

REVIEW

New concepts in calcium-sensing receptor pharmacology and signalling

Donald T Ward¹ and Daniela Riccardi²

¹Faculty of Life Sciences, The University of Manchester, Manchester, UK, and ²School of Biosciences, Cardiff University, Cardiff, UK

Correspondence

Dr Donald Ward, Faculty of Life Sciences, The University of Manchester, Core Technology Facility, 46 Grafton Street, Manchester M13 9NT, UK.
E-mail: d.ward@manchester.ac.uk
Dr Daniela Riccardi, School of Pathophysiology and Repair, Cardiff University, Museum Avenue, Cardiff CF10 3AX, UK.
E-mail: riccardi@cf.ac.uk

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The calcium-sensing receptor (CaR) is the key controller of extracellular calcium (Ca²⁺) homeostasis via its regulation of parathyroid hormone (PTH) secretion and renal Ca²⁺ reabsorption. The CaR-selective calcimimetic drug Cinacalcet stimulates the CaR to suppress PTH secretion in chronic kidney disease and represents the world's first clinically available receptor positive allosteric modulator (PAM). Negative CaR allosteric modulators (NAMs), known as calcilytics, can increase PTH secretion and are being investigated as possible bone anabolic treatments against age-related osteoporosis. Here we address the current state of development and clinical use of a series of positive and negative CaR modulators. In addition, clinical CaR mutations and transgenic mice carrying tissue-specific CaR deletions have provided a novel understanding of the relative functional importance of CaR in both calciotropic tissues and those elsewhere in the body. The development of CaR-selective modulators and signalling reagents have provided us with a more detailed appreciation of how the CaR signals *in vivo*. Thus, both of these areas of CaR research will be reviewed.

Abbreviations

ADH, autosomal dominant hypocalcaemia; CaR, calcium-sensing receptor; ECD, extracellular domain; FHH, familial hypocalcaemic hypercalcaemia; ICD, intracellular domain; NAM, negative allosteric modulator; NSHPT, neonatal severe hyperparathyroidism; PAM, positive allosteric modulator; PMA, phorbol-12-myristate-13-acetate; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein

Introduction

The principal physiological action of the CaR has been well defined *in vivo*, both in clinical studies of human CaR mutations (Thakker, 2004), as well as in transgenic animal studies in which CaR is underexpressed in particular tissues (Ho *et al.*, 1995; Chang *et al.*, 2008). The CaR (also commonly referred to as CaSR) has been given the provisional nomenclature CaS by NC-IUPHAR, but this should not be confused with the plant calcium receptor, CaS (Han *et al.*, 2003; Tang *et al.*, 2007; Vainonen *et al.*, 2008), with which the animal receptor bears no sequence or structural relation. CaR is a class C

GPCR located on chromosome 3 in humans (Aida *et al.*, 1995) and, with the exceptions described below, it is expressed on the cell membrane as a disulphide-linked constitutive homodimer (Bai *et al.*, 1998a; Ward *et al.*, 1998).

Clinical and transgenic evidence for the central role of CaR in calcium homeostasis

The two tissues in which CaR expression is highest are the parathyroid glands, where CaR activation suppresses

parathyroid hormone (PTH) secretion (Brown *et al.*, 1993), and the kidneys (Riccardi *et al.*, 1998), where CaR limits Ca^{2+} reabsorption to protect against hypercalcaemia (Kantham *et al.*, 2009). Heterozygous, loss-of-function CaR mutation results in mild-to-moderate increases in PTH secretion (familial hypocalciuric hypercalcaemia, FHH), whereas homozygous loss-of-function CaR mutations can cause life-threatening elevations in PTH secretion and blood calcium levels (neonatal severe hyperparathyroidism, NSHPT) (reviewed by Thakker, 2004; Egbuna and Brown, 2008). An FHH-like condition can also result from CaR autoantibodies (Kifor *et al.*, 2003; Pallais *et al.*, 2004; Brown, 2009) directed against the N-terminal region of the receptor's extracellular domain (ECD) as demonstrated using a phage-display library of CaR peptides (Kemp *et al.*, 2009). The original CaR ($-/-$) knockout mouse (produced by deleting CaR exon 5) displayed an NSHPT-like phenotype with greatly elevated neonatal PTH levels causing growth retardation and early death (Ho *et al.*, 1995). The heterozygous CaR knockout ($+/-$) more closely resembled FHH in humans with a more moderate increase in PTH secretion. The lethal rise in PTH secretion and blood calcium levels in these mice can be overcome in double knockout mice in which the CaR knockout is combined with either a PTH ($-/-$) knockout (Kos *et al.*, 2003), or, a Glial cell missing-2 (Gcm-2) ($-/-$) knockout in which the parathyroid glands fail to develop (Tu *et al.*, 2003). Again, this replicates the situation in humans whereby early parathyroidectomy in NSHPT saves the life of the infant.

In fact, the Exon-5 CaR knockout is a 'hypomorph' as it retains some function in cartilage (Rodriguez *et al.*, 2005), in keratinocyte (Oda *et al.*, 2000) and lung (Finney *et al.*, 2011). Thus, an alternative murine model has been produced by deleting Exon-7, which knocks out the entire transmembrane region and intracellular domain (ICD), allowing for the transcription and translation of a non-functional secreted fragment corresponding to amino acid 1–577 of the ECD (Chang *et al.*, 2008). Indeed, a parathyroid-specific Exon-7 CaR knockout mouse exhibits even greater elevations in PTH levels (Chang *et al.*, 2008), that is an even more severe phenotype, than the partial Exon-5 CaR knockout (Ho *et al.*, 1995). Furthermore, in mice where the Exon-7 was selectively deleted from the osteoblasts, severe bone defects were observed thus demonstrating the specific role of CaR in bone, independent of its effects in parathyroid gland and kidney (Chang *et al.*, 2008).

Together, these *in vivo* observations demonstrate consistently the crucial role of CaR in maintaining stable blood calcium levels. This is further supported by clinical and transgenic studies in which CaR activity is elevated resulting in hypoparathyroid conditions and hypocalcaemia (Thakker, 2004; Egbuna and Brown, 2008). Gain-of-function CaR mutations result in autosomal dominant hypocalcaemia (ADH) and many such gain-of-function (and indeed loss-of-function) CaR mutations have been collated at <http://www.casrdb.mcgill.ca/>. These gain-of-function mutations are generally, although not exclusively, clustered either close to the two cysteine residues responsible for homodimerization, namely Cys-129 and 131 (Zhang *et al.*, 2001), or, in the transmembrane domain. Again, this condition can be mimicked by autoantibodies that bind the CaR except that here they elicit a gain-of-function effect by stabilizing activating con-

formations of the receptor (Brown, 2009). One such condition is autoimmune polyglandular syndrome type 1 (APS1) in which autoantibodies bind the N-terminal region of the CaR ECD leading to elevated CaR activity and thus hypoparathyroidism with characteristic hypocalcaemia and hyperphosphataemia (Brown, 2009; Kemp *et al.*, 2009). Furthermore, there are two mouse models of CaR gain-of-function; the first is the Nuf mouse, which expresses a CaR mutation (L723Q) resulting from a chemical mutagenesis study (Hough *et al.*, 2004). This mouse exhibits ectopic calcification, hypocalcaemia, hyperphosphataemia and inappropriately reduced levels of plasma PTH consistent with ADH in humans. In the second model, 5-point mutations were introduced to the CaR ECD but targeted to mature osteoblasts using the 3.5 kb osteocalcin promoter (Dvorak *et al.*, 2007). The resulting mouse displayed normal PTH and calcium levels but with enhanced bone resorption, further supporting a tissue-specific role for CaR in bone.

Calcium-sensing receptor pharmacology

Most GPCRs respond to modified amino acids or polypeptides making it possible to predict and define their agonist binding sites. However, for elemental calcium and CaR this has proven very difficult. The Hill coefficient for CaR indicates the cooperative binding of three to five Ca^{2+} ions to the receptor (Bai, 2004). It has long been recognized that clusters of negatively charged extracellular amino acids most likely account for CaR agonist binding; however, the lack of a solved 3D structure for CaR and the low affinity of Ca^{2+} binding have seriously hindered further investigation of this. Recent modelling work, employing the crystal structure for mGluR, has identified at least three (Huang *et al.*, 2007) and as many as five potential Ca^{2+} binding sites in CaR (Huang *et al.*, 2009b). Two such regions, Gly222–Ile235 and Gly383–Ile408 (each containing five to six glutamate or aspartate residues) were individually inserted into a scaffold protein that exhibited no prior cation-binding capacity. Introduction of either of the two D/E-rich sequences gave the new protein the ability to bind calcium (Huang *et al.*, 2007). Furthermore, selective substitution of either Glu-224, Glu-228/229 or Glu398/399 for isoleucine each resulted in loss of CaR function supporting these observations. Indeed, the Glu398/399Ile mutation had perhaps the greatest effect at reducing CaR Ca^{2+} sensitivity and maximal response (Huang *et al.*, 2007). Such a steep agonist concentration/effect relationship is a critical feature of CaR function given the physiological necessity to maintain ionized serum Ca^{2+} concentration between 1.1 and 1.3 mM. Even moderate hypocalcaemia or hypercalcaemia can cause altered neuromuscular activity leading to tetany or arrhythmias respectively. Therefore, further studies are necessary to elucidate the mechanistic basis for such cooperativity.

Studies of GPCR oligomerization challenge the notion that receptors always exhibit a simple linear relationship between receptor and ligand with a 1:1 stoichiometry (Park and Palczewski, 2005). For instance, serotonin/glutamate receptor complexes respond to hallucinogenic drugs and are

new potential drug targets for psychosis (Gonzalez-Maeso *et al.*, 2008). Furthermore, in cerebellar Purkinje cells, type B γ -aminobutyric acid receptors (GABA_BR) and type 1 metabotropic glutamate receptor (mGluR1) colocalize in the dendritic spines of excitatory synapses. Here, conformational changes evoked by elevated Ca²⁺_o concentration onto GABA_BR lead to a constitutive increase in the glutamate sensitivity of mGluR1 (Tabata *et al.*, 2004). The evidence that heterodimerization within group C GPCRs produces new types of receptors with entirely diverse pharmacological profiles has spurred a number of studies concerning CaR oligomerization. It has long been known that the CaR exists in the plasma membrane as a functional homodimer (Ward *et al.*, 1998), but there is more recent evidence that CaR may also oligomerize with other family C GPCR members. For instance, in hippocampal and cerebellar neurons, the CaR heterodimerizes not only with mGluR1 α and mGluR5 (Gama *et al.*, 2001), but also with R1 and R2 GABA_B receptors (Chang *et al.*, 2007). In both circumstances, formation of the heteromeric complex leads to profound changes in CaR expression levels, agonist sensitivity and cell signalling. Furthermore, CaR heterodimerization with other Group C GPCRs involved in nutrient sensing has been reported, which would allow for differential sensing of food intake (Wellendorph *et al.*, 2010).

Although Ca²⁺_o-sensing is a crucial aspect of CaR function, many other divalent (Mg²⁺), trivalent (lanthanides such as Gd³⁺), polyvalent cations [e.g. poly-L-lysine, poly-arginine, protamine (Nemeth, 2002)] and the aminoglycoside antibiotics (Ward *et al.*, 2002; 2005; Gibbons *et al.*, 2008), are all agonists of the CaR (reviewed by Brown and MacLeod, 2001). These are orthosteric, type I agonists, in that they can activate the CaR in the absence of Ca²⁺_o. In addition, compounds that modify the endogenous affinity of the receptor for Ca²⁺_o, or allosteric modulators, have been identified. Some of these modulators are present in our body under physiological conditions including L-aromatic amino acids, glutathione, ionic strength and alkalization (Bandyopadhyay *et al.*, 2010). For example, such type II agonists have been shown to produce biologically relevant effects such as the modulation of PTH secretion by dietary protein intake (Conigrave *et al.*, 2004; Broadhead *et al.*, 2010), regulation of gastric function (Cheng *et al.*, 1999), cholecystokinin secretion (Liou *et al.*, 2011) and alterations in urinary Ca²⁺_o excretion (Riccardi and Brown, 2010).

PTH plays an essential role in Ca²⁺_o and inorganic phosphate (Pi) homeostasis and deviations in plasma PTH have a profound impact on mineral ion metabolism. For instance, prolonged increases in circulating PTH levels, such as those seen during primary hyperparathyroidism or hyperparathyroidism secondary to kidney failure, are associated with increase risks of cardiovascular morbidity and mortality (Block *et al.*, 2004a) and activate bone-resorbing cells, the osteoclasts, causing net bone loss (Antonsen *et al.*, 1998). In contrast, short-lived bursts in plasma PTH activate bone-forming cells, the osteoblasts, with an induction of bone formation markers and an improvement in bone mineral density in post-menopausal women (Dempster *et al.*, 1993). Therefore, in an ageing population, where the incidence of chronic kidney disease and osteoporosis are on the rise, manoeuvres aimed at modifying circulating plasma PTH represent an area of great therapeutic potential. As it was well

established that the parathyroid CaR represents the master regulator of PTH secretion, the preclinical development of compounds that could modulate its secretion by the parathyroid glands was already ongoing even before the receptor was molecularly identified in 1993 (Nemeth, 2002; 2006). Knowledge of the molecular structure of the CaR confirmed what was already known, that because of the low affinity of Ca²⁺_o and other inorganic cations at the CaR (Brown *et al.*, 1993), it was unlikely that orthosteric ligands could provide the desired specificity and potency. Therefore, Nemeth and colleagues at NPS Pharmaceuticals, Inc., set out to identify compounds that could modify the concentration–response curves of the CaR for Ca²⁺_o. This approach proved to be successful, and led to the identification of two small organic molecules that can mimic Ca²⁺_o or potentiate its receptor affinity, but that are not polycations. These ligands are termed ‘type II calcimimetics’, while orthosteric agonists are considered ‘type I calcimimetics’ (Nemeth *et al.*, 1998). On the other hand, compounds that reduce the CaR affinity for Ca²⁺_o have also been identified and are named ‘calcilytics’ (Nemeth *et al.*, 2001). As for all positive allosteric modulators (PAM) and negative allosteric modulators (NAM), calcimimetics and calcilytics offer the advantage that: (i) they do not alter the plasma concentrations of the endogenous ligands, nor do they compete with them and bind to different sites from the orthosteric agonists; (ii) they enhance or inhibit endogenous physiological responses, that is, they do not evoke supra-maximal ones or activate *de novo* signalling pathways; and (iii) their effects are saturable, that is, they are maximally active at EC₅₀ values for Ca²⁺_o and are ineffective at minimal or maximal concentrations of the physiological ligand (Christopoulos and Kenakin, 2002). A list of the main CaR allosteric modulators with their EC₅₀ or IC₅₀ values is presented in Table 1.

Calcimimetics: positive CaR modulation and chronic kidney disease

Most current calcimimetics are phenylalkylamines and are derived from Ca²⁺ channel blockers, such as verapamil or TMB-8 (Nemeth, 2002). An initial screening of this class of drug demonstrated that the calcium channel blocker fendiline could evoke a substantial mobilization of Ca²⁺_i in isolated bovine parathyroid cells. Two of these compounds, NPS-467 and its chlorinated derivative, NPS-568, were identified and the racemic mixture separated into the R- and S-enantiomers for further pharmacological studies in bovine parathyroid cells. The R-enantiomer was shown to be 10-fold (NPS-568) or 100-fold (NPS-467) more potent than the S-enantiomer. Selectivity studies showed that, at concentrations below 10 μ M, these two compounds did not activate nor affect the agonist responses of a variety of other GPCRs, including mGluRs. However, at high concentrations they did induce β adrenergic receptor-dependent second messenger production, an effect which is also mimicked by high (>5 mM) Ca²⁺_o (Chen *et al.*, 1989). Therefore, calcimimetics can evoke significant off-target pharmacology at high concentrations (>1 μ M). However, specific effects on the CaR can be investigated by: (i) using drug concentrations at or below

Table 1

The table includes the calcimimetics and calcilytics discussed in the review, with EC₅₀ (calcimimetics) or IC₅₀ (calcilytics) values reported in heterologous expression systems (i.e. cells overexpressing the human CaR) or in cells endogenously expressing the CaR (generally, although not exclusively, parathyroid cells). The table also includes information concerning drug testing *in vivo* (n.a.: information not available)

	EC ₅₀ or IC ₅₀ (<i>in vitro</i> , recombinant) (nM)	EC ₅₀ or IC ₅₀ (<i>in vitro</i> , native) (nM)	PTH modulation <i>in vivo</i>	References
Calcimimetics				
NPS 467 (1a)	60	60	yes	Nemeth <i>et al.</i> , 1998
NPS R 568 (1b)	30	27	yes	Nemeth <i>et al.</i> , 1998
Sensipar (Cinacalcet HCl)	51	28	yes	Nemeth <i>et al.</i> , 2004
Calindol	310	n.a.	n.a.	Petrel <i>et al.</i> , 2004
AC-265347	10	n.a.	yes	Ma <i>et al.</i> , 2011
Calcilytics				
NPS 2143 (SB-262470)	43	41	yes	Nemeth <i>et al.</i> , 2001
NPS 53574 (quinazolin-4-ones)	3500	97	yes	Shcherbakova <i>et al.</i> , 2005
Ronacaleret (1b, SB-751689)	320	✓	yes	Fitzpatrick <i>et al.</i> , 2011; Balan <i>et al.</i> , 2009
Calhex 231	330	n.a.	n.a.	Petrel <i>et al.</i> , 2004; Kessler <i>et al.</i> , 2006

1 µM; (ii) performing stereoselectivity experiments (the R-enantiomer being 10- to 100-fold more potent than the S-enantiomer); and (iii) using Ca²⁺ concentrations close to its EC₅₀ value for the biological function to be assessed (as allosteric modulators, calcimimetics are ineffective at minimal and maximal Ca²⁺ concentrations – see above). It should be noted that when the calcimimetics are used outside of these experimental conditions, the resulting responses cannot be assumed to be true CaR-mediated events.

Calcimimetics are the first example of receptor PAMs to have received regulatory approval. Because extensive knowledge of the pharmacology of orthosteric and allosteric agonists in native parathyroid cells preceded the cloning of the CaR (Brown *et al.*, 1993), the calcimimetic Cinacalcet® received Food and Drug Administration (FDA) approval 11 years after the CaR was cloned. Marketed as Sensipar® in the USA, Mimpara in the EU and Regpara in Japan, the calcimimetics have been approved for the treatment of hyperparathyroidism secondary to chronic kidney disease (Block *et al.*, 2004b) and for parathyroid carcinoma (Silverberg *et al.*, 2007). In addition, several other 'off label' applications have been reported in which Cinacalcet treatment aims at rectifying hypercalcaemic/hypophosphataemic states of various nature. These include, but are not limited to, hypercalcaemia after renal transplantation (Kruse *et al.*, 2005), lithium-induced hyperparathyroidism (Sloand and Shelly, 2006), X-linked hypophosphataemia (Alon *et al.*, 2008) and oncogenic osteomalacia (Geller *et al.*, 2007). In addition, recent studies suggest the possibility of using calcimimetics to rescue CaR mutants, which result in protein misfolding (Huang *et al.*, 2007).

Calcimimetics bind to the transmembrane region of the CaR (Miedlich *et al.*, 2002; 2004; Petrel *et al.*, 2004), and are believed to act by stabilizing CaR in an active state. In both rodent models and human subjects with end-stage chronic

kidney disease, treatment with the marketed calcimimetic, Cinacalcet HCl, lowers PTH with the nadir after approximately 2–4 h, before PTH levels rise slowly again until ~12 h post-dosing (Nemeth *et al.*, 2004). Studies in patients indicate that Cinacalcet treatment not only helps achieve the stringent targets set for an effective management of advanced chronic kidney disease, but it also provides a better control of plasma Pi and the calcium x inorganic phosphate (CaxPi) product, disturbances of which are associated with an increase in cardiovascular morbidity and mortality (Block *et al.*, 1998; 2004a). A recent prospective study carried out on a large cohort of dialysis patients over 26 months has demonstrated that calcimimetic treatment improves all-cause and cardiovascular survival (Block *et al.*, 2010).

The blood pressure-lowering effects of a high calcium diet in spontaneously hypertensive rats and humans has been known for more than 40 years (Ayachi, 1979). However, it is only 14 years since the late Dr Bukoski first hypothesized that a CaR in the perivascular nerve network might provide the molecular transducer for the effects of hypercalcaemia on blood pressure (Bukoski *et al.*, 1997). These studies spurred a number of laboratories to elaborate on this initial idea, and thus identify the potential importance of the CaR in the vasculature. Thus, the existence of a functional CaR has been demonstrated in intact arteries (Ohanian *et al.*, 2005), endothelial (Weston *et al.*, 2005) and smooth muscle cells (Wonneberger *et al.*, 2000; Smajilovic *et al.*, 2006) and in intact human arteries (Molostvov *et al.*, 2007; Alam *et al.*, 2009). As some patients with advanced chronic kidney disease also manifest with profound cardiovascular complications and most of them take Cinacalcet, we set out to investigate the role of the CaR and potential clinical utility of the calcimimetics in the vasculature. Our observations have shown that loss of CaR expression is associated with an increase in vascular calcification both *in vitro* and *in vivo*

(Alam *et al.*, 2009), and that the calcimimetics delay smooth muscle cell calcification *in vitro* (Alam *et al.*, 2009). In addition, other investigators have shown that the calcimimetics appear to protect against the vascular remodelling, which occurs during advanced chronic kidney disease (Koleganova *et al.*, 2009) and to delay calcification and atherosclerosis, which occur in apolipoprotein E-deficient mice (Ivanovski *et al.*, 2009), either alone or in combination with vitamin D (Rodriguez *et al.*, 2008). The first evidence of an effect of calcimimetics on the cardiovascular system in humans derives from a recent publication by Block *et al.* (2010). In a prospective study involving a large cohort of patients with advanced chronic kidney disease which required haemodialysis, the authors have demonstrated that 2 year calcimimetic treatment reduces all-cause cardiovascular mortality. It remains to be determined whether these beneficial effects of calcimimetics on patient survival are mediated directly through the vascular CaR. Alternatively, they could be ascribed to calcium channel inhibition by these compounds (see above) or, as the recent literature seems to suggest, through inhibition of renin secretion by the juxtaglomerular apparatus of the kidney (Beierwaltes, 2010), which would result in blood pressure reduction. While hypotensive effects of calcimimetics have been demonstrated in normal rats *in vivo*, they are not equally present in all blood vessel types (i.e. they are more prominent in the mesenteric, rather than the renal artery), tend to be detected at concentrations of the drug well above those used to inhibit PTH secretion and do not appear to be stereoselective. These studies suggest that the effect of the calcimimetics may be mediated through an inhibition of calcium channels, rather than to an activation of the CaR (Nakagawa *et al.*, 2009). The latter conclusion is supported by a recent study by Thakore and Ho (2011), who also used a calcimimetic other than Cinacalcet, namely calindol, and that ascribed its vasorelaxant properties to L-type Ca²⁺ channel blockade. Whatever their molecular target (i.e. whether Ca²⁺ channel inhibition or CaR activation), the improvement of all-cause mortality by calcimimetics in patients with advanced chronic kidney disease reported in the Block *et al.* (2010) study remains a significant positive side-effect of these drugs. A randomized clinical trial in haemodialysis patients taking Cinacalcet is now due and it will no doubt shed light on the potential vasorelaxant effects of this drug.

The relative therapeutic success of Cinacalcet has led to the development of other calcimimetic compounds, for example the structurally novel benzothiazole compounds reported recently by Acadia Pharmaceuticals (Ma *et al.*, 2011). These compounds reportedly exhibit greater CaSR potency than the phenylalkylamine calcimimetics, at least *in vitro*, and may have a different binding site (Ma *et al.*, 2011).

Calcilytics: negative modulation and osteoporosis

PTH is a potent regulator of skeletal metabolism owing to its unique pharmacokinetic profile, which allows this hormone to evoke both anabolic and catabolic effects (Rizzoli *et al.*, 1992). It is the osteoanabolic action of this hormone, which

has led to the testing of intact PTH (1–84, Preos/Preotact) or one of its biologically active fragments (1–34, Forteo) in pre-clinical models of hormone-deficient bone loss and, subsequently, in post-menopausal women. On the basis of the ability to reduce fracture risk and to increase bone mineral density (Rittmaster *et al.*, 2000; Neer *et al.*, 2001; Greenspan *et al.*, 2007), PTH (1–34) and intact PTH (1–84) were granted FDA and European Medicines Agency (EMA) approval to treat hormone-deficient osteoporosis in 2002 and 2006 respectively. However, PTH treatment carries a 'black box' label and has only been approved for 2 years based on the evidence that it increased the risk of osteosarcoma in rats (Vahle *et al.*, 2002). These findings, together with an inconvenient route of administration (daily subcutaneous injections) and the elevated costs, suggested that identification of specific, potent CaR antagonists capable of evoking endogenous bursts in plasma PTH could overcome some of the issues associated with exogenous PTH treatment. If successful, this strategy has the additional advantage that CaR modulation can only evoke changes in plasma PTH within the physiological range, rather than supra-maximal effects potentially achieved with exogenous PTH administration. At the same time as the calcimimetic studies were carried out, NPS Pharmaceuticals, Inc., together with the then SmithKline Beecham (now GlaxoSmithKline) had already initiated a drug discovery programme for the identification of pharmacological inhibitors of the CaR (Nemeth, 2002), which could evoke bone anabolic effects without inducing sustained PTH-dependent bone loss. That is, the ideal drug had to achieve rapid plasma PTH C_{MAX}, followed by a rapid return to baseline levels shortly after administration of the compound (Nemeth, 2002).

A first screen identified a weak CaR antagonist, which had off-target pharmacology at the β -adrenoceptors, the cardiac human ether-a-go-go related gene (hHERG) ion channel and cytochrome P450 2D6 (CYP2D6) (Nemeth *et al.*, 2001; Marquis *et al.*, 2009b). Subsequent modifications of this compound yielded the first calcilytic, NPS-2143, an orally active compound with greater potency and specificity for the CaR (Nemeth *et al.*, 2001; Marquis *et al.*, 2009a).

Mutagenesis and homology modelling studies have subsequently demonstrated that the calcilytic binding site is located within the transmembrane domain of the CaR molecule, where it partly overlaps with the calcimimetic (NPS-R568) binding site (Miedlich *et al.*, 2004). Initial functional studies carried out in recombinant systems demonstrated that NPS-2143 suppressed CaR activity. *In vivo*, NPS-2143 altered plasma PTH levels with a good overlap with its *in vitro* potency, with EC₅₀ values of ~40 nM (Nemeth *et al.*, 2001). Treating rats with NPS-2143 evoked an increase in plasma PTH which was comparable to that observed following exogenous PTH administration (Nemeth *et al.*, 2001). These *in vivo* effects on plasma PTH have never been tested for other calcilytics, including Calhex 231 (Petrel *et al.*, 2003; Kessler *et al.*, 2006), for which the therapeutic benefits are unknown, as thus far efficacy, potency and safety studies *in vivo* are unavailable.

Following NPS-2143 treatment bone mineral density did not increase, due to the long half-life of NPS-2143, which resulted in an increase in plasma PTH, a bone catabolic stimulus. In support of this hypothesis, blocking the sustained,

PTH osteocatabolic effects with estrogens resulted in formation of new bone (Gowen *et al.*, 2000). Thus, these observations suggested feasibility in producing short bursts in plasma PTH as an anti-osteoporotic treatment, an effect that could be achieved by rapid and transient inhibition of the parathyroid CaR. Efforts to improve the pharmacokinetics of this amino alcohol chemotype led to the identification of compounds with better PK, but with poor oral bioavailability. To obviate this problem, a 'pro-drug' approach was used. An ester form of NPS-2143 was produced (SB-423557) which, following a rapid absorption, was quickly cleaved into the free acid form (SB-423562) before entering the systemic circulation (Nemeth, 2008). In a rat model of osteoporosis, treatment with SB-423557 caused a transient increase (two- to threefold) in plasma PTH concentration, which was maximal after 20 min and that had returned to baseline 2 h post-treatment (Kumar *et al.*, 2010). These effects were accompanied by an increase in bone mineral density and an activation of markers of bone formation. Early clinical studies in healthy volunteers infused with the pro-drug or free acid showed a dose-dependent increase in plasma PTH of twofold or greater for up to 8 h, and that SB-423557 was safe and well tolerated (Kumar *et al.*, 2010).

Further developments have led to the synthesis of another orally bioavailable amino alcohol chemotype, ronacaleret hydrochloride (SB-751689) (Nemeth, 2008), with improved specificity and pharmacokinetic profiles. Although ronacaleret reached phase 2 and treatment in postmenopausal women did activate bone turnover markers, its development was terminated in 2008 due to lack of efficacy (Balan *et al.*, 2009).

Subsequent screening of an internal compound library at Novartis led to the identification of compound '2a', which, following a series of optimization steps to improve its PK, led to 1H-quinazolin-2-ones derivatives which were found to act as calcilytics. Of these, the orally bioavailable compounds '7h' and '11m' evoked a very rapid (within minutes) and short-lasting (an hour or so) peak in plasma PTH at subnanomolar concentrations (Widler *et al.*, 2010). While these observations sound promising, clinical studies are necessary to assess the efficacy, potency and safety profiles of these compounds.

Recently Japan Tobacco Inc. has synthesized a novel calcilytic, JTT-305 (now licensed to Merck, Inc., as MK-5442) (Balan *et al.*, 2009). It is a similar chemotype of ronacaleret and, in phase 2 clinical trials, it shows a significant improvement of bone mineral density (Fukumoto, 2011).

Calcium-sensing receptor-mediated intracellular signalling

The CaR exhibits pleiotropic interaction with $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ in a variety of cell types (reviewed in Ward, 2004; Khan and Conigrave, 2009; Magno *et al.*, 2010) and even to $G\alpha_s$ in mammary epithelial tumour cells (Mamillapalli *et al.*, 2008; Magno *et al.*, 2010) and murine pituitary corticotroph-derived, AtT-20 cells (Mamillapalli and Wysolmerski, 2010). Such apparent promiscuity may be necessary to provide selective regulation of the wide array of cellular effects associated

with the CaR both within and without the calcium homeostatic system. Such interactions may be cell- and context-dependent; however, it is nevertheless possible to identify particular pathways as being responsible for mediating certain CaR functions. For example, in mice lacking either $G\alpha_q$ or $G\alpha_{11}$ in their parathyroid glands, there is a modest increase in PTH secretion; however, where both $G\alpha_q$ and $G\alpha_{11}$ are missing, PTH levels are greatly elevated (Wettschureck *et al.*, 2007). Therefore, it would appear that, for PTH secretion control at least, CaR-induced $G\alpha_{q/11}$ activation contributes significantly to the response.

Calcium-sensing receptor-induced Ca^{2+}_i oscillations

To understand the mechanism of CaR-mediated signalling we must look at the activity of the receptor in individual cells. Ca^{2+}_i imaging methods that measure only total fluorescence from multiple cells (e.g. the cuvette system) tend to show a standard, if steep, sigmoidal agonist concentration – effect relationship for CaR. However, single cell Ca^{2+}_i imaging reveals significant cell-to-cell variability in the dynamics of the Ca^{2+}_i mobilization ranging from transient and oscillatory responses to sustained responses. It is essential therefore to understand how these signals are shaped in order to understand both the mechanism of CaR signalling *per se* but also the reason for the steepness of the CaR agonist concentration–effect curve in particular. This cell-to-cell variability does mean though that significant care must be taken when reporting single cell studies to determine precisely what constitutes a 'representative cell'. This means categorizing the various CaR responses where possible (Bruce *et al.*, 1999; Davies *et al.*, 2007) as well as developing mathematical descriptions of the oscillations (Szekely *et al.*, 2009).

Stimulation of CaR expressed in HEK-293 cells elicits Ca^{2+}_i mobilization from intracellular stores (reviewed in Breitwieser, 2006) rather than Ca^{2+}_o influx *per se*, although it should be noted that modest increases in Ca^{2+}_i concentration can follow Ca^{2+}_o elevation even in non-transfected HEK-293 cells, especially following exposure to low Ca^{2+}_o or Ca^{2+}_o -free buffers (Nemeth *et al.*, 2004). CaR-induced Ca^{2+}_i mobilization has also been reported in parathyroid cells (Ridefelt *et al.*, 1995) and other cell types where CaR is expressed endogenously (Shorte *et al.*, 1995; Adebajo *et al.*, 1998; Ward *et al.*, 2002) and thus a number of studies have explored the mechanistic basis and functional consequences of CaR-induced Ca^{2+}_i oscillations.

Phosphorylation of the CaR intracellular tail regulates receptor activity

Most CaR-HEK cell studies employ baseline Ca^{2+}_o concentrations of 0.5 or 1 mM in the experimental buffer and at these concentrations no cells are activated. However, Ca^{2+}_i mobilization is observed at ~2 mM Ca^{2+}_o mostly in the form of transient responses or low frequency oscillations. At ~3 mM

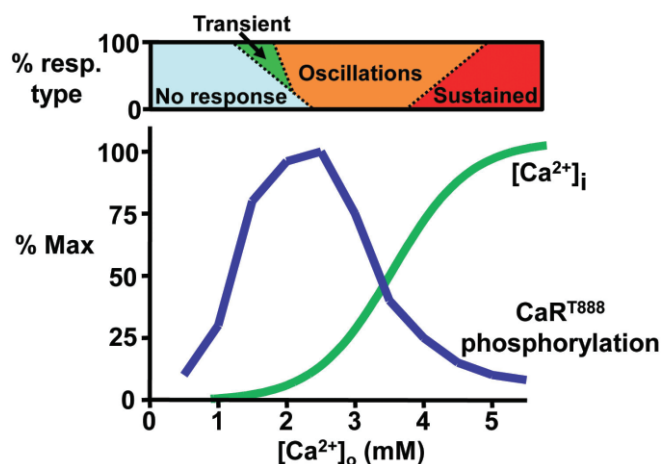


Figure 1

Schematic representation of the Ca^{2+}_o concentration dependence of CaR^{T888} phosphorylation and CaR-induced Ca^{2+}_i mobilization. The classic sigmoidal concentration dependence of CaR-induced Ca^{2+}_i mobilization is not matched by a sigmoidal relationship for CaR^{T888} phosphorylation but by a biphasic (bell-shaped) relationship. Therefore, at higher Ca^{2+}_o concentrations, CaR^{T888} phosphorylation is low or absent (at least after a brief initial rise), thus permitting the sustained responses seen for Ca^{2+}_i mobilization. The relative concentration dependence of the transient (green), oscillatory (orange) and sustained (red) responses normally observed represent approximations based on data from Davies *et al.* (2007) and McCormick *et al.* (2010). This bar is intended to indicate the heterogeneity of the cellular responses at each concentration (see also Nash *et al.*, 2002).

Ca^{2+}_o most responses are high frequency oscillations, while at 5 mM Ca^{2+}_o most responses are sustained (Breitwieser and Gama, 2001; Davies *et al.*, 2006; 2007) (Figure 1). It should be noted that the actual concentrations at which these 'transitions' occur are subject to multiple factors such as temperature, receptor expression levels and buffer ingredients but the trend is broadly true under most conditions. What is interesting is that the linear part of the Ca^{2+}_o concentration–effect curve comprises each of these response types and thus any signal or condition that may independently modulate oscillation frequency will tend to alter the EC_{50} accordingly without necessarily affecting agonist binding. This is interesting both from a molecular perspective and also as a means of altering CaR efficacy. The single most important CaR residue for controlling these oscillations is threonine-888 (in humans) and this serves as a highly conserved protein kinase C phosphorylation site. Cloning and sequencing of the bovine CaR and then human CaR, revealed at least five conserved PKC consensus sequences in the intracellular loops (T646 and S794) and intracellular tail (T888, S895 and S915) of the receptor. Mutating each putative PKC site in turn to a non-phosphorylatable residue, Bai *et al.* (1998b) found that the two intracellular loop residues do not contribute to regulation of CaR Ca^{2+}_o sensitivity and that mutation of residues S895 and S915 elicit only modest effects on the EC_{50} for Ca^{2+}_o or on responsiveness to the phorbol ester phorbol-12-myristate-13-acetate (PMA). In contrast, mutation of T888 (to valine) elicited a substantial leftward shift in the Ca^{2+}_o

concentration–effect curve for Ca^{2+}_i mobilization as well as substantially reducing the inhibitory effect of PMA on the CaR response. Furthermore, CaR^{T888D} and CaR^{T888E}, whose mutations are phosphomimetic, revealed a significant rightward-shift in Ca^{2+}_o concentration dependence (Jiang *et al.*, 2002). Together, these data suggested that CaR^{T888} is central to the control of CaR signalling. This idea was advanced by single cell imaging which revealed that CaR^{T888A} tends to elicit sustained rather than oscillatory Ca^{2+}_i mobilization (Young *et al.*, 2002) which can be mimicked in cells expressing wild-type CaR by inhibiting PKC. This suggested that for each Ca^{2+}_i oscillation, PKC-mediated phosphorylation on CaR^{T888} terminated the Ca^{2+}_i mobilization caused by receptor activation (Figure 2) and a phospho-specific antibody for CaR^{T888} did indeed reveal that CaR can be phosphorylated by PKC in that way (Davies *et al.*, 2007). The concept of dynamic phosphorylation as a mechanism of class C GPCR regulation was first proposed by Nash *et al.* (2001; 2002) with specific regard to the metabotropic glutamate receptor mGluR5. The residue phosphorylated dynamically in this case is mGluR5^{S839} (Kim *et al.*, 2005), this residue being 13 amino acids closer to the transmembrane region than CaR^{T888}. Indeed, there are both interesting parallels as well as specific differences between the dynamic phosphorylation of the two receptors.

CaR^{T888} dephosphorylation shapes downstream signalling

There is a significant problem, however, with the idea that CaR activation leads to CaR^{T888} phosphorylation and thus to receptor inhibition. The most striking problem is that at the top end of the Ca^{2+}_o concentration effect curve, the responses of single cells tend to change from oscillatory into sustained, whereas one might expect their CaRs to be maximally phosphorylated and thus maximally inhibited. To resolve this issue the actual concentration dependence of Ca^{2+}_o on CaR^{T888} phosphorylation was examined by immunoblotting (10 min agonist treatment) and instead of a sigmoidal curve, a biphasic, bell-shaped profile was observed. That is, at low to moderate Ca^{2+}_o concentrations, there is a concentration-dependent increase in CaR^{T888} phosphorylation, which peaks around the Ca^{2+}_o concentration at which oscillatory Ca^{2+}_i mobilization occurs. However, as Ca^{2+}_o concentrations are increased further, the amount of CaR^{T888} phosphorylation observed after 10 min declines consistent with the increased oscillation frequency seen at those concentrations. Finally, at ~5 mM Ca^{2+}_o little CaR^{T888} phosphorylation is observed after 10 min consistent with the sustained, that is, uninhibited Ca^{2+}_i mobilization observed at those concentrations. This implies that, at moderate Ca^{2+}_o concentrations, PKC-mediated phosphorylation of CaR^{T888} matches its rate of dephosphorylation over time, albeit in an alternating manner so as to elicit stable oscillations. However, at higher Ca^{2+}_o concentrations, CaR preferentially elicits differentially greater levels of phosphatase activity rendering the receptor less phosphorylated and thus able to elicit either high frequency oscillations or even sustained Ca^{2+}_i mobilization (Figure 1). In support of this, the calcimimetic NPS-R467 (1 μ M; a high potency PAM)

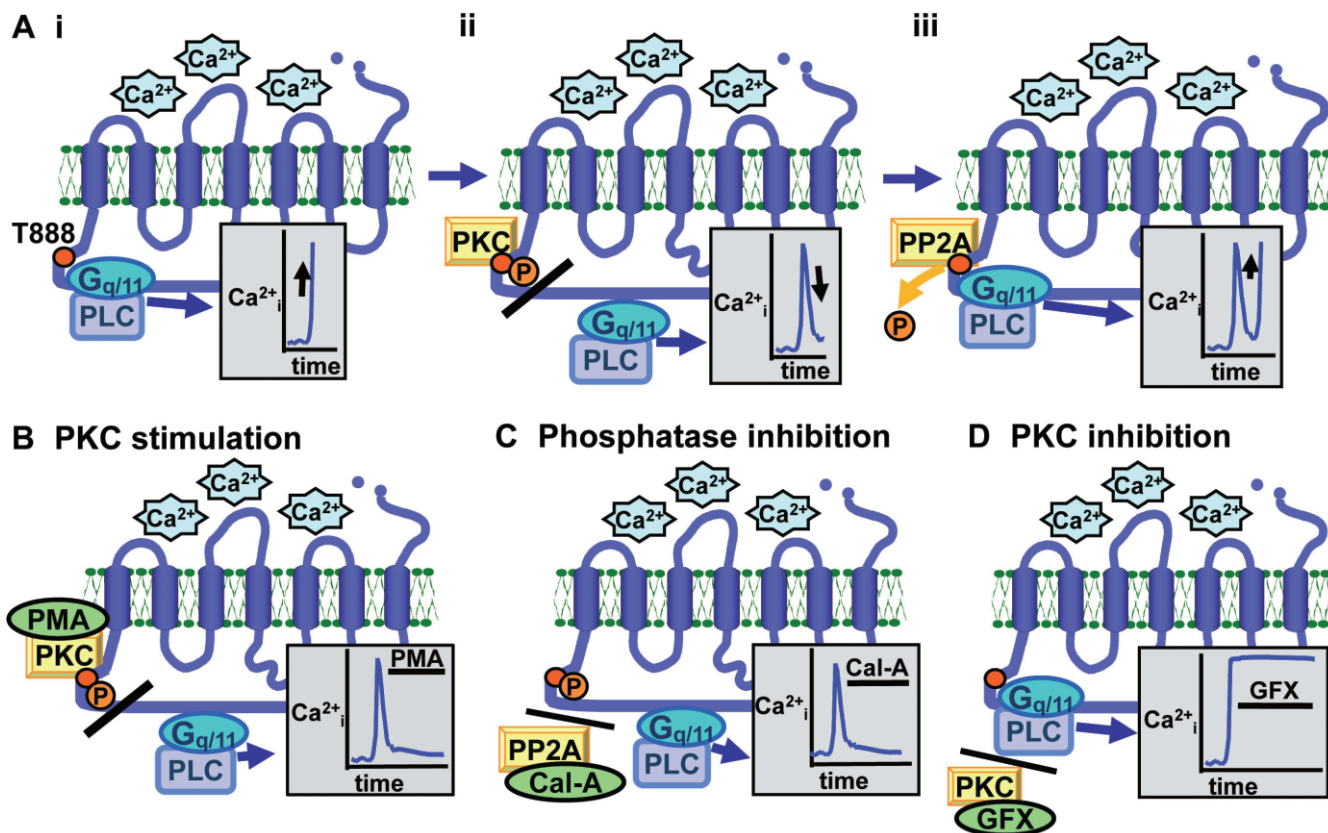


Figure 2

Schematic representation of the generation of CaR-induced Ca^{2+}_i oscillations based on dynamic phosphorylation of CaR^{T888} . Panel A indicates the three components of each oscillatory cycle including (i) CaR activation eliciting Ca^{2+}_i mobilization, (ii) CaR^{T888} phosphorylation uncoupling the pathway components and (iii) CaR^{T888} dephosphorylation leading to further Ca^{2+}_i mobilization. This model therefore explains the suppressive effects of phorbol ester (PMA, Panel B) and calyculin-A (Cal-A, Panel C) and the stimulatory effect of GF109203X (GFX, Panel D) on CaR-induced Ca^{2+}_i mobilization.

elicited rapid but transient CaR^{T888} phosphorylation and thus sustained Ca^{2+}_i mobilization, in a manner similar to high Ca^{2+}_o concentration (McCormick *et al.*, 2010). In contrast, the L-aromatic amino acids (10 mM; lower potency PAM) elicited sustained CaR^{T888} phosphorylation and oscillatory Ca^{2+}_i mobilization similarly to the effects of moderate Ca^{2+}_o concentration.

The identity of the phosphatase responsible remains unknown although some evidence points towards PP2A being involved. For example, the PP1/PP2A inhibitor calyculin-A prevents dephosphorylation of CaR^{T888} and inhibits CaR-induced Ca^{2+}_i mobilization in CaR-HEK and PT cells and abolishes the inhibitory effect of CaR on PTH secretion in human isolated PT cells. Furthermore, PP2A catalytic subunit colocalizes with phosphorylated CaR and PP2A activity is elevated following CaR activation in HEK-293 cells (Davies *et al.*, 2007; McCormick *et al.*, 2010).

Together, this model of differential CaR^{T888} phosphorylation explains both the suppressive effect of phorbol esters and calyculin-A and stimulatory effect of PKC inhibition on CaR-induced Ca^{2+}_i mobilization (Figure 2), and the transition from oscillatory to sustained CaR signalling (Figure 1).

Functional importance of CaR^{T888} phosphorylation

Whilst mutation of CaR^{T888} to non-phosphorylatable residues such as alanine and valine produces a leftward shift in Ca^{2+}_o concentration dependence in HEK-293 cells, that is, *in vitro*, it is necessary to determine whether this has any consequence *in vivo*. Indeed, we have recently identified a case of ADH resulting from the mutation $\text{CaR}^{\text{T888M}}$ (Lazarus *et al.*, 2010). The index case and his father both exhibited hypocalcaemia, inappropriately normal PTH concentrations and relative hypercalcaemia typical of ADH. When recapitulated *in vitro*, $\text{CaR}^{\text{T888M}}$ exhibited a leftward shift in Ca^{2+}_o concentration dependence and resistance to signalling inhibition by PMA (Lazarus *et al.*, 2010). This would suggest that, under normal conditions, the human CaR is subject to feedback inhibition by phosphorylation on Thr-888 raising the possibility that PTH secretion and thus Ca^{2+}_o homeostasis may be controlled not only by pharmacological modulation of the ECD of the receptor but also by interfering with those intracellular signalling pathways responsible for the mediation of CaR sig-

nalling. Together, these molecular and clinical data explain functional observations first made ~20 years ago showing that phorbol esters inhibit the suppressive effects of high Ca^{2+}_o concentration on PTH secretion (Nemeth *et al.*, 1986; Kobayashi *et al.*, 1988; Morrissey, 1988; Membreno *et al.*, 1989).

Mitogen-activated protein kinase activation

CaR is known to activate an array of protein kinase pathways most notably the mitogen-activated protein (MAP) kinases ERK and p38^{MAPK} (reviewed in Ward, 2004; Khan and Conigrave, 2009; Magno *et al.*, 2010). The mechanism of CaR-induced ERK activation, at least in culture, involves triple-pass signalling whereby CaR stimulates an extracellular matrix metalloproteinase to elicit the extracellular release of an EGF-like peptide, which then stimulates EGF receptor (EGFR)-mediated signalling (MacLeod *et al.*, 2004). In CaR-expressing HEK-293 cells, chronic receptor activation stimulates PTH-related protein (PTHrP) secretion into the medium (MacLeod *et al.*, 2004), an effect also observed in PC-3 human prostate cancer cells (Yano *et al.*, 2004) and H-500 rat Leydig cells (Sanders *et al.*, 2000), and in each case the release of PTHrP is dependent on ERK activation resulting from transactivation of the EGFR (Yano *et al.*, 2004). Similarly, matrix metalloproteinase and EGFR activation also result from CaR activation in human MCF-7 breast cancer cells (El Hiani *et al.*, 2009). Indeed, this mechanism may contribute to the putative oncogenic effects of CaR in breast and prostate cancers – whereas CaR is associated with tumour suppression in colon and parathyroid tumours (Saidak *et al.*, 2009).

Calcium-sensing receptor and the cytoskeleton

Another possible mechanism by which the CaR might limit PTH secretion is to attenuate the trafficking of secretory granules through the cytoskeletal network. The exocytotic barrier function of cortical actin has been described in endocrine cells such as adrenal chromaffin cells and pancreatic beta-cells (Burgoyne and Morgan, 2003) but may also exist in parathyroid cells (Quinn *et al.*, 2007). Significantly, the cortical actin barrier is not simply a constitutive brake on secretion but can be regulated via extracellular signals causing actin assembly/disassembly (Burgoyne and Morgan, 2003). In dispersed bovine parathyroid cells, the filamentous actin-severing compounds, latrunculin and cytochalasin, permitted increased PTH secretion, whereas the actin polymerizing agent, jasplakinolide, substantially inhibited PTH secretion (Quinn *et al.*, 2007). In addition, CaR activation elicits ρ -mediated actin filament formation in HEK-293 cells (Davies *et al.*, 2006) and thus could be directly involved in cytoskeletal alteration as a mechanism for secretory modulation. This idea is further supported by the fact that CaR binds the cytoskeletal protein filamin-A, via the receptor's ICD and disruption of this interaction inhibits CaR-mediated ERK acti-

vation (Hjalm *et al.*, 2001). Also, CaR-induced $\text{G}\alpha_{12/13}$ and ρ activation has been reported in Madin-Darby canine kidney cells (Huang *et al.*, 2004) and in breast cancer cells (Huang *et al.*, 2009a). Together, $\text{G}\alpha_{12/13}$ proteins have been implicated in a variety of cellular functions including regulation of platelet activation and smooth muscle contraction, as well as embryonic angiogenesis, cell migration in the immune system and metastatic spread in tumour cells (Worzfeld *et al.*, 2008). Therefore, it will be interesting to determine the extent to which CaR-induced $\text{G}\alpha_{12/13}$ activation may influence these processes in cells in which the CaR is expressed.

Conclusions

Because of their ability to modulate PTH secretion, CaR-based therapeutics constitute an important and novel toolkit for the treatment of disturbances in Ca^{2+}_o metabolism. Calcimimetics are the first class of receptor PAMs on the market and are being used increasingly for the treatment of hyperparathyroidism and of other hypercalcaemic disorders. Indeed, recent clinical observations suggest that they also ameliorate cardiovascular complications associated with advanced chronic kidney disease and might allow for a reduction in vascular calcification and an improvement in blood pressure control in patients suffering from this disease. As for calcilytics, they improve bone mineral density and induce markers of bone formation in ovariectomized rats and have reached phase III clinical trials for the treatment of post-menopausal osteoporosis, a disease which affects 1:3 women and 1:10 men above the age of 50, worldwide. Finally, basic science research has made significant advances in understanding CaR-mediated cell signalling *in vivo*. Further studies must go hand-in-hand with these drug discovery programmes to establish how best to achieve an inhibition of the CaR to mobilize the pool of PTH-containing vesicles, in order to evoke short-lived osteoanabolic bursts of this hormone without its undesired effects on bone breakdown.

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Conflict of interest

Both authors declare no conflict of interest.

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