive to the slightest changes in extracellular Ca^{2+} ($Ca^{2+}{}_o$) (Brown et al., 1993; Brown and MacLeod, 2001). When a decrease in $Ca^{2+}{}_o$ is sensed, PTH secretion increases, through an as yet poorly defined mechanism, likely involving cytoskeletal components F-actin and caveolin-1 (Quinn et al., 2007). The resultant increase in circulating PTH normalizes the $Ca^{2+}{}_o$ levels by its actions on the kidneys, bones and, indirectly, intestines (Brown, 1991; Kurokawa, 1994). The opposite effect on PTH secretion is observed when an increase in $Ca^{2+}{}_o$ is sensed through the CaR, leading to a decrease in PTH release. In contrast to its actions on the PTH, activation of the CaR by $Ca^{2+}{}_o$ has a stimulatory effect on calcitonin secretion from C cells of the thyroid (Garrett et al., 1995). Thus, through a refined, bidirectional mechanism, the CaR regulates the secretion of PTH and calcitonin, playing a primary role in the maintenance of $Ca^{2+}{}_o$ homeostasis.

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Many questions about the physiological significance of the CaR have been answered through the identification of mutations of the CaR gene, that were demonstrated to cause several inherited disorders of Ca^{2+}_{o} sensing. Familial hypocalciuric hypercalcemia (FHH) (Pollak et al., 1993) and neonatal severe hyperparathyroidism (NSHPT) (Pollak et al., 1993) arise from loss-of-function mutations of the CaR gene, which right-shift the set-point for Ca^{2+}_{o} inhibition of PTH secretion and for stimulation of urinary Ca^{2+} excretion, whereas autosomal dominant hypocalcemia (ADH) and Bartter Syndrome type V (Watanabe et al., 2002) are caused by gain-of-function mutations of the CaR (Pollak et al., 1994; Zhao et al., 1999). Identification that these disorders are caused by CaR mutations definitively confirmed the involvement of this receptor in Ca^{2+}_{o} homeostasis (Brown, 1997).

The CaR is widely expressed and apart from maintaining constant blood Ca^{2+} levels, it has numerous other functions in different tissues. CaR expression has of course been confirmed in cell types involved in Ca^{2+}_{o} homeostasis, including the parathyroid (Brown et al., 1993), the thyroid (Garrett et al., 1995), kidneys (Riccardi et al., 1996; Riccardi et al., 1995) and the bone (Chang et al., 2008; Kameda et al., 1998; Mentaverri et al., 2006), as well as in tissues not involved in Ca^{2+}_{o} homeostasis, such as the brain (Ruat et al., 1995), the large intestine (Sheinin et al., 2000), lens epithelial cells (Chattopadhyay et al., 1997), the pancreas (Squires et al., 2000), the liver (Canaff et al., 2001), antral gastrin cells of the stomach (Ray et al., 1997), and cells of the cardiovascular system (Wonneberger et al., 2000; Ziegelstein et al., 2006), influencing numerous physiological processes, including gastric acid secretion (Dufner et al., 2005), hepatic bile secretion (Canaff et al., 2001) and insulin release from the β cells of the pancreas (Squires et al., 2000) to name a few, as well as pathological processes such as vascular calcification and atherosclerosis (Alam et al., 2009).

2. Agonists and allosteric modulators of the CaR

CaR ligands are normally classified as orthosteric agonists (type I agonists), that are capable of activating the CaR on their own, and allosteric modulators, that bind to allosteric sites on the CaR and require the binding of an orthosteric agonist to the receptor to produce their effects. Clearly, Ca^{2+}_{o} is the primary orthosteric agonist of the CaR, and it is the only ligand of this receptor with an incontestable physiological role through the CaR. Other orthosteric agonists include divalent and trivalent cations, including Mg^{2+}, Al^{3+}, Sr^{2+}, Mn^{2+}, Ni^{2+}, Gd^{3+} and Ba^{2+} (Brown, 2007; McGehee et al., 1997), aminoglycoside antibiotics (AGAs) (McLarnon et al., 2002) and polyamines (Cheng et al., 2004), all of which are positively charged. In general, CaR agonists with a high positive charge density tend to have higher potency. An orthosteric antagonist of the CaR has not yet been identified.

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Both positive (type II agonists) and negative allosteric modulators of the CaR have been identified. Aromatic L-amino acids have been demonstrated to act as positive allosteric modulators of the CaR (Lee et al., 2007), and they enhance $Ca^{2+}{}_o$ signaling (Conigrave et al., 2000). Synthetic positive allosteric modulators of the CaR, known as calcimimetics, just like aromatic L-amino acids, shift the concentration-response curve of $Ca^{2+}{}_o$ to the left. Negative allosteric modulators of the CaR are known as calcilytics, and they right-shift the concentration-response curve of $Ca^{2+}{}_o$. In addition to the various ligands of the CaR, recent studies have demonstrated that extracellular pH (pH_o) and ionic strength are also capable of modulating the activity of this receptor (Quinn et al., 2004; Quinn et al., 1998).

Modulation of CaR signaling by exogenous ligands is of great therapeutic interest. For example Sr^{2+} (in a ranelic acid form) has been demonstrated to be effective for the treatment of osteoporosis (Kendler et al., 2009), and it is currently used for this purpose, although whether its beneficial effects are produced through the CaR still remains to be confirmed. The recent discovery of calcimimetics and calcilytics has created a lot of excitement, as their use allows for a more specific regulation of the CaR in numerous diseases associated with decreased and increased CaR signaling, respectively. Calcimimetics have been described to be effective in the treatment of hyperparathyroidism (HPT) (Quarles et al., 2003) and investigations are currently under way to determine the therapeutic potential of calcilytics in the treatment of osteoporosis (Brown, 2007). Therefore, understanding the precise signaling of the various CaR ligands is of great importance, in order to minimize the potential adverse effects that may arise through mishandling of a receptor involved in the crucial physiological function of $Ca^{2+}{}_o$ homeostasis. The therapeutic applications of various CaR ligands are discussed in further detail below.

a. Differential binding sites of various CaR ligands on the CaR

The identification of activating and inactivating mutations of the CaR involved in disorders of $Ca^{2+}{}_o$ sensing has greatly helped in elucidating the role of individual residues of the CaR in ligand binding and subsequent signaling. Early *in vitro* studies have demonstrated the causative role of the inactivating mutations of the CaR (for example P55L, N178D, R185Q, Y218S, P221S, R227L, R680C, P747fs, V817I) in FHH and NSHPT, by observing increased EC_{50} values at activating intracellular pathways through the mutant receptors (Bai et al., 1996; Pearce et al., 1996). In contrast, mutations associated with ADH (for example F128L, T151M, and E191K) lead to reduced EC_{50} for $Ca^{2+}{}_o$ (Pearce et al., 1996). It was hypothesized that the modified signaling of mutant receptors was either due to altered affinity of the receptor for its agonists, or due to a failure or

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facilitation to couple and/or activate the available, suitable G proteins, as a result of a modified receptor conformation. In addition, modified cell surface trafficking of receptors could also be a contributing factor (D'Souza-Li et al., 2002).

The precise localization of Ca^{2+}_o binding has been hampered by the unsuccessful attempts to crystallize the CaR. However, recent studies by Huang et al (Huang et al., 2009; Huang et al., 2007) which have utilized homology modeling using the metabotropic glutamate receptor type 1 (mGluR1) as a model, have greatly helped in this quest and they have reported the existence of five distinct Ca^{2+}_o binding sites (sites 1-5) located in the VFTM of the CaR, confirming the generally held belief that the ECD is the principal binding site for Ca^{2+}_o (Huang et al., 2009). The residues involved in each site are demonstrated in Figure 2. Some of the residues identified in these studies correspond to previously-described activating and inactivating mutations (for example Y218) or are located in close proximity to known mutations (for example E228). While sites 4 and 5 do not correspond to any known naturally-occurring mutations, a previous study has described that double mutant receptors, involving residues in these sites (E378I/E379I and E398I/E399I) display altered Ca^{2+}_o-induced Ca^{2+}_i stimulation (Huang et al., 2007). The presence of five Ca^{2+}_o binding sites in the VFTM of the CaR may explain the reported high (around 3-4) Hill coefficient of CaR activation. Mun et al (2004) have shown that the VFTM of the CaR is also involved in aromatic L-amino acid sensing (Mun et al., 2004). It was demonstrated that chimeric receptor constructs of CaR-mGluR1 that retained the VFTM domain of CaR, retained amino acid sensing. While, a CaR lacking residues 1-599 of the ECD but with an intact TM region and a functional but truncated C terminus (T903 CaR) failed to respond to aromatic L-amino acids but retained responsiveness to the calcimimetic NPS R-467 (Mun et al., 2004). Additionally, the binding of aromatic L-amino acids to the ECD was supported using a series of receptor mutants involving the VFTM (Zhang et al., 2002).

However, the VFTM is not the only site for orthosteric binding. As described above, CaR mutations at residues outside of the VFTM lead to reduced Ca^{2+}_o signaling; therefore, it is possible that other binding sites for Ca^{2+}_o exist. Ray and Northup (2002) have created a mutant CaR, lacking most of the C terminal and N terminal domains, and they have demonstrated that Ca^{2+}, Mg^{2+}, Gd^{3+}, spermine and poly-L-arginine produced intracellular signaling in HEK293 cells, showing that some of the binding sites for these ligands are also located in the seven TM domains (Ray and Northup, 2002).

Calcimimetic and calcilytic binding sites have previously been shown to be located in the TM helices and extracellular loops of the CaR (Miedlich et al., 2004; Petrel et al., 2004; Petrel et al., 2003) (Figure 2). Miedlich and colleagues (2004) have shown that mutations of certain residues present in the TM helices (Phe-668, Phe-684 and Glu-837) reduced the effects of both the calcilytic

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NPS 2143 and the calcimimetic NPS R-568. While another residue, Arg-680, was involved only in NPS 2143 binding, possibly as a result of structural differences between the two ligands due to the presence of an alkyl bridge hydroxyl group in NPS 2143 (Figure 3B). Further studies (Petrel et al., 2004; Petrel et al., 2003) have identified additional residues involved in calcilytic and calcimimetic binding located in TM VI and TM VII (Trp-818, Phe-821, and Ile-841). Thus the binding pockets of calcilytics and calcimimetics are partially overlapping but non-identical (Figure 2).

b. Orthosteric agonists of the CaR

i. Other divalent and trivalent cations as agonists of the CaR

Magnesium (Mg^{2+})

It has been recognized for years that in contrast to an increase in PTH secretion observed at low Ca^{2+}_o levels, a paradoxical reduction in PTH secretion occurs in hypomagnesemic patients (Anast et al., 1972; Duran et al., 1984; Mennes et al., 1978). This phenomenon has been confirmed *in vitro* and the release of PTH from parathyroid cells was shown to decrease at low Mg^{2+} (Quitterer et al., 2001). Whether the CaR is implicated in this effect was investigated by creating CaR mutants involving residues described to be involved in extracellular Mg^{2+} (Mg^{2+}_o) binding (R185Q, F128L, R795W) (Quitterer et al., 2001). This, however, was without effect and the Mg^{2+} response remained unaltered using the mutant receptors. In addition to its extracellular functions, Mg^{2+} is known to have a vital intracellular role in modulating the activity of heterotrimeric G proteins by inhibiting guanine nucleotide exchange (Higashijima et al., 1987; La Piana et al., 2008; Quitterer et al., 2001). Thus, modulation of the activity of Gα subunits by Mg^{2+} was proposed as a possible explanation for the paradox, and indeed it was demonstrated that when Gα with reduced affinity for Mg^{2+} was used, CaR activation was no longer affected by Mg^{2+} deficiency (Quitterer et al., 2001). However, despite the paradoxical findings in severely hypomagnesemic patients, under normal situations, the secretion of PTH from the parathyroid can also be negatively controlled by Mg^{2+} (Navarro et al., 1999), in a manner similar to Ca^{2+}. Furthermore, early studies have shown that patients with FHH and NSHPT and mice lacking the CaR, have elevated serum Mg^{2+} levels, showing that the CaR also acts as a Mg^{2+}-sensing receptor *in vivo* (Brown, 1997; Ho et al., 1995).

While in general Mg^{2+}_o produces similar effects through the CaR to those of Ca^{2+}, inconsistent sensitivity of the CaR to Mg^{2+}_o has been reported in different cells and tissues. In the parathyroid, Mg^{2+} has a potency comparable to that of Ca^{2+}_o, whereas sheep parafollicular cells of the thyroid are

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many-fold less sensitive to Mg^{2+}_o compared to other divalent cations, with a rank order: Gd^{3+}>Ba^{2+}>Ca^{2+}>>Mg^{2+} (McGehee et al., 1997). The insensitivity of these cells to Mg^{2+}_o has been suggested to be due to species differences in the CaR (Garrett et al., 1995), possibly as a result of differential post-translational modifications or expression of accessory proteins (McGehee et al., 1997). Similar insensitivity of the CaR to Mg^{2+}_o was reported by Yamashita (1990) using rat calcitonin-secreting cell line rMTC (Yamashita and Hagiwara, 1990), suggesting that differences exist in CaR signaling by different cations depending on cell type.

Aluminium (Al^{3+})

Physiologically, Al^{3+} is present only in trace amounts in biological systems and normal serum concentrations of Al^{3+} are less than 0.4 μM (Lajeunesse et al., 1998). However, accumulation of Al^{3+} occurs in certain diseases, for example in end stage renal disease, with Al^{3+} levels at around 5 μM (Lajeunesse et al., 1998). Increased serum Al^{3+} concentrations produce various adverse effects including central nervous system dysfunction, decreased bone mineral content and renal failure (Jespersen et al., 1991; Quarles and Drezner, 1991).

It has been suggested previously that Al^{3+} may produce its physiological effects through the CaR (Lau et al., 1991; Quarles et al., 1997). However, Al^{3+} is a weak agonist of the CaR and Spurney and colleagues (1999) have described that in HEK293 cells transfected with the CaR, Ca^{2+}, Mg^{2+} and Gd^{3+} produced an increase in Ca^{2+}_i levels and IP_3 generation in a concentration-dependent manner, but Al^{3+} had no effect at concentrations lower than 1 mM (Spurney et al., 1999). These findings were corroborated by Mailland et al (1997), who also reported a lack of Al^{3+} activity at micromolar concentrations on IP_3 production in CCL39 fibroblasts transfected with the CaR (Mailland et al., 1997).

However, using Al^{3+} concentrations similar to the serum Ca^{2+} concentrations (0.5-2 mM), a suppression of PTH was observed in an *in vitro* study using bovine parathyroid cells (Morrissey et al., 1983). This was supported in a study by Gonzalez-Suarez et al (2003), where Al^{3+} was reported to decrease PTH secretion and cell proliferation in parathyroid glands in nephrectomized Wistar rats with chronic renal failure, treated for 8 weeks with $AlCl_3$ (Gonzalez-Suarez et al., 2003). In addition, Al^{3+} was also demonstrated to inhibit PTH gene expression by a postranscriptional mechanism (Gonzalez-Suarez et al., 2005). Thus, at millimolar concentrations Al^{3+} produces effects similar to those of Ca^{2+}. However, the physiological significance of these findings is debatable, since serum Al^{3+} levels are very low, even in disease states.

Concentrations of Al^{3+} in the micromolar range have been described to modify various signaling pathways *in vitro*, including stimulation of PKC and modulation of cAMP production in

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osteoblasts (Hartle et al., 1996; Quarles et al., 1994). *In vivo* studies have suggested that at around 50 μM Al^{3+}, *de novo* bone formation is stimulated and *in vitro* studies have shown that osteoblast proliferation increases (Hartle et al., 1996; Quarles et al., 1988; Quarles et al., 1994). However, since it has been demonstrated that Al^{3+} is a weak agonist through the CaR and produces its effect only at concentrations greater than 1 mM (Mailland et al., 1997; Spurney et al., 1999), the reported effects of Al^{3+} in the micromolar range are likely produced through a different mechanism.

Strontium (Sr^{2+})

Most studies investigating the effects of Sr^{2+} on the CaR have utilized a ranelic acid form of the cation, primarily focusing on its effects in osteoclasts and osteoblasts, due to the reported affinity of Sr^{2+} for bone, where it is taken up into the bone matrix crystals. The specific target of Sr^{2+} in bone cells has not been clear. While it has been demonstrated that the effects of Sr^{2+}, such as G protein activation, are maintained in osteoblasts that lack the CaR, suggestive of the involvement of another GPCR in mediating the responses of Sr^{2+} in bone (Pi and Quarles, 2004), it is now believed that the CaR is one of the mediators of Sr^{2+}'s effects in bone cells (Fromigue et al., 2009; Hurtel-Lemaire et al., 2009). Recently, it has been described that Sr^{2+} stimulates the differentiation of pre-osteoblasts to osteoblasts through the activation of the CaR, thus increasing bone formation (Bonnelye et al., 2008). It also stimulates the secretion of osteoprotegerin from osteoblasts, leading to an inhibition of the formation of osteoclasts from pre-osteoclasts, by modulating the osteoprotegerin/Receptor Activator for NF-κB (RANK) system, which leads to a decrease in bone resorption (Atkins et al., 2009).

Hurtel-Lemaire et al (2009) have demonstrated that Sr^{2+} stimulates the apoptosis of primary mature rabbit osteoclasts in a concentration-dependent manner (Hurtel-Lemaire et al., 2009). Both Ca^{2+}_o and Sr^{2+} produced a stimulation of PLC and nuclear translocation of NF-κB in mature osteoclasts through the activation of the CaR. However, the authors have observed a difference between the intracellular effects produced by Ca^{2+}_o and Sr^{2+}, showing that Sr^{2+}-induced osteoclast apoptosis was depend on PKCβII activation and independent of IP_3 signaling, while the effects produced by Ca^{2+}_o were independent of the PKCβII pathway and dependent on IP_3. The differential activation of intracellular signaling pathways by Sr^{2+} and Ca^{2+}_o allows for an additive effect of the combination of the two agents on osteoclast apoptosis, due to a non-competitive nature of their signaling through the CaR. It is likely, therefore, that in osteoporosis patients treated with strontium ranelate, Sr^{2+} and Ca^{2+}_o act together to inhibit bone resorption (Hurtel-Lemaire et al., 2009).

The idea that Sr^{2+} mediates its effects through the activation of the CaR has recently also been supported in a study by Fromigue et al (2009), where it was shown that Sr^{2+} (in a ranelic acid form)

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rapidly increases ERK1/2 phosphorylation in osteoblasts expressing the CaR but not in osteoblasts from CaR knock-out mice (Fromigue et al., 2009). Interestingly however, Ca^{2+}_{o} and Sr^{2+} increased cell replication and prevented cell apoptosis in osteoblasts from both CaR knock-out mice and wild-type mice, indicating that Sr^{2+} can act independently of the CaR/ERK1/2 cascade to promote osteoblast proliferation. Additionally, Sr^{2+} was shown to activate the Akt pro-survival pathway in osteoblasts from both wild-type and CaR knock-out mice and both the proliferative and anti-apoptotic effects of Sr^{2+} were abrogated by selective inhibition of COX-2, showing that in cells of the osteoblast lineage, in addition to the CaR, Sr^{2+} produces its effects through other pathways (Fromigue et al., 2009).

ii. Polyamines

Polyamines, including spermine, spermidine and putrescine (Figure 3A), are cationic compounds known to activate the CaR, as has been demonstrated for instance in bovine parathyroid cells, where spermine (200 μM) was shown to inhibit PTH secretion by 50% (Quinn et al., 1997). Polyamines are found in a wide variety of tissues, and are involved in stabilizing nucleic acid helical structure, having a role in cellular metabolism, growth and differentiation. Polyamines also play a role in neurotransmission, producing their effects through modulation of the N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptors and by blocking inward rectifying potassium channels (de Vera et al., 2008; Kurata et al., 2007; Shin et al., 2005).

Another important physiological role of polyamines is in the maintenance of the function of gastrointestinal epithelia, described to occur through the activation of the CaR expressed in different cell types present in the gastrointestinal tract (Cheng et al., 2004; Ray et al., 1997). Polyamines were described to mediate an increase in intracellular IP_3 and Ca^{2+}_{i} accumulation in perfused colonic crypts, with the rank order spermine > spermidine > putrescine (Cheng et al., 2004), and in HEK293 cells transfected with the CaR (Quinn et al., 1997). In perfused colonic crypts, spermine was shown to inhibit both basal and forskolin-induced fluid secretion (Cheng et al., 2004). Increased Ca^{2+}_{o} concentrations were described to shift the EC_{50} for spermine to the left and, interestingly, sub-threshold concentrations of spermine increased the sensitivity of CaR-expressing HEK293 cells to Ca^{2+}_{o}, strongly suggestive of the involvement of the CaR (Cheng et al., 2004; Quinn et al., 1997). In the study by Cheng et al (2004), it was reported that polyamine-induced effects required the presence of Ca^{2+}_{o}. However, the requirement for Ca^{2+}_{o} was not supported in a study by Canaff et al (2001), where spermine (1.25-20 mM) produced a concentration-dependent increase in Ca^{2+}_{i} in rat hepatocytes through the activation of the CaR in the absence of Ca^{2+}_{o} (Canaff et al., 2001),

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suggestive of being an orthosteric agonist. In addition to their actions in the colon, polyamines have also been described to produce Ca^{2+}_i mobilisation and ERK1/2 activation using the human esophageal epithelial cell line (HET-1A) and in basal cells of the human esophagus, through the activation of the CaR (Justinich et al., 2008). Moreover, in G cells of the stomach, which express the CaR, spermine was shown to stimulate gastrin release in a concentration-dependent manner (Ray et al., 1997).

iii. Aminoglycoside antibiotics (AGAs)

Neomycin, gentamicin and tobramycin (Figure 3A), which belong to the group of AGAs, are known to activate the CaR. McLarnon et al (2002) have compared the relative effects of different AGAs on Ca^{2+}_i increase using HEK293 cells transfected with the CaR (McLarnon et al., 2002). Neomycin, gentamicin and tobramycin produced a concentration-dependent increase in Ca^{2+}_i with EC_{50} values of 43, 258 and 177 μM, respectively. These compounds were without effect in non-transfected cells. However, CaR agonism was shown not to be a feature common to all AGAs and kanamycin was ineffective at concentrations <1 mM. Interestingly, the rank order of potencies of the different AGAs correlates positively with the number of their attached amino groups, which may explain the lack of effect of kanamycin, which has the fewest attached amino groups (four, compared to five for gentamicin and trobamycin, and six for neomycin) (McLarnon et al., 2002).

It is important to understand the precise signaling of AGAs through the CaR, since these compounds produce an important adverse effect - nephrotoxicity. It has previously been described that the CaR contributes to this phenomenon (Ward et al., 2002). The proximal tubule-derived opossum kidney cells were shown to respond to elevated Ca^{2+}_o, neomycin and gentamicin, with an increase in Ca^{2+}_i, ERK1/2 activation, and phosphoinositide 3-kinase-dependent phosphorylation of Akt, glycogen synthase kinase 3β and p38 MAPK (Ward et al., 2005; Ward et al., 2002). After a 4-day treatment with gentamicin, or other CaR agonists, the cells underwent cell death. Furthermore, gentamicin elicited significantly more cell death in HEK293 cells transfected with the CaR than in non-transfected cells (Ward et al., 2005). These results imply that the CaR is likely to contribute to signaling underlying the renal toxicity of AGAs.

c. Allosteric modulators of the CaR

i. Aromatic L-amino acids

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A feature common to all family 3 GPCRs, including the CaR, is the presence of amino acid binding sites in the VFTM (Conigrave et al., 2007). The family 3 of GPCRs includes receptors whose primary ligands are amino acids, such as the glutamate and GABA receptors and several receptors that act as broad-spectrum amino acid sensors (Wellendorph and Brauner-Osborne, 2009). The aromatic L-amino acids that have been reported to activate the CaR include L-phenylalanine (L-phe), L-tryptophan (L-trp), L-tyrosine (L-tyr) and L-histidine (Figure 3B) (Conigrave et al., 2007), producing an allosteric modulation of CaR signaling in the presence of $Ca^{2+}{}_o$. Some aliphatic L-amino acids also bind the CaR, such as for example L-leucine and poly-L-arginine (Brown et al., 1991; Busque et al., 2005).

The existence of an interaction between the CaR and amino acids is not very surprising, since it has been known for years that $Ca^{2+}{}_o$ concentration and amino acid metabolism are linked. People with increased intake of aromatic amino acids have increased urinary Ca^{2+} excretion, compared to people with similar increases in intake of branched-chain amino acids (Dawson-Hughes et al., 2007). Additionally, secondary HPT has been reported in subjects consuming low-protein diets, suggestive of an amino acid sensing mechanism linked to the control of urinary Ca^{2+} excretion and PTH release (Conigrave et al., 2002).

The organ in which aromatic L-amino acids come into contact with the CaR, is the gastrointestinal tract, where these compounds regulate various functions, including the rate of gastric acid secretion from G-cells of the stomach and acid-secreting parietal cells (Busque et al., 2005; Conigrave et al., 2002). Busque and colleagues (2005) have reported that L-phe, L-trp, and L-leucine produce a decrease in gastric luminal pH in *ex vivo* stomach preparations at physiological $Ca^{2+}{}_o$ concentrations. Hira et al (2008) have demonstrated in *in vivo* and *in vitro* studies that L-phe stimulates cholecystokinin secretion and $Ca^{2+}{}_i$ mobilization in enteroendocrine STC-1 cells, which express the CaR, in a concentration-dependent manner (Hira et al., 2008). This effect was augmented at elevated $Ca^{2+}{}_o$ concentrations and inhibited by NPS 2143, a negative allosteric modulator of the CaR, suggestive of the involvement of the CaR (Hira et al., 2008). A confirmation that the CaR is involved in the functions of aromatic L-amino acids was described in a recent study by Lee et al (2007) using HEK293 cells transfected with the CaR (Lee et al., 2007). This study has shown that L-phe and L-trp enhance the $Ca^{2+}{}_o$-induced increase in $Ca^{2+}{}_i$ mobilization in CaR transfected cells, as well as in normal human parathyroid cells, but not in non-transfected HEK293 cells. L-phe and L-trp were also shown to induce a small, but physiologically significant, enhancement of $Ca^{2+}{}_o$-dependent suppression of PTH secretion and ERK1/2 activation in parathyroid cells, producing a decrease in the EC_{50} of $Ca^{2+}{}_o$ (Lee et al., 2007). However, due to the small effects produced by aromatic L-

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amino acids on the various physiological effects, it is believed that these compounds are likely involved only in fine-tuning of CaR signaling.

ii. Synthetic allosteric modulators of the CaR

Synthetic allosteric modulators of the CaR have recently been identified, belonging to the phenylalkylamine group of compounds, with structural similarities to the naturally occurring CaR agonists, such as the aromatic L-amino acids, with an aromatic ring and positively charged amine groups (Figure 3B). These ligands were synthesized with the aim to regulate PTH secretion in patients with HPT. Calcimimetics and calcilytics do not activate the wild-type CaR directly, but rather shift the concentration-response curves of $Ca^{2+}{}_o$ and other orthosteric agonists to the left or right, respectively (Ferry et al., 1997). As mentioned earlier, allosteric modulators of the CaR bind to the seven TM region of the CaR, not the ECD which is the binding site for orthosteric ligands. CaR allosteric modulators that have been studied in some detail include the calcimimetics cinacalcet (Evenepoel, 2008), NPS R-568 (Harrington and Fotsch, 2007) and calindol (Petrel et al., 2004), and the calcilytics NPS 2143 (Gowen et al., 2000) and Calhex 231 (Petrel et al., 2003) (Figure 3B). The identification of calcimimetics and calcilytics led to the realization that these compounds have a therapeutic potential for the treatment of various disorders associated with CaR malfunction, such as HPT and possibly osteoporosis, respectively. Calcilytic and calcimimetic compounds have advantages over conventional CaR ligands in that their effects are more specific due to their more limited range of targets. Other CaR agonists, such as divalent and trivalent cations, aromatic L-amino acids and polyamines, produce their effects in addition to the CaR, via ion channels and various other receptors, such as GPRC6A, NMDA and AMPA.

d. The effect of pH and ionic forces on the signaling of the CaR

It has recently been described that the activity of the CaR is modulated by pH_o (Doroszewicz et al., 2005; Quinn et al., 2004) and ionic strength (Quinn et al., 1998). This is not surprising, since all CaR agonists are cationic compounds and their receptor binding occurs through electrostatic interactions with charged residues in the ECD of the CaR, including glutamic and aspartic acid residues, which can be modulated by physicochemical conditions of the extracellular milieu (Quinn et al., 2004).

The effects of pH_o on CaR's sensitivity to $Ca^{2+}{}_o$ were observed at both decreased and increased pH_o, deviating from the physiological pH_o of 7.4. At increased pH_o, the CaR was more sensitive to activation by $Ca^{2+}{}_o$ and $Mg^{2+}{}_o$, whereas a decrease in pH_o produced lower sensitivity (Doroszewicz

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et al., 2005; Quinn et al., 2004). However, when pH_o was decreased even further, to less than 5.5, CaR sensitivity to $Ca^{2+}{}_o$ was partially recovered. The pH_o-induced effect was CaR specific, and another GPCR, the thrombin receptor, was shown to be insensitive to changes in pH_o (Quinn et al., 2004).

In contrast, McLarnon and colleagues (2002), have shown that reducing pH_o from 7.4 to 6.9, to mimic the luminal pH of the renal proximal tubule, which express the CaR on the apical surface, enhanced the sensitivity of the CaR to tobramycin and elicited a greater increase in $Ca^{2+}{}_i$ (McLarnon et al., 2002). These findings suggest that decreasing pH_o has an opposite effect on AGAs compared to $Ca^{2+}{}_o$ and $Mg^{2+}{}_o$. Furthermore, these results imply that AGAs may be more potent CaR agonists in the proximal tubule than anywhere else (McLarnon et al., 2002).

The discrepancy between the pH_o-induced effects on divalent cation signaling versus AGA signaling through the CaR may be explained by the resistance of the charges on Ca^{2+} and Mg^{2+} to pH. Thus in this case, the pH_o-induced effect is mainly produced by modulation of the charged residues on the CaR, for example the modulation of the positively charged histidine residues, which are present at high density in the ECD, as may occur at elevated pH_o. Whereas, in case of polycationic compounds, such as AGAs, as well as polyamines and aromatic L-amino acids, which contain several primary amino groups, decreased pH_o may lead to their protonation, making the compounds more positively charged, thus activating the CaR to a greater extent.

In addition, decreased pH_o, on its own, in the absence of agonists is capable of activating the CaR, suggesting that $H^+{}_o$ is itself an agonist of this receptor (Quinn et al., 2004). Moreover, $H^+{}_o$ may be capable of shifting the balance between active and inactive receptor states, favouring a receptor conformation, which produces constitutive activation. Thus, the sensitivity of the CaR to pH_o and the subsequent modulation of signaling may have physiological relevance in tissues that experience changes in pH_o, including the stomach, the kidney, bone, and the brain, where an additional function of the CaR may be as a pH_o sensor.

In addition to pH_o, ionic strength of the extracellular milieu also influences CaR activity. A study by Quinn et al (1998) has described that changes in the concentration of external NaCl (or other salts) changed the activation of the CaR by $Ca^{2+}{}_o$ and spermine. Ionic strength had an inverse effect on the sensitivity of CaR to its agonists (Quinn et al., 1998); at lower ionic strength the CaR was more sensitive to activation by $Ca^{2+}{}_o$ and at higher ionic strength it was less sensitive. In parathyroid cells, addition of 40 mM NaCl shifted the EC_{50} for $Ca^{2+}{}_o$ inhibition of PTH to the right by more than 0.5 mM. Thus physiologically, in addition to being a pH_o sensor, the CaR may also act as an ionic strength sensor (Quinn et al., 1998).

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e. Agonist-directed signaling through the CaR

It is now widely accepted that through the activation of the same receptor, different agonists are capable of producing agonist-specific active receptor states, leading to a bias in G protein selection and intracellular pathway activation, resulting in various physiological effects (Aloyo et al., 2009; Kenakin, 2007; Michel and Alewijnse, 2007). This concept is often referred to as 'agonist-directed signaling' or 'agonist-directed stimulus trafficking'.

As described above, due to the different binding sites of the various CaR ligands to the CaR, it is conceivable that differential CaR active conformations may be formed with preferential activation of different intracellular pathways (Figure 4). For example, as noted earlier, the sensitivity of the CaR to $Mg^{2+}{}_o$ changes depending on the cell type under study. While in the parathyroid $Mg^{2+}{}_o$ produces effects comparable to $Ca^{2+}{}_o$, in the thyroid it is much less efficacious in comparison (Garrett et al., 1995). A possible explanation for this may be the unavailability or differential expression levels of the preferred G protein subtypes in certain cell types, or the strength of stimulus, and weak signal through the CaR may result in the activation of only the most efficiently coupled G proteins.

Chatthopadhyay and colleagues (2007) have reported that in HEK293 cells transfected with the CaR, the potency of Sr^{2+} varied depending on the biological response tested. Sr^{2+} was less potent than $Ca^{2+}{}_o$ at stimulating IP_3 accumulation and at increasing $Ca^{2+}{}_i$, but was comparable to $Ca^{2+}{}_o$ at stimulating ERK phosphorylation and opening of non-selective cation channel (Chattopadhyay et al., 2007). Whether differential modulation of the CaR by $Ca^{2+}{}_o$ and Sr^{2+} is responsible for these differences is not certain and differential activity of the two cations at ion channels could offer an alternative explanation (Chattopadhyay et al., 2007). Furthermore, as described above, differences in Sr^{2+} and $Ca^{2+}{}_o$ signaling through the CaR have been described to occur in primary mature rabbit osteoclasts, where Sr^{2+}-induced apoptosis was shown to be depend on PKCβII activation, whereas $Ca^{2+}{}_o$-induced effects were not. Furthermore, Sr^{2+} signaling was shown to be independent of the IP3 pathway, in contrast to $Ca^{2+}{}_o$ signaling. Both agents produced stimulation of PLC and nuclear translocation of NF-κB (Hurtel-Lemaire et al., 2009).

Another example of differential CaR signaling with various agonists was proposed by Ziegelstein et al (2006) who showed that human aortic endothelial cells responded to spermine, leading to the release of $Ca^{2+}{}_i$ and an increase in nitric oxide production, whereas $Ca^{2+}{}_o$, $Gd^{3+}{}_o$ and neomycin were ineffective (Ziegelstein et al., 2006). The participation of the CaR in the spermine-induced effects was confirmed by the use of siRNA directed against the CaR, which abolished the response (Ziegelstein et al., 2006). Furthermore, with the addition of cinacalcet, a concentration-

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dependent vasodilatation was reported in the pre-contracted aorta in a study by Smajilovic et al (2007), whereas the CaR agonists, neomycin and Gd^{3+} had no effect (Smajilovic et al., 2007). Since the involvement of the CaR was not confirmed in this study, it is possible that this vasodilatory effect of cinacalcet may be mediated through a different mechanism, for example by a direct action on ion channels (Smajilovic et al., 2007). Another example of this phenomenon was demonstrated in a study by Bruce et al (1999), where stimulation of pancreatic acinar cells with Ca^{2+}_o and Gd^{3+} produced Ca^{2+}_i release, while neomycin had no effect (Bruce et al., 1999).

Other differences in CaR signaling by different CaR agonists include the nature of Ca^{2+}_i release. When compared to elevated Ca^{2+}_o, which stimulates PLC-mediated production of IP_3 and causes sinusoidal oscillations in Ca^{2+}_i, aromatic L-amino acid-induced CaR activation does not stimulate PLC but promotes transient oscillations in Ca^{2+}_i through a distinct mechanism, involving the transient receptor potential cation channel 1 (TRPC1) (Rey et al., 2006). Selective abolition of TRPC1 by siRNAs or using an antibody that binds the pore region of the channel abolished the aromatic L-amino acid-induced Ca^{2+}_i oscillations, as did PKC inhibitors, siRNA directed against PKCα or an impairment of calmodulin function (Rey et al., 2006). These differences between Ca^{2+}_o and aromatic L-amino acid signaling through the CaR are also indicative of agonist-directed signaling.

3. Current uses and potential therapeutic applications of CaR ligands

a. Sr^{2+}

Strontium ranelate, a Sr^{2+} salt of ranelic acid, is a newly developed drug for the treatment of osteoporosis, currently marketed as Protelos® or Protos® by Servier. Ranelate in strontium ranelate acts as a vector for the introduction of Sr^{2+} into the organism, allowing Sr^{2+} to replace Ca^{2+} (1/10) in the hydroxyapatite crystals. This drug is atypical among osteoporosis treatments in that it both increases deposition of new bone by osteoblasts and decreases bone resorption by osteoclasts, thus favoring bone formation. The effectiveness of this drug has been demonstrated by numerous previous studies (Roux et al., 2008; Seeman et al., 2008; Tournis et al., 2006). Roux et al (2008) have reported that the risk of vertebral fractures is reduced by 35% in postmenopausal women, aged between 50 and 65 years, treated with strontium ranelate during a 4 year period. Similar results were reported in another study, using an older population with a mean age of 69 years, which showed that the risk of developing new vertebral fractures decreased by 41% over a 3 year period of strontium ranelate treatment, and this effect was apparent in the first year of treatment with a 49% risk reduction (Tournis et al., 2006). Additionally, in patients above

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the age of 74 years, the incidence of non-vertebral fractures was 16% lower in the strontium ranelate-treated group compared to the placebo group, and the risk of hip fractures diminished by 36% (Tournis et al., 2006). Furthermore, recent long-term studies of 8 years and 5 years, have reported a continued increase in bone mass density in strontium ranelate treated postmenopausal women and a reduced risk of fractures (Meunier et al., 2009; Reginster et al., 2009).

b. Calcimimetics

It has been shown that calcimimetic drugs, which amplify the sensitivity of the CaR to Ca^{2+}_o, can suppress PTH levels in a concentration-dependent manner, leading to a fall in blood Ca^{2+} in different forms of HPT (Block et al., 2004; Peacock et al., 2005). Thus, they are likely to become a major therapy for the treatment of primary HPT in certain patients with parathyroid adenomas and carcinomas, and secondary HPT associated with renal failure.

HPT is characterized by several features, including increased secretion of PTH and an increase in the mass of parathyroid tissue. Primary HPT is the result of an increase in the mass of the parathyroid gland, which causes increased PTH secretion and subsequently hypercalcemia, for example in parathyroid tumors, and it can often be cured by the removal of the parathyroid gland. Secondary HPT, which occurs in chronic kidney disease, develops as a result of reduced Ca^{2+}_o and calcitriol concentrations in serum and an increase in phosphorous concentrations, which in combination, consequently lead to increased PTH secretion as a compensatory response. The treatments for secondary HPT that have been available until now include vitamin D and calcium-containing phosphate binders (Komaba et al., 2008). While these treatments have beneficial effects, they also have many disadvantages. Calcium-containing phosphate binders raise Ca^{2+}_o and lower phosphate, while vitamin D increases serum concentrations of Ca^{2+}_o and phosphate and lowers PTH. Ca^{2+}_o has an antiproliferative effect in the parathyroid, leading to decreased proliferation of parathyroid cells. However, patients treated with high doses of these compounds often display hypercalcemia, and in case of vitamin D also hyperphosphatemia. In haemodialysis patients, an imperfect management of serum PTH, Ca^{2+}_o, phosphorous and the Ca^{2+}_o x phosphorous product can lead to serious consequences, especially an increased risk of cardiovascular and other soft tissue calcifications (Goodman, 2004). Therefore, better therapeutic agents are required for the treatment of HPT associated with chronic renal failure. Phenylalkylamine calcimimetics may be the answer to this problem. Due to the increase in the sensitivity of the CaR to Ca^{2+}_o by calcimimetics, these compounds may be very beneficial under the circumstances of both primary and secondary HPT, which are characterized by decreased sensitivity of CaR to Ca^{2+}_o (Tfelt-Hansen and Brown, 2005).

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Initial studies have investigated the efficacy of the calcimimetic NPS R-568 for the treatment of HPT. Silverberg SJ et al (1997) have demonstrated that this compound reduces both serum PTH and Ca^{2+}_o in patients with primary HPT in a concentration-dependent manner (Silverberg et al., 1997). In parallel, in rats it has been demonstrated that NPS R-568 also reduces proliferation of parathyroid cells in uremic HPT. *In vitro* studies have shown that in the presence of 1.3 mM Ca^{2+}, the potency of NPS R-568 at augmenting the IP_3 response in both CHO cells transfected with rat brain CaR and AtT-20 cells with endogenous CaR was in the micromolar range. In both cell types, the IP_3 concentration-response curves of NPS R-568 were shifted to the left in the presence of increasing Ca^{2+}_o, indicating that the potency of the drug is dependent on Ca^{2+}_o (Ferry et al., 1997). Unfortunately, the clinical tests on this drug were discontinued due to its low bioavailability and high inter- and intra-individual variability (Shoback et al., 2003). Following this, cinacalcet (AMG 073) was developed and clinical research has focused on this drug due to its superior pharmacokinetic properties. The efficacy of cinacalcet for the treatment of primary (Peacock et al., 2005; Shoback et al., 2003), and secondary HPT (Block et al., 2004) was demonstrated, as described below.

In 2003, in a study by Shoback et al, the effectiveness of cinacalcet was confirmed in a short term study of 15 days in patients with primary HPT, showing that serum Ca^{2+} was normalized after 1 day of cinacalcet treatment and remained in the normal range for the duration of the study. Additionally, reduced PTH levels were also reported (Shoback et al., 2003). These results were later confirmed by Peacock et al (2005) in a long-term double-blind placebo-controlled study. Oral cinacalcet was shown to rapidly normalize serum Ca^{2+}_o and produce a small decrease in PTH (7.6% decrease compared to 7.7% increase in placebo patients) in primary HPT patients (Peacock et al., 2005). These effects were maintained for the duration of the 52-week long study. In addition, serum phosphorous was also increased and cinacalcet was shown to decrease tubular Ca^{2+} reabsorption (Peacock et al., 2005). A recent study by Marcocci et al (2009) has demonstrated that cinacalcet also produces beneficial effects in patients with persistent primary HPT that have undergone parathyroidectomy, leading to a decrease in serum Ca^{2+} levels, thus showing its applicability in this setting (Marcocci et al., 2009). Thus, cinacalcet treatment may be especially interesting for the treatment of primary HPT, since this disorder, while being accompanied by increased serum Ca^{2+} levels, can otherwise be asymptomatic. Therefore, calcimimetics may provide a non-invasive way to normalize serum Ca^{2+} levels, providing a non-surgical alternative.

The effectiveness of cinacalcet for the treatment of secondary HPT has also been described (Goodman et al., 2002; Lindberg et al., 2003; Quarles et al., 2003; Sprague et al., 2009). Cinacalcet was shown to be effective for this purpose in short term studies (Goodman et al., 2002; Quarles et

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al., 2003). After conducting a 14-week study, Block et al (2004) have reported a 43% decrease in serum PTH in patients with secondary HPT receiving cinacalcet, compared with a 9% increase in the placebo group (Block et al., 2004). A 15% decrease in serum Ca^{2+} x phosphorus product was seen in the cinacalcet group, compared to no change in the placebo group. In another 18-week long study, in which secondary HPT patients with end stage renal disease were treated with up to 100 mg cinacalcet daily, the mean level of PTH in serum decreased by 33% compared to a slight increase (3%) in PTH in placebo patients (Quarles et al., 2003). In addition, the Ca^{2+} x phosphorous product decreased by 7.9% in cinacalcet-treated patients compared to a 11.3% increase in placebo treated patients (Quarles et al., 2003). The decrease in Ca^{2+} x phosphorus product was confirmed by Lindberg et al (2003), who reported an 11.9% decrease in cinacalcet-treated patients, compared to a 10.9% increase in placebo subjects after 18 weeks of treatment (Lindberg et al., 2003). The authors also reported a 26% reduction in PTH in cinacalcet-treated (50 mg/day), compared to a 22% increase in the placebo group of hemodialysis patients with secondary HPT. A recent study by Chonchol et al (2009) has reported a 43.1% decrease in intact PTH in cinacalcet treated patients in a double-blind 32-week study (Chonchol et al., 2009). Additionally, another recent study has demonstrated that the beneficial effects of cinacalcet are maintained over three years, with decreased PTH and Ca^{2+} x phosphorous levels (Sprague et al., 2009). The confirmed decrease in Ca^{2+} x phosphorus product implies that cinacalcet may produce beneficial effects in HPT by decreasing vascular calcifications (Aladren Regidor, 2009; Block et al., 2004; Quarles et al., 2003). The reduction in the Ca^{2+} x phosphorous product is probably due to decreased PTH-driven Ca^{2+} and phosphorous efflux from bone. Since PTH is capable of directly stimulating RANK ligand-mediated osteoclast maturation, its decrease will result in reduced bone resorption. However, since the CaR is expressed in bone cells, osteoclasts and osteoblasts, a direct effect of cinacalcet on the CaR expressed by these cells may provide another explanation for the reduced Ca^{2+} and phosphorous levels.

These cumulative studies have led to the approval of cinacalcet in 2004 in Europe, North America and Australia for the treatment of HPT, with trade names “Mimpara” in Europe and “Sensipar” in North America and Australia. It is the first positive allosteric modulator of any GPCR to be approved for therapeutic use. From the studies conducted so far, cinacalcet appears to be safe with no major side-effects. Other calcimimetic agents from novel families of compounds have also been described (Kessler et al., 2004), thus while the research on calcimimetics continues, there is already ample evidence that these compounds have an important role in the treatment of HPT, and possibly other diseases where CaR sensing is dysregulated, such as FHH and NSHPT.

c. Calcilytics

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On the other hand, calcilytics, such as NPS 2143 and Calhex 231, shift the concentration-response curves of Ca^{2+} to the right (Huang and Breitwieser, 2007; Kessler et al., 2006) and they directly increase PTH secretion, indirectly raise plasma Ca^{2+} concentrations and urinary phosphate excretion. Therefore, due to the anabolic effects of PTH on bone, calcilytics have been suggested to have a potential in the prevention and treatment of osteoporosis (Arey et al., 2005). Calcilytics are currently being assessed for their ability to induce a "pulse" in the serum PTH concentration, thus mimicking the “pulse” resulting from injection of PTH, a known anabolic form of treatment for osteoporosis (Brown, 2007).

Calcilytics may also have a role in the treatment of hypoparathyroidism, as occurs in patients with an underactive parathyroid gland and in ADH. In addition, it has recently been hypothesized that these compounds may have a role in the treatment of certain cancers. For example, it has been described that the CaR is involved in bone metastases of prostate and breast cancer cells (Liao et al., 2006; Mihai et al., 2006), in part due to its role in the perpetuation of the vicious cycle in the bone, created by the parathyroid hormone-related peptide (PTHrP) (Powell et al., 1991; Sanders et al., 2001). Activation of the CaR by Ca^{2+}_o leads to the release of PTHrP, which is believed to be a mediator in around 70% of malignant osteolysis in breast and prostate cancers, resulting in further release of Ca^{2+}_o and tumor growth promoting factors. Thus, in this setting antagonizing the CaR by the use of calcilytics may decrease the incidence of bone lesions, which is the primary contributor to the mortality of these cancers (Coleman, 1997). However, due to the contrasting effects of these agents compared to calcimimetics, one of the adverse effects that may arise through the use of calcilytics is an increased risk of developing vascular calcifications.

4. Conclusions

The CaR is ubiquitously expressed and has numerous physiological functions in addition to its primary role in the maintenance of constant blood Ca^{2+} levels. The large number of compounds capable of binding and modulating the activity of this receptor as well as the effects of extracellular pH and ionic strength on its functionality add to the complexity of physiological CaR signaling. The recent approval of cinacalcet, a CaR modulator, for the treatment of HPT and the efficacy of Sr^{2+} (in a ranelic acid form) for the treatment of osteoporosis (where Sr^{2+} may produce its effects through the CaR) demonstrate the potential of this receptor as a therapeutic target. The list of therapeutically used drugs targeting the CaR will likely expand further in the future, since recent findings point to the beneficial effects of calcilytics in the treatment of osteoporosis and possibly bone metastases. The signaling of CaR ligands is complicated even further by the ability of the GPRC6A receptor to

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bind many of these ligands, including L-amino acids, Ca^{2+}, Mg^{2+}, Sr^{2+}, Al^{3+}, Gd^{3+} and calcimimetics (Christiansen et al., 2007; Pi et al., 2005; Wellendorph and Brauner-Osborne, 2004), as well as by the activity of different cations through various ion channels (Li et al., 2007; Numata and Okada, 2008). Despite the rather promiscuous nature of most of these CaR ligands, the discovery of calcimimetic and calcilytic compounds has improved the situation considerably, due to their more limited range of receptor targets. These compounds will be of great service in future research. Novel allosteric modulators of the CaR are constantly being identified (Gavai et al., 2005; Yang et al., 2009), thus the research on these very important compounds continues, and may provide novel treatments for disorders where CaR sensing is dysregulated. Nevertheless, further targeting methods need to be identified and the specificity of CaR ligands increased, in order to reduce the secondary effects that may occur with the administration of these ligands.

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Figure legends

Figure 1. A schematic representation of the CaR in a dimeric form, with disulphide bridges at Cys129 and Cys131 linking the VFTMs of the monomeric receptors. The activated CaR transduces the signal through its interaction with G proteins, leading to stimulation of PLCβ and MAPK through the activation of $G\alpha_{q/11}$, and an inhibition of adenylate cyclase activity and subsequent decrease in intracellular cAMP levels via $G\alpha_i$.

Figure 2. A schematic representation of the 1078 amino acid-long human CaR. The predicted orthosteric Ca^{2+}_o-binding site 1 involving the residues S147, S170, D190, Y218 and E297 is shown in purple. Site 2 which consists of residues D215, L242, S244, D248 and Q253 is shown in yellow. Site 3 consisting of residues E224, E228, E229, E231 and E232 is demonstrated in pink. Site 4 (E350, E353, E354, N386, S388) and site 5 (E378, E379, T396, D398, E399) are shown in green and blue, respectively (Huang et al., 2009). In three dimensions, the indicated residues form 5 separate Ca^{2+}_o binding pockets in the ECD, as predicted with homology modeling using the mGluR1 (Huang et al., 2009). Residues involved in both calcimimetic and calcilytic binding are shown in black (F668, F684, W818, F821, E837, I841). Residues described to play part in calcilytic binding only are shown in orange (R680, F688, L776) (Miedlich et al., 2004; Petrel et al., 2004; Petrel et al., 2003).

Figure 3. Chemical structures of polycationic orthosteric agonists of the CaR, aminoglycoside antibiotics and polyamines (A), and allosteric modulators of the CaR, aromatic L-amino acids, calcimimetics and calcilytics (B) are shown.

Figure 4. Agonist-directed signaling through the CaR. A schematic representation of the various conformations of the intracellular regions of the CaR that result upon binding of different ligands. Depending on the conformation, differential activation of the available G proteins is mediated. The diagram represents the reported differences in CaR signaling upon activation by Ca^{2+}_o, Sr^{2+}_o, Ca^{2+}_o/cinacalcet or Ca^{2+}_o/NPS 2143. Based on the results obtained in the mature osteoclast, Ca^{2+}_o and Sr^{2+}_o may be able to trigger different intracellular signaling through the CaR. Ca^{2+}_o was shown to activate the PLCβ pathway with subsequent signaling via the IP_3; in contrast, Sr^{2+}_o produced its effects in these cells through the PLCβ/DAG/PKCβII pathway, independently of the IP_3 (Hurtel-Lemaire et al., 2009). Using various cell types, it has been shown that calcimimetics (cinacalcet) and calcilytics (NPS 2143) increase and decrease Ca^{2+}_o-signaling through the CaR,

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respectively. The thickness of the arrows signifies the strength of pathway activation. While this diagram is hypothetical, the G protein heterotrimers designated as G1 and G2, likely represent two different $G_{q/11}$ heterotrimers.

Figure 1

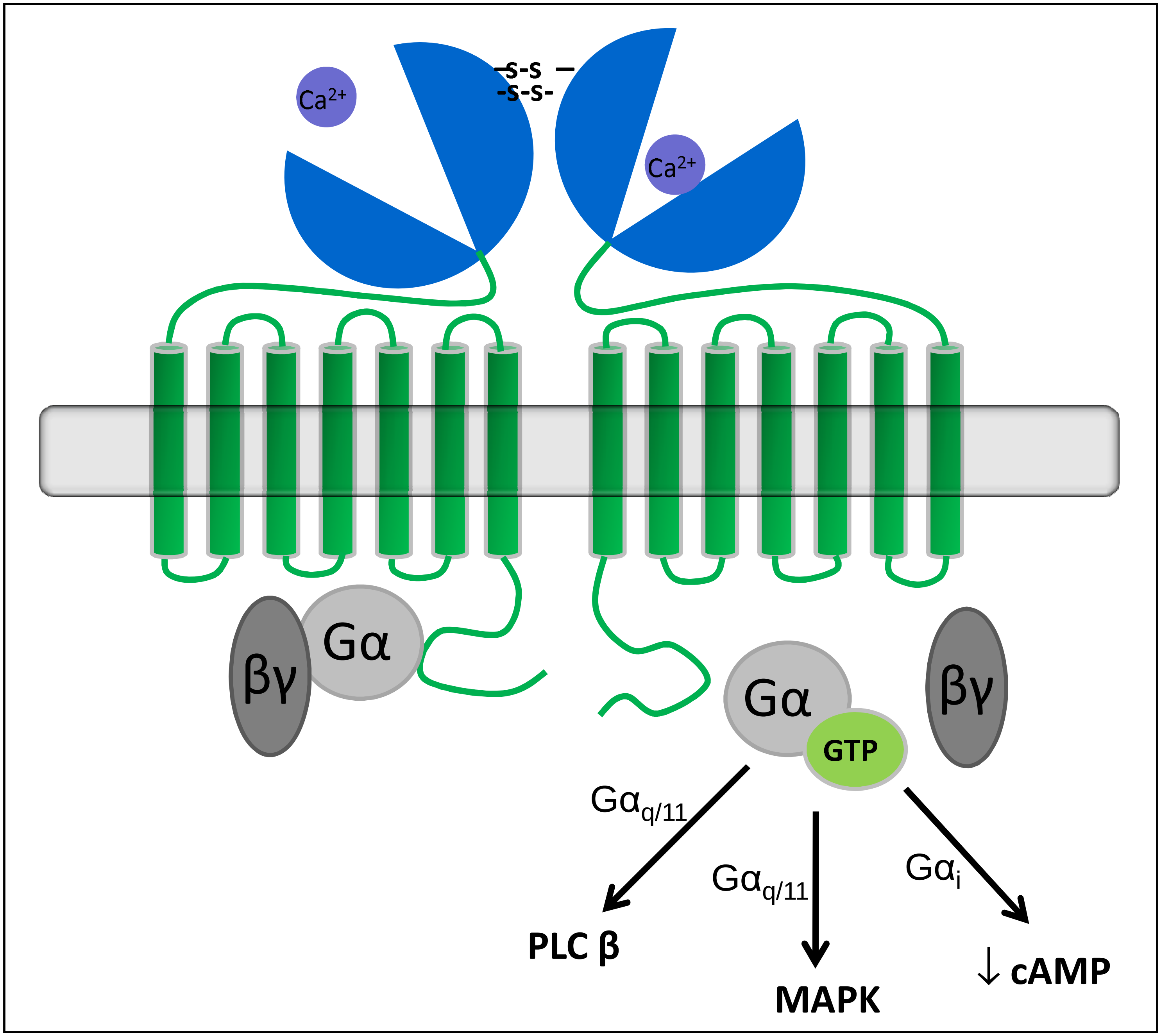

Figure 2

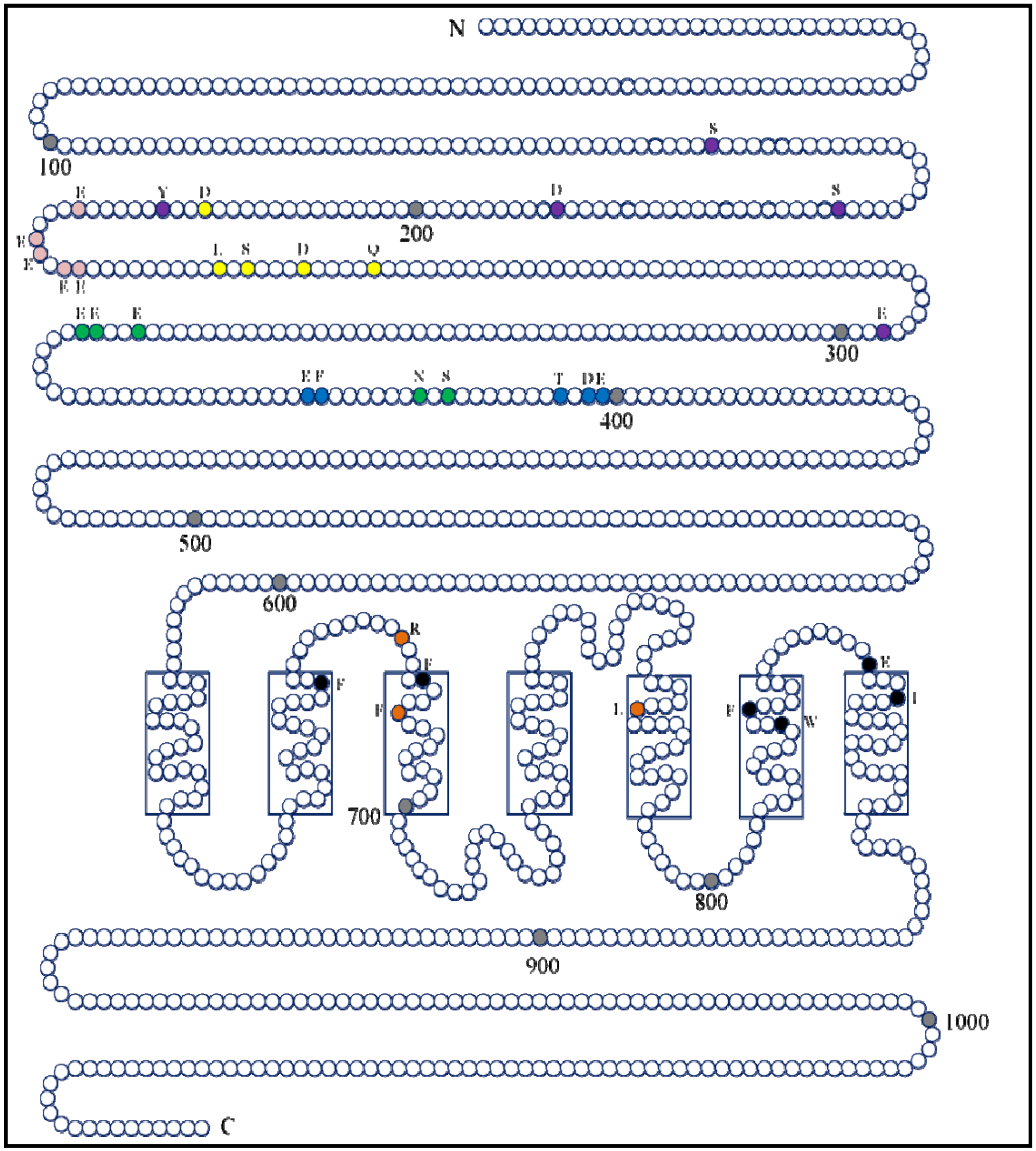

Figure 3

A.

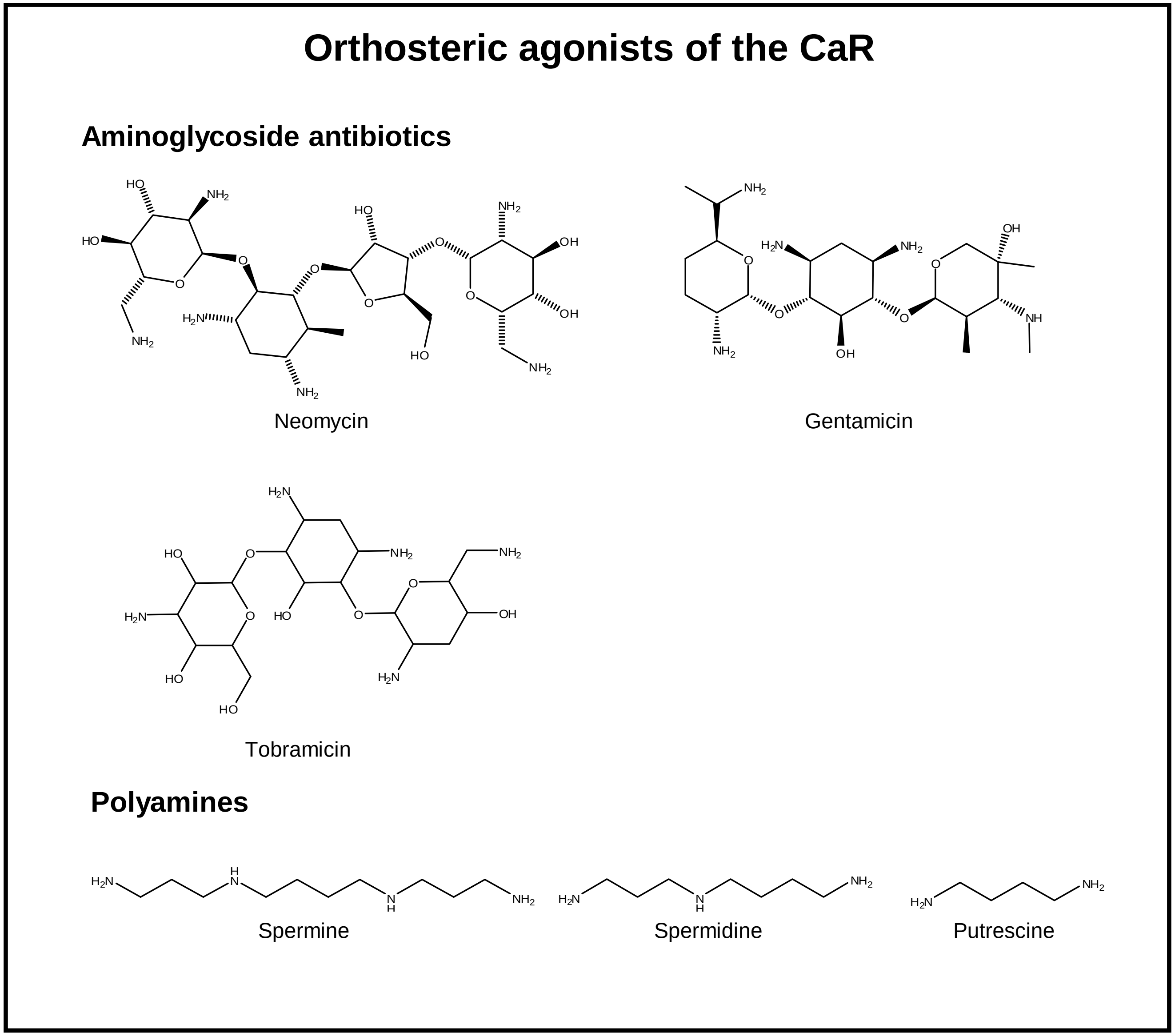

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Figure 3
B.

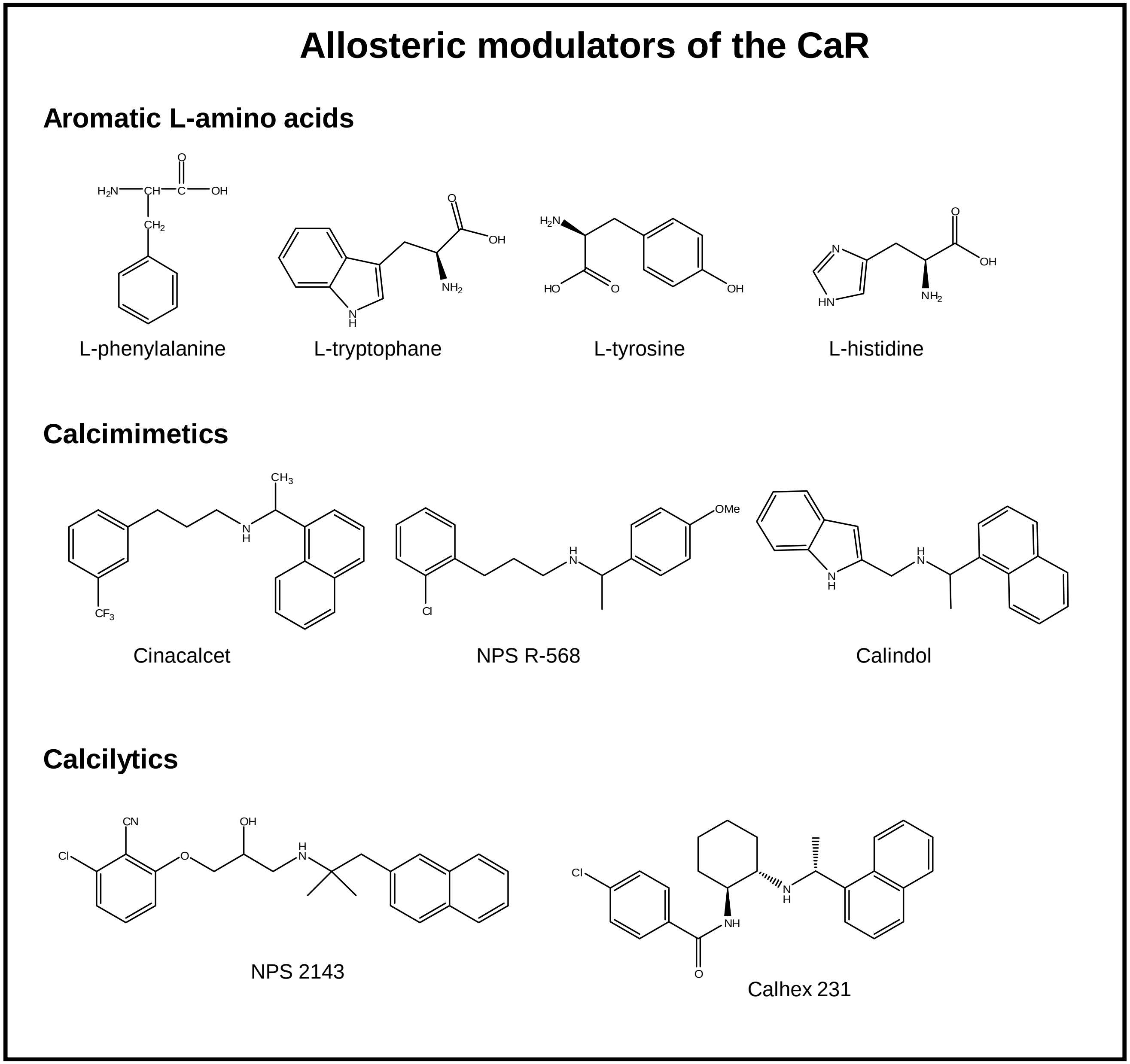
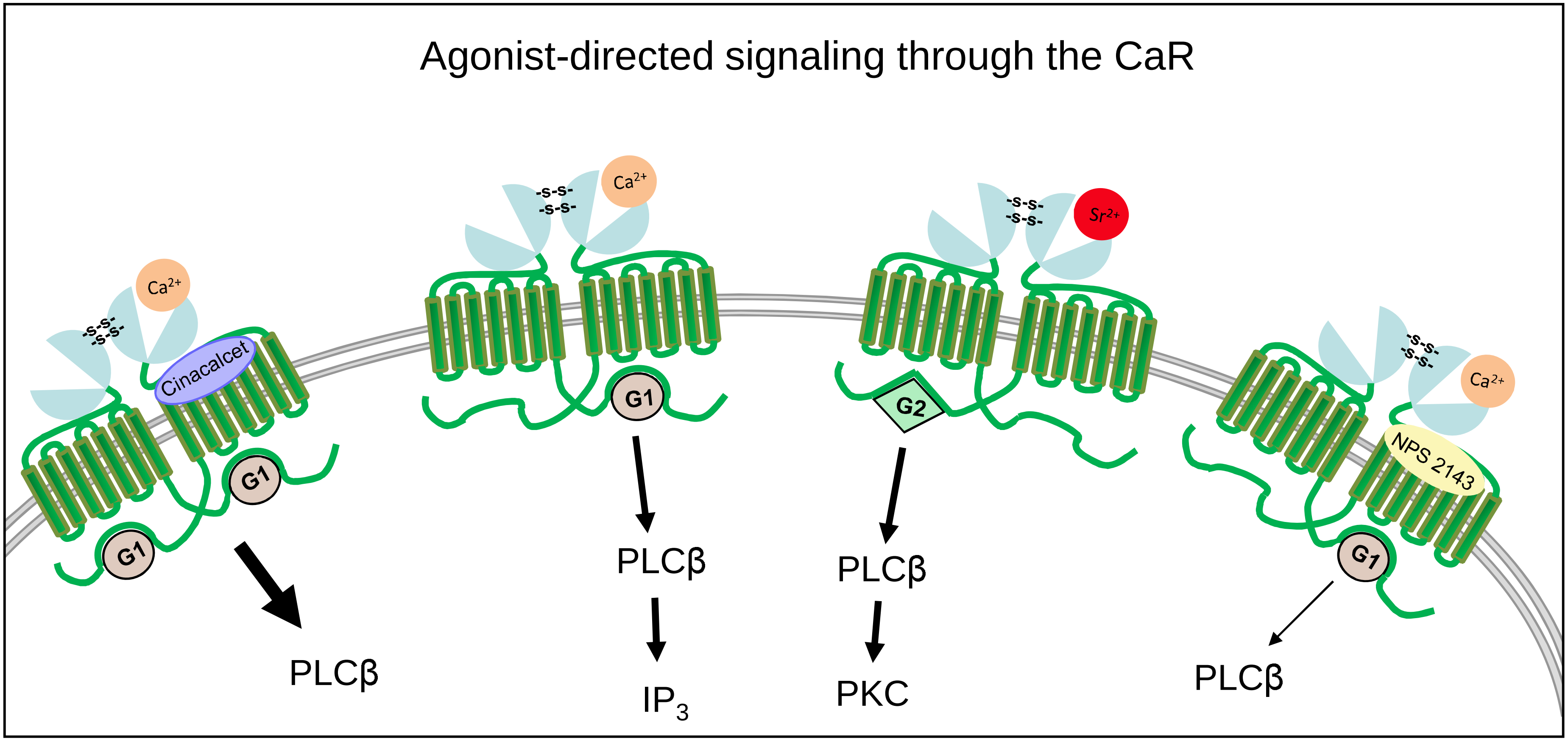

Allosteric modulators of the CaR
Aromatic L-amino acids
L-phenylalanine
L-tryptophane
L-tyrosine
L-histidine
Calcimimetics
Cinacalcet
NPS R-568
Calindol
Calcilytics
NPS 2143
Calhex 231

Figure 4



Agonist-directed signaling through the CaR
Ca2+
-S-S-
-S-S-
Cinacalcet
G1
G1
PLCβ
Ca2+
-S-S-
-S-S-
G1
PLCβ
IP3
Sr2+
-S-S-
-S-S-
G2
PLCβ
PKC
-S-S-
-S-S-
Ca2+
NPS 2143
G1
PLCβ