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REVIEW

Engendering biased signalling from the calcium-sensing receptor for the pharmacotherapy of diverse disorders

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The human calcium-sensing receptor (CaSR) is widely expressed in the body, where its activity is regulated by multiple orthosteric and endogenous allosteric ligands. Each ligand stabilizes a unique subset of conformational states, which enables the CaSR to couple to distinct intracellular signalling pathways depending on the extracellular milieu in which it is bathed. Differential signalling arising from distinct receptor conformations favoured by each ligand is referred to as biased signalling. The outcome of CaSR activation also depends on the cell type in which it is expressed. Thus, the same ligand may activate diverse pathways in distinct cell types. Given that the CaSR is implicated in numerous physiological and pathophysiological processes, it is an ideal target for biased ligands that could be rationally designed to selectively regulate desired signalling pathways in preferred cell types.

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Abbreviations
ADIS, agonist-driven insertional signalling; CaSR, calcium-sensing receptor; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; IP_3, inositol 1,4,5-trisphosphate; L-Phe, L-phenylalanine; PC-PLC, phosphatidylcholine-specific PLC; PI 3-kinase, phosphoinositide 3-kinase; PI-PLC, phosphatidylinositol-specific PLC; PTH, parathyroid hormone; RANKL/TNFSF11, receptor activator of NFκB ligand; SNP, single nucleotide polymorphism; TRPC, transient receptor potential cation

The CaSR protein and its ligands

The extracellular calcium-sensing receptor (CaSR) is a family C GPCR that plays a pivotal role in maintaining extracellular calcium (Ca^{2+}_o) homeostasis. [Drug/molecular target nomenclature throughout this manuscript conforms to BJP's Concise Guide to PHARMACOLOGY (Alexander *et al.*, 2013)]. It is composed of 1078 amino acids in four main domains beyond the signal peptide (http://www.casrdb.mcgill.ca). These are an N-terminal extracellular Venus FlyTrap (VFT) domain (residues 22–528) linked via a cysteine-rich domain (residues 529–612) to the heptahelical signalling domain (residues 613–862) and a C-terminal intracellular domain (residues 863–1078). The VFT domain provides binding sites for the endogenous (orthosteric) agonists Ca^{2+}_o and Mg^{2+}_o, although Ca^{2+}_o can also activate the CaSR via the heptahelical or extracellular loop domains, as evidenced by its activity at 'headless' CaSR constructs that lack the N-terminal domain of the receptor (Ray and Northup, 2002; Mun *et al.*, 2004; Ray *et al.*, 2005). The CaSR also responds to various di- and tervalent cations, including Gd^{3+}, Al^{3+}, Sr^{2+}, Ba^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} and Pb^{2+} (McGehee *et al.*, 1997; Handlogten *et al.*, 2000). The VFT is additionally the site of action of endogenous allosteric agonists, including cationic polyamines, such as spermine and spermidine (Quinn *et al.*, 1997), and endogenous allosteric modulators, including L-amino acids and glutathione analogues (for a review, see Conigrave and Hampson, 2010). Aminoglycoside antibiotics, such as neomycin, gentamicin and tobramycin, also activate the CaSR (McLarnon *et al.*, 2002; Ward *et al.*, 2002), although their binding site is yet to

be identified. Synthetic allosteric modulators that bind in the heptahelical domain and extracellular loops of the CaSR have also been identified, which include calcimimetics, such as cinacalcet (and related phenylalkylamines) (Nemeth *et al.*, 1998), and calcilytics, such as NPS 2143 (Nemeth, 2002).

Biased signalling from the CaSR

The CaSR couples to $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ (Huang *et al.*, 2004; Davey *et al.*, 2012) and even G_s in some cell contexts (Mamillapalli and Wysolmerski, 2010). Stimulation of distinct effectors downstream of these G proteins mediates cellular responses to CaSR activation (Figures 1–3). Its promiscuous binding to numerous endogenous ligands, and its propensity to couple to multiple G proteins and downstream signalling pathways, make the CaSR an ideal candidate for biased signalling (also known as stimulus bias, ligand-directed trafficking of receptor stimulus, functional selectivity or biased agonism). Biased signalling arises from the ability of different ligands to favour distinct conformational receptor states, each possessing its own coupling preferences to downstream signalling pathways (Kenakin, 2011).

Biased signalling operates in response to various endogenous CaSR agonists and modulators. In HEK293 cells, for instance, when stimulated with Ca^{2+}_o, the CaSR preferentially couples to inhibition of cAMP production and stimulation of inositol 1,4,5-trisphosphate (IP_3) accumulation over phosphorylation of ERK 1 and 2 (ERK1/2) (Thomsen *et al.*, 2012a). Spermine, however, strongly favours ERK1/2 phosphorylation (Thomsen *et al.*, 2012a). Similarly, although both Ca^{2+}_o and L-phenylalanine (L-Phe) stimulate CaSR-mediated Ca^{2+}_i release, Ca^{2+}_o promotes higher frequency Ca^{2+}_i oscillations (up to 4 min^{-1}), whereas L-Phe mediates lower frequency oscillations (up to 2 min^{-1}) (Rey *et al.*, 2010). Ca^{2+}_o-mediated sinusoidal Ca^{2+}_i release is facilitated by activation of PLC, which promotes the release of IP_3 and DAG from the parent phospholipid phosphatidylinositol 4,5-bisphosphate. IP_3 causes release of Ca^{2+}_i from intracellular stores and DAG activates PKC, which subsequently phosphorylates the CaSR and attenuates signalling (Bai *et al.*, 1998; Davies *et al.*, 2007). In contrast, activation of the CaSR by L-Phe is reported to promote its interaction with $G_{12/13}$, Rho, filamin-A and transient receptor potential cation (TRPC) 1 channels, resulting in TRPC1 channel opening and Ca^{2+} influx from the extracellular fluid (Rey *et al.*, 2005; 2006; 2010). Intriguingly, higher frequency Ca^{2+}_o-stimulated Ca^{2+}_i oscillations and lower frequency L-Phe-stimulated Ca^{2+}_i oscillations have also been observed in human proximal tubule epithelial cells (Rey *et al.*, 2005). These results suggest that the cellular location of CaSR expression and its subsequent exposure to a subset of ligands that are present in specific compartments (e.g. in the luminal compartments of the gastrointestinal tract or renal tubules) may govern its signalling, enabling selective activation of intracellular signalling pathways depending on which ligand binds the receptor.

This review will summarize our current understanding of the physiological roles of the human CaSR, the implications of biased signalling arising from the CaSR and how biased signalling may be engendered to manipulate CaSR function in pathological states.

The CaSR's roles in calcium homeostasis

Two well-characterized actions of the CaSR in response to a rise in systemic Ca^{2+}_o level are (i) suppression of parathyroid hormone (PTH) secretion and (ii) suppression of renal calcium re-absorption arising, respectively, from receptors

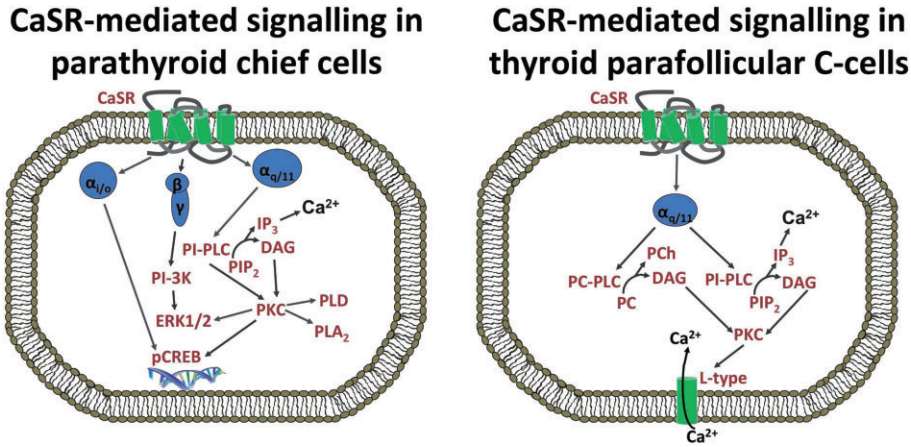

Figure 1

CaSR-mediated regulation of PTH and calcitonin release. Suppression of PTH release from parathyroid chief cells is dependent on CaSR activation of $G_{q/11}$-mediated pathways and possibly the suppression of cAMP synthesis (not shown). CaSR activation of PI-PLC and phosphorylation of ERK1/2 leads to changes in the immediate secretion of PTH. CaSR activity has additionally been linked to changes in PTH gene transcription and subsequent PTH synthesis. Calcitonin release from parafollicular C-cells of the thyroid gland has been linked to $G_{q/11}$-mediated stimulation of PI-PLC, PC-PLC and the opening of L-type Ca^{2+} channels but calcitonin release is independent of ERK1/2 signalling and inhibition of cAMP. PIP_2, phospholipid phosphatidylinositol 4,5-bisphosphate.

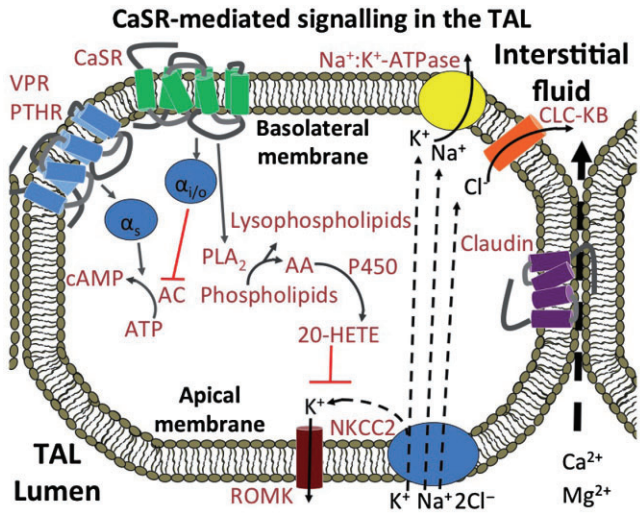

Figure 2

CaSR-mediated control of ion transport across the thick ascending limb. On the basolateral membrane of cells in the cortical thick ascending limb (cTAL), Ca^{2+}_o-induced suppression of transepithelial Ca^{2+} transport is dependent on PLA_2-mediated inhibition of renal outer medullary potassium (ROMK) channels (Wang *et al.*, 1997; Huang *et al.*, 2007; Cha *et al.*, 2011), which contributes to enhanced urinary excretion of Na^+, Ca^{2+} and Mg^{2+}. In addition, Ca^{2+}_o suppresses transcellular Na^+ and Cl^- re-absorption secondary to $G_{i/o}$-mediated inhibition of PTH- and vasopressin-stimulated cAMP production (de Jesus Ferreira *et al.*, 1998; for review, see Gamba and Friedman, 2009). Blockade of K^+ recycling across the luminal membrane and impaired NaCl reabsorption induce an attendant reduction in the lumen positive transepithelial potential that drives Ca^{2+}_o re-absorption. In consequence, paracellular transport of Ca^{2+} (and Mg^{2+}) is impaired resulting in enhanced urinary excretion. In addition to this acute mechanism for Ca^{2+}_o-dependent inhibition of Ca^{2+} re-absorption, the CaSR has recently been reported to up-regulate the expression of claudin-14, a key inhibitor of a claudin-16/19-dependent divalent cation selective paracellular pathway in the thick ascending limb (Gong *et al.*, 2012).

expressed by parathyroid chief cells and renal cortical thick ascending limb cells of Henle's loop. Indeed, in response to hypercalcaemia, the CaSR mediates enhanced renal calcium excretion, independent of changes in PTH levels (Kantham *et al.*, 2009; Loupy *et al.*, 2012). This effect requires CaSR-mediated inhibition of Ca^{2+} re-absorption via both paracellular and transcellular pathways (Figure 2) (for a review, see Riccardi and Brown, 2010). Together, these effects rapidly stimulate renal calcium excretion and lower the serum calcium level. In addition, the CaSR appears to mediate three further responses to elevated Ca^{2+}_o: (i) suppression of $1,25(OH)_2D_3$ synthesis, which lowers the drive for intestinal Ca^{2+} absorption; (ii) negative modulation of PTH-induced phosphate excretion to raise the serum inorganic phosphate level; and (iii) decreased osteoclastic-dependent bone resorption. CaSR-dependent suppression of bone resorption is mediated via receptors on osteoblasts, which suppress osteoclastogenesis, via receptors on thyroid C-cells, which promote the release of calcitonin, and via receptors on osteoclasts themselves, which suppress resorptive activity (for a review, see Riccardi and Kemp, 2012). The concerted effect of these various CaSR-mediated responses is a pronounced decrease in ionized Ca^{2+}_o concentration and a more modest increase in serum phosphate level.

The CaSR also facilitates the transport of Ca^{2+} across the placenta in support of fetal skeletal development and growth (Kovacs *et al.*, 1998) and promotes Ca^{2+} transport into milk during lactation via its expression in mammary duct epithelial cells (Cheng *et al.*, 1998; VanHouten, 2005). Recently, selective knockout of the CaSR in the lactating mouse mammary gland confirmed that the mammary gland receptor promotes calcium transport into milk and, in turn, calcium accrual in suckling neonates (Mamillapalli *et al.*, 2013). Furthermore, CaSR-null lactating dams from this model exhibited hypercalcaemia and appropriately suppressed serum PTH levels, demonstrating that the mammary gland CaSR has a physiological role in maintaining normal maternal serum calcium levels during lactation (Mamillapalli *et al.*, 2013).

Roles of the CaSR in bone and cartilage cells

The CaSR has been implicated in bone mineralization and linear bone growth. In osteoblasts, the CaSR modulates the expression of genes that promote bone mineralization and osteoblast differentiation. Activation of the CaSR in osteoblasts by Sr^{2+}, for instance, stimulates phosphoinositide 3-kinase (PI 3-kinase) and consequent Akt phosphorylation (Rybchyn *et al.*, 2011). CaSR activation also promotes Wnt-dependent β-catenin translocation to the nucleus and attendant osteoblast differentiation (Rybchyn *et al.*, 2011). The CaSR promotes the differentiation of cartilage-producing chrondrocytes in the growth plate, leading to the growth of the long bones during skeletal development (Chang *et al.*, 2008).

The CaSR in cells of the osteoblast–osteocyte lineage also suppresses osteoclastogenesis by inducing down-regulation of receptor activator of NFκB ligand (TNFSF11) and up-regulation of its decoy receptor osteoprotegerin (Brennan *et al.*, 2009; Saidak and Marie, 2012). Reduced expression of TNFSF11 results in reduced osteoclast number, reduced osteoclast activity and reduced bone resorption (Dvorak-Ewell *et al.*, 2011). In addition, CaSRs expressed on osteoclasts appear to mediate high Ca^{2+}_o-induced apoptosis (Kanatani *et al.*, 1999; Mentaverri *et al.*, 2006), which is dependent on PLC, IP_3, nuclear translocation of NF-κB and enhanced caspase activity (Mentaverri *et al.*, 2006).

Some of the effects of elevated Ca^{2+}_o in bone are mediated not only by the CaSR but also by other family C GPCRs, including $GPRC_6$, which mediates some of the responses to Ca^{2+}_o in osteoblasts and thereby contributes to the control of bone mineralization (Pi *et al.*, 2010).

Roles of the CaSR in tissues that are not involved in calcium homeostasis

Perhaps surprisingly, the CaSR is also expressed in tissues that are not involved in Ca^{2+}_o homeostasis. In the CVS, for

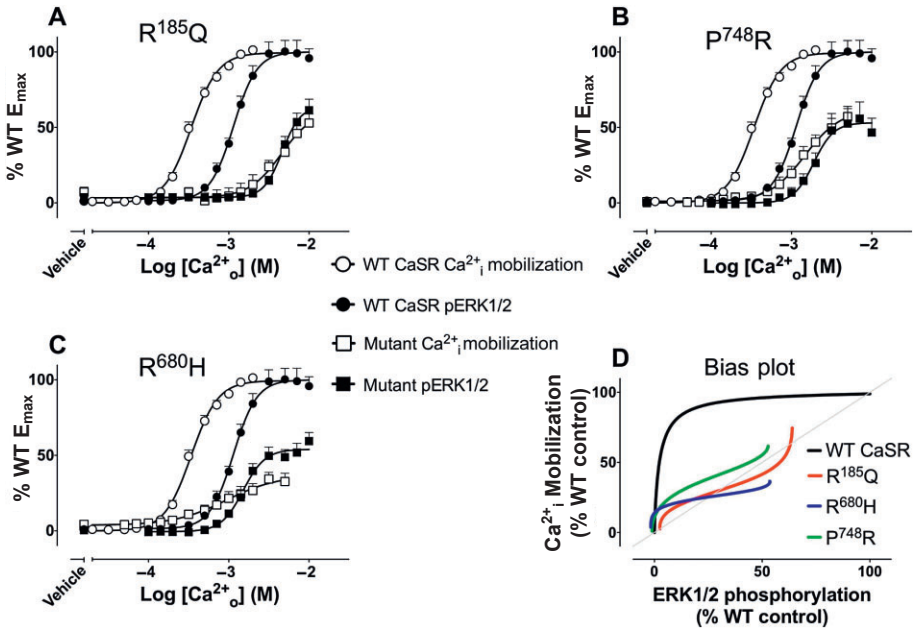

Figure 3

Biased signalling engendered by naturally occurring CaSR mutations. Concentration–response curves (A–C) to Ca^{2+}_o in Ca^{2+}_i mobilization assays or ERK1/2 phosphorylation assays at the WT CaSR or mutant CaSRs (the mutation is stated in the title of each graph) and bias plots (D) corresponding to these curves. The bias plot depicts the response of cells expressing the receptor to equimolar concentrations of Ca^{2+}_o measured in Ca^{2+}_i mobilization assays (y axis) and ERK1/2 phosphorylation assays (x axis). If the receptor shows no preference for either pathway, points on the bias plots are coincident and the plots overlap with the line of identity (grey line). If it favours one of the pathways, the points fall away from this line towards the preferred pathway. Thus, naturally occurring mutations can engender stimulus bias.

example, CaSR expression in the heart and blood vessels has been linked to the modulation of BP (see Smajilovic *et al.*, 2011 for a review) and protection against vascular calcification (Alam *et al.*, 2009), raising the possibility that a drug that selectively modulates CaSR function in this setting might be effective at lowering BP and/or reducing or even reversing calcification.

In the CNS, the CaSR is widely expressed in neurons and glial cells and is subject to developmental regulation (Rogers *et al.*, 1997). For example, it promotes neuronal differentiation, myelination and growth during development (Ferry *et al.*, 2000; Chattopadhyay *et al.*, 2008; Vizard *et al.*, 2008), and modulates neurotransmission in post-natal life (Phillips *et al.*, 2008).

In addition, the CaSR acts as an ionic strength sensor in the subfornical organ linked to the control of BP and whole body salt and water metabolism (Washburn *et al.*, 1999). It also directly modulates salt and water transport in the colon (Geibel and Hebert, 2009) and renal tubules (for a review, see Riccardi and Brown, 2010).

CaSR expression has also been demonstrated in monocytes and macrophages (Yamaguchi *et al.*, 1998; Olszak *et al.*, 2000), in which, alongside $GPRC_6$, it activates the key inflammatory mediator NLRP3 (Lee *et al.*, 2012; Rossol *et al.*, 2012), and in keratinocytes it acts as a mediator of differentiation (Tu *et al.*, 2008). Consistent with this latter idea, keratinocyte-specific CaSR null mice demonstrate disordered skin development (Bikle *et al.*, 2012; Tu *et al.*, 2012).

Modulation of macronutrient digestion, absorption, nutrient disposition and storage

The CaSR has multiple roles in the gastrointestinal tract dependent primarily on its expression by enteroendocrine cells as well as epithelial cells such as gastric parietal cells (for a review, see Conigrave and Brown, 2006). Thus, CaSRs expressed by enteroendocrine G-cells detect nutrient L-amino acids (Geibel and Hebert, 2009) to stimulate the release of gastrin and thus gastric acid secretion (Feng *et al.*, 2010), and CaSRs expressed by I-cells mediate cholecystokinin (CCK) release (Liou *et al.*, 2011; Wang *et al.*, 2011) to support the digestion and absorption of macronutrients. In addition, CaSRs expressed by K- and L-cells mediate amino acid–induced release of gastric inhibitory peptide and glucagon-like peptide 1 (GLP-1), respectively (Mace *et al.*, 2012), to provide a mechanism by which gut luminal contents facilitate nutrient-dependent insulin release.

Consistent with its effects on gut hormone release and gastric acid secretion, CaSRs expressed in hepatocytes promote bile flow (Canaff *et al.*, 2001) to facilitate digestion and absorption of fats and CaSRs expressed on adipocytes, in turn, facilitate fat storage by promoting adipocyte differentiation and adipogenesis (Cifuentes and Rojas, 2008; He *et al.*, 2011; 2012; Reyes *et al.*, 2012). CaSR activation in adipose tissue also elevates the production of cytokines and chemokines, including IL-6, IL-1β, TNF-α and CC chemokine

2 (Cifuentes *et al.*, 2012) and pro-inflammatory cytokines increase CaSR expression in adipocytes (Cifuentes *et al.*, 2010), suggesting the existence of a positive feedback loop. CaSR-mediated release of GLP-1 and the appetite reducing peptide, peptide YY (Mace *et al.*, 2012), as well as CCK provide satiety signals to the hypothalamus for the suppression of further feeding. Collectively, these findings suggest that the CaSR plays a fundamental role in the regulation of appetite and nutrient disposal.

Finally, the CaSR has been detected in pancreatic islet beta-cells, with evidence suggesting it modulates insulin release (Squires *et al.*, 2000; Leech and Habener, 2003; Gray *et al.*, 2006; Parkash and Asotra, 2011).

Modulation of colonic epithelial cell differentiation and proliferation

The human colonic epithelium is composed of tubular invaginations known as 'crypts', at the base of which, stem cells proliferate rapidly and rise progressively towards the luminal surface. At the crypt base, CaSR expression is barely detectable, but it increases substantially as epithelial cells move towards the crypt apex promoting differentiation and suppressing proliferation (for a review, see Peterlik *et al.*, 2013) dependent, in part, on Ca^{2+}_i mobilization and ERK1/2 activation (Bhagavathula *et al.*, 2005; Rey *et al.*, 2010). Evidence that dietary calcium protects against colon cancer in human populations (for a review, see Peterlik *et al.*, 2013) and that CaSR expression is markedly down-regulated in colon cancer (Kallay *et al.*, 2003; Chakrabarty *et al.*, 2005; Hizaki *et al.*, 2011; Singh *et al.*, 2012) has led to the hypothesis that the CaSR acts as a colonic tumour suppressor (for reviews, see Brennan *et al.*, 2012; Singh *et al.*, 2013). Consistent with this idea, CaSR activators promote Wnt5a secretion to suppress inappropriate canonical Wnt signalling in colon cancer cells (MacLeod *et al.*, 2007).

Broader implications of the CaSR as a drug target

Given that the CaSR mediates diverse sensing and signalling functions and associated cellular responses in a surprising diversity of tissues and physiological contexts, it may be a suitable target for the treatment of disorders in which appropriate up- or down-regulated expression or increased or decreased receptor function could be engendered in a tissue and/or signal pathway selective manner.

Impact of CaSR mutations and polymorphisms in disorders of calcium metabolism and other disorders

The broad nature of the CaSR's physiological roles is highlighted by the effects of many naturally occurring mutations or single nucleotide polymorphisms (SNPs) that occur in the CaSR gene. These include, as might be expected, disorders of calcium homeostasis, including familial hypocalciuric hypercalcaemia and neonatal severe hyperparathyroidism, both of which arise from inactivating mutations, as well as autosomal dominant hypocalcaemia, which arises from activating mutations (Brown and MacLeod, 2001). In addition, more severe activating mutations of the CaSR induce the salt-wasting disorder Bartter syndrome type V (Watanabe *et al.*, 2002). These disorders result from mutation-specific impairments in receptor function, which include deficiencies in signalling capacity, trafficking to the cell surface and/or dimerization (Pidasheva *et al.*, 2006; Grant *et al.*, 2012; Leach *et al.*, 2012).

Other disorders that have been linked to CaSR mutations and/or SNPs include some forms of nephrolithiasis (Vezzoli *et al.*, 2012), idiopathic epilepsy (Kapoor *et al.*, 2008), Alzheimer's disease (Conley *et al.*, 2009), chronic pancreatitis (Muddana *et al.*, 2008), coronary heart disease and myocardial infarction (Marz *et al.*, 2007), prostate cancer (Shui *et al.*, 2013) and rectal cancer (Speer *et al.*, 2002).

The majority of mutations that cause loss or gain in CaSR function and/or expression encode amino acid substitutions affecting the receptor's 19 residue signal peptide, its N-terminus, heptahelical domains and C-terminal tail (http://www.casrdb.mcgill.ca). Frameshift mutations (e.g. Ma *et al.*, 2008) or nonsense mutations that introduce premature stop codons and thereby truncate the receptor protein (e.g. Rodrigues *et al.*, 2011; Ward *et al.*, 2013) also induce loss of function and/or expression. Receptor truncation can also arise from mutations in acceptor splice sites (D'Souza-Li *et al.*, 2001) or from the insertion of Alu elements (Janicic *et al.*, 1995). The incidence of mutations in untranslated regions of the CaSR gene and their significance for disorders of calcium homeostasis are currently unknown.

Mutations that result in amino acid substitutions in the CaSR protein cause a range of functional outcomes, most commonly altering the signalling capacity of the receptor and/or reducing receptor expression. In fact, many 'loss-of-function' CaSR mutations cause reductions in cell surface expression, which limit signalling output in response to ligands (Leach *et al.*, 2012). As noted earlier, a reduction in CaSR expression has also been reported in the context of colonic tumourigenesis (Hizaki *et al.*, 2011; Singh *et al.*, 2012) and is a prominent feature of primary (Kifor *et al.*, 1996; Cetani *et al.*, 2000) and secondary (Kifor *et al.*, 1996; Gogusev *et al.*, 1997; Chikatsu *et al.*, 2000; Yano *et al.*, 2000) hyperparathyroidism. In these disease states, reduced CaSR expression is correlated with increased cell proliferation and decreased differentiation. Intriguingly, in rats treated with streptozotocin, which induces type I diabetes by destroying pancreatic islet beta-cells, CaSR expression was reduced in cardiomyocytes (Bai *et al.*, 2012), and up-regulated in the pancreas, liver and kidney (Haligur *et al.*, 2012). Thus, aberrant CaSR expression is a hallmark of various proliferative and metabolic disorders.

Engendering biased signalling at the CaSR

Given that endogenous ligands promote biased signalling at the CaSR, it is possible that synthetic drugs that bias CaSR

signalling towards a particular 'signature' cluster of pathways to the exclusion of others will provide unique treatment opportunities for specific CaSR-mediated disorders. Likely design characteristics will be discussed in the following sections.

At present, strontium ranelate and cinacalcet are the only drugs in clinical practice that target the CaSR. Strontium ions are presumed to bind, like Ca^{2+}_o, in the VFT domain, thereby stimulating receptor signalling in osteoblasts and osteoclasts. In osteoblasts, Sr^{2+} stimulates the expression of genes that promote cell proliferation and differentiation and down-regulates expression of the pro-osteoclastogenic signal TNFSF11 (RANKL) and up-regulates expression of its decoy receptor osteoprotegerin (Brennan *et al.*, 2009; for a review, see Saidak and Marie, 2012). The outcome is reduced bone resorption together with enhanced bone formation. These effects appear to be primary contributors to the therapeutic efficacy of strontium ranelate in osteoporosis.

Cinacalcet, in contrast, binds in the receptor's heptahelical domain and acts as a positive allosteric modulator. Therapeutically, cinacalcet enhances Ca^{2+}_o-mediated receptor activation, which results in normalization of PTH levels in hyperparathyroidism via enhanced inhibition of PTH secretion from the parathyroid glands. Cinacalcet has also been used successfully to correct serum Ca^{2+}_o concentrations in patients possessing loss-of-function CaSR mutations by lowering the Ca^{2+}_o set point in the parathyroid to normal levels (Timmers *et al.*, 2006; Reh *et al.*, 2011; Wilhelm-Bals *et al.*, 2012). Furthermore, we and others have shown that phenylalkylamine calcimimetics such as cinacalcet are efficient pharmacochaperones that promote trafficking of loss-of-expression CaSR mutants to the cell surface (White *et al.*, 2009; Leach *et al.*, 2013), indicating that allosteric modulators could be used to regulate CaSR expression levels in diseases where its expression is attenuated. However, cinacalcet and other early generation phenylalkylamines frequently cause hypocalcaemia (Chonchol *et al.*, 2009). Thus, its use is currently limited to patients with hyperparathyroidism in the context of end-stage kidney disease, parathyroid cancer or patients with moderate–severe primary hyperparathyroidism who cannot undergo parathyroidectomy. Cinacalcet causes a transient increase in the serum calcitonin level in both haemodialysis patients and in patients that have undergone renal transplantation (Serra *et al.*, 2008; Arenas *et al.*, 2013). Following cinacalcet administration, the serum calcitonin level peaks after 2–3 h and normalizes after 6–12 h and may thus exacerbate the hypocalcaemic effect of calcimimetics. Calcitonin inhibits bone resorption (Hosking *et al.*, 1981) and has traditionally been thought to decrease renal Ca^{2+} re-absorption (Haas *et al.*, 1971), thus lowering the serum Ca^{2+}_o concentration. Although recent work suggests that calcitonin may, in fact, stimulate renal Ca^{2+} re-absorption (Hsu *et al.*, 2010), suggesting calcitonin acts to redirect Ca^{2+} from urine to bone rather than directly lowering serum Ca^{2+} through enhanced secretion. Drugs that bias CaSR activity towards signalling pathways, which selectively suppress PTH secretion to the exclusion of others that promote calcitonin release, may reduce the incidence of symptomatic hypocalcaemic episodes. Proximal signalling events that link CaSR to the inhibition of PTH release and activation of calcitonin release have been identified (Figure 1) and will be described later.

Desired properties of CaSR modulators that selectively target PTH release

PTH secretion is promoted by GPCR-mediated stimulation of AC (for a review, see Brown and MacLeod, 2001). Elevated Ca^{2+}_o suppresses the release of PTH from prepackaged vesicles and enhances PTH degradation in parathyroid cells (Habener *et al.*, 1975), and these effects are likely mediated by the CaSR. Activation of the CaSR by elevated Ca^{2+}_o suppresses AC activity and subsequent intracellular cAMP levels, for example, in dopamine-stimulated bovine parathyroid cells (Chen *et al.*, 1989). However, although overnight exposure to pertussis toxin attenuates CaSR-mediated suppression of cAMP levels demonstrating a role for $G_{i/o}$ in the mechanism, $G_{i/o}$ is not required for Ca^{2+}_o-dependent suppression of PTH secretion (Brown *et al.*, 1992; for a review, see Brown and MacLeod, 2001), pointing to the existence of key roles for other G-proteins in PTH secretion control. Consistent with this, mice, in which $G\alpha_q$ was selectively deleted in parathyroid cells on a global $G\alpha_{11}$ null background, exhibited a phenotype that closely resembled that described for CaSR exon-5 null mice (Ho *et al.*, 1995), including growth retardation, parathyroid gland hyperplasia, hyperparathyroidism and severe hypercalcaemia (Wettschureck *et al.*, 2007). The first CaSR knockout strategy described employed a deletion of 20 bps in exon 5, which encodes residues 460–536 in the N-terminal extracellular domain, rendering the succeeding sequence out of frame (Ho *et al.*, 1995). It is now known that an exon 5-less CaSR splice variant is expressed and functional in several tissues including keratinocytes (Oda *et al.*, 1998) and fetal lung (Finney *et al.*, 2011). More recently, tissue-specific deletion of CaSR exon 7 has revealed more severe phenotypes including developmental disturbances of cartilage and bone (Chang *et al.*, 2008). CaSR-mediated suppression of PTH release via $G_{q/11}$ has been linked to activation of phosphatidylinositol-specific PLC (PI-PLC) resulting in the release of IP_3 and DAG with consequent elevation of Ca^{2+}_i and activation of PKC (Corbetta *et al.*, 2002). PKC stimulates ERK1/2 phosphorylation and inhibition of either PKC or ERK1/2 signalling abolished the inhibitory effect of Ca^{2+}_o on PTH release (Corbetta *et al.*, 2002). PKC additionally activates PLD and PLA_2 (Kifor *et al.*, 1997). CaSR-mediated PI 3-kinase activity also appears to contribute to the inhibitory control of PTH secretion (Corbetta *et al.*, 2002). Although it seems paradoxical that CaSR-mediated activation of pathways that lead to increased Ca^{2+}_i should suppress PTH secretion rather than activate it, as observed for other hormones that are released via exocytosis (Gustavsson *et al.*, 2012), it is possible that $G_{q/11}$ contributes to CaSR-mediated suppression of intracellular cAMP levels via Ca^{2+}_i-inihibited isoforms of AC (5, 6 or 9) as described for CaSR-expressing HEK-293 cells (Gerbino *et al.*, 2005) and renin-secreting renal juxtaglomerular cells (Atchison and Beierwaltes, 2013).

Both $G_{i/o}$ and $G_{q/11}$ have been implicated in the control of gene expression that modulates PTH production (Thiel *et al.*, 2012; Avlani *et al.*, 2013) and release of Ca^{2+}_i has been linked to changes in PTH gene transcription (Ritter *et al.*, 2008).

CaSR-mediated release of calcitonin from thyroid parafollicular C-cells has also been linked to PLC-dependent signalling and appears to occur independently of the activation

of ERK1/2 signalling and suppression of cAMP synthesis (Thomsen *et al.*, 2012b). However, the signalling mechanisms that couple CaSR activation to calcitonin release appear to be different in various cell models pointing to the need for a more robust human C-cell model. Thus, in sheep C-cells in primary culture, the CaSR was reported to signal via a pathway dependent on Gβγ-mediated stimulation of PI 3-kinase followed by the activation of PKCζ and PLC (McGehee *et al.*, 1997; Liu *et al.*, 2003). However, in cultured rat 6–23 medullary thyroid carcinoma cells, PI 3-kinase was not required for Ca^{2+}_o-stimulated calcitonin secretion. Instead, it was driven by phosphatidylcholine-specific PLC (PC-PLC) as well as PI-PLC-dependent signalling pathways coupled to Ca^{2+}_i mobilization (Thomsen *et al.*, 2012b). Ca^{2+}_o-induced elevations in Ca^{2+}_i were mediated by L-type Ca^{2+} channels activated downstream of PC-PLC.

Interestingly, unlike Ca^{2+}_o, calcitonin secretion induced by the closely related alkaline earth metal Sr^{2+}_o was driven solely by PI-PLC, and Sr^{2+}_o-mediated elevations of Ca^{2+}_i did not require L-type Ca^{2+} channels (Thomsen *et al.*, 2012b). The observed differences in CaSR-mediated signalling and calcitonin release in response to Ca^{2+}_o versus Sr^{2+}_o point to the existence of distinct classes of divalent cation binding sites linked to the activation of discrete signalling mechanisms. In keeping with this notion, distinct Ca^{2+}_o binding sites have been described in the CaSR VFT domain (Huang *et al.*, 2009) as well as the HH domain (Ray and Northup, 2002; Mun *et al.*, 2004). In addition, a binding site for the tervalent cation Gd^{3+} has been identified at the dimeric interface of neighbouring VFT domains in the crystal structure of the rat type-1 metabotropic glutamate receptor, a CaSR homologue (Tsuchiya *et al.*, 2002).

Thus, CaSR-acting drugs that selectively promote $G_{q/11}$-dependent activation of PI-PLC and ERK1/2 and, perhaps, promote the inhibition of cAMP formation (Chen *et al.*, 1989) may suppress PTH secretion without disturbing calcitonin secretion. Since the development of cinacalcet and related calcimimetics, several classes of novel positive allosteric CaSR modulators have been identified (Harrington *et al.*, 2010; Kiefer *et al.*, 2010; Ma *et al.*, 2011; Deprez *et al.*, 2013). Of these compounds, calcimimetic B (Amgen, Thousand Oaks, CA, USA), when compared with NPS R568, has reduced potency for the promotion of calcitonin release relative to its ability to inhibit PTH secretion in rats. In addition, in human TT medullary thyroid carcinoma C-cells, calcimimetic B exhibits reduced ability to stimulate calcitonin secretion when compared with the first generation phenylalkylamine, NPS R568 (Henley *et al.*, 2011). If these differences in selectivity are retained *in vivo* in treated patients, calcimimetic B or a related compound with reduced propensity to stimulate calcitonin release might reduce the frequency and/or severity of hypocalcaemic episodes.

Biased drugs that correct defective signalling engendered by naturally occurring mutations

Although CaSR mutations have traditionally been classified as 'loss-' or 'gain-of-function', their effects are not transmitted equally across all pathways. Some CaSR mutations differentially alter receptor signalling, resulting in a change in coupling preference (Leach *et al.*, 2012). Thus, naturally occurring mutations engender CaSR signalling bias. This has implications for the treatment of patients harbouring such mutations. For instance, when overexpressed in HEK293 cells, the wild-type (WT) CaSR couples preferentially to Ca^{2+}_i mobilization over ERK1/2 phosphorylation, highlighted by a higher Ca^{2+}_o potency for Ca^{2+}_i mobilization (Davey *et al.*, 2012; Leach *et al.*, 2012). In addition, we identified three naturally occurring mutations in which this coupling preference was lost, that is, both pathways were equally sensitive to Ca^{2+}_o (Leach *et al.*, 2012) (Figure 3). Thus, a drug that preferentially enhanced CaSR signalling via Ca^{2+}_i mobilization could be used to restore the natural signalling bias.

Type-II calcimimetics (positive modulators), including cinacalcet and NPS R568 (Nemeth *et al.*, 1998), and the calcilytic, NPS 2143 (Nemeth, 2002), also engender biased signalling at the CaSR. These small-molecule drugs manifest greater allosteric modulation of Ca^{2+}_i mobilization relative to ERK1/2 phosphorylation and possess higher potency and estimated affinity for receptor states that mediate plasma membrane ruffling relative to either of the other two pathways investigated (Davey *et al.*, 2012; Leach *et al.*, 2013). Thus, for mutants whose coupling to Ca^{2+}_i mobilization is compromised, cinacalcet corrects their signalling bias towards the coupling preference of the WT receptor.

Interestingly, some naturally occurring mutations of the CaSR alter the stimulus bias engendered by allosteric modulators. Positive allosteric modulation of the CaSR mutant $R^{680}C$ by cinacalcet, for example, is approximately equal in both Ca^{2+}_i mobilization and ERK1/2 phosphorylation assays. In the case of the CaSR mutant, $V^{817}I$, however, cinacalcet exhibits a bias towards Ca^{2+}_i mobilization that is greater than that observed for the WT CaSR (Leach *et al.*, 2013).

Compared with WT, where NPS 2143 negatively modulates the potency of Ca^{2+}_o approximately 1.3-fold more in Ca^{2+}_i mobilization assays than in ERK1/2 phosphorylation assays, negative modulation by NPS 2143 of the mutant $E^{767}K$ was approximately fourfold greater for Ca^{2+}_i mobilization. However, in the case of $V^{836}L$, little difference was observed between the modulation of either pathway (Leach *et al.*, 2013). These changes can be visualized on a 'bias plot' that depicts the relationship between changes in agonist potency in two dimensions (corresponding to two distinct receptor-dependent signalling pathways) in the presence of equimolar concentrations of modulator (Figure 4). For instance, the bias plots shown in Figure 4 depict the change in Ca^{2+}_o potency in the presence of equimolar concentrations of allosteric modulator measured in Ca^{2+}_i mobilization assays (y axis) and ERK1/2 phosphorylation assays (x axis). If the allosteric modulator exerts greater cooperativity on one pathway, the points fall on the corresponding side of the line of identity.

An understanding of how naturally occurring mutations alter the pharmacoregulation of the CaSR protein may permit predictions of the clinical responses to specific drug treatments in different tissues. Consistent with this idea, a small study in patients with secondary hyperparathyroidism in the context of end-stage renal failure reported that cinacalcet-mediated suppression of PTH was markedly enhanced in a patient homozygous for the polymorphism $R^{990}G$ (Rothe *et al.*, 2005).

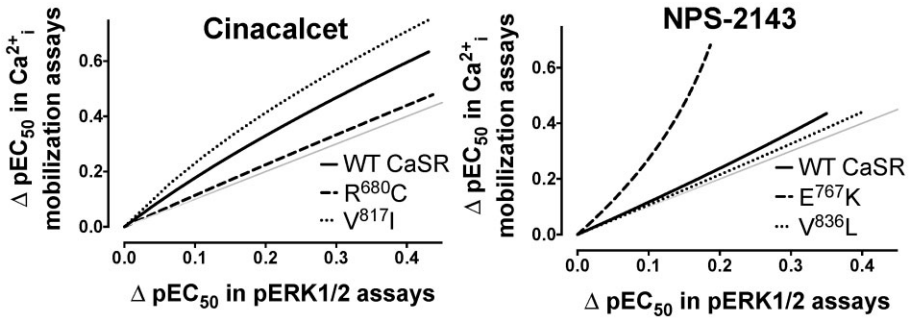

Figure 4

Allosteric stimulus bias is altered by naturally occurring CaSR mutations. Cooperativity bias plots highlight that allosteric compounds show bias in their modulation of Ca^{2+}_i mobilization and ERK1/2 phosphorylation, but that certain mutations alter this bias. The pEC_{50} of Ca^{2+}_o in the absence and presence of modulator in Ca^{2+}_i mobilization and ERK1/2 phosphorylation assays was first fitted to an allosteric ternary complex model and 150 XY coordinates of points that defined the curve that best fit the equation were determined. Next, the XY coordinates for the two pathways were plotted against one another, with Ca^{2+}_i mobilization data on the y axis against ERK1/2 phosphorylation data on the x axis. These plots thus represent the change (Δ) in Ca^{2+}_o pEC_{50} in the presence of equimolar concentrations of allosteric modulator across the two pathways.

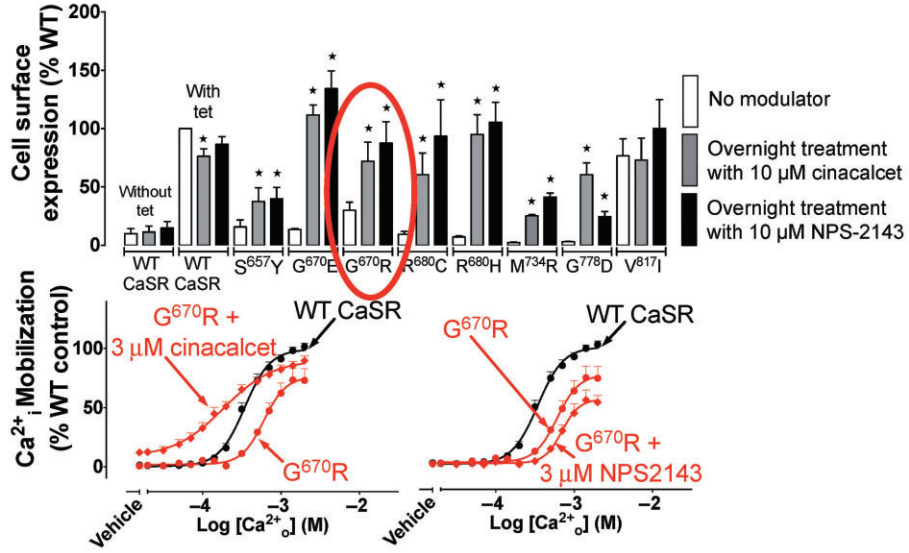

Figure 5

Stimulus bias engendered by NPS 2143. Both cinacalcet and NPS 2143 modulate trafficking of loss-of-expression mutant CaSRs to the cell surface, but whereas cinacalcet is a positive modulator of CaSR signalling, NPS 2143 is a negative allosteric modulator of signalling. For mutants such as $G^{670}R$, positive modulation of trafficking and signalling may lead to overstimulation of the receptor, whereas NPS 2143 may be more appropriate for balancing expression versus signalling. *Significantly different from value obtained in the absence of modulator (no modulator), one-way ANOVA.

Drugs that bias trafficking versus signalling

As noted earlier, various CaSR mutations reduce cell surface expression (Leach *et al.*, 2012), and the expression of some of these mutants is rescued by cinacalcet and related calcimimetics (White *et al.*, 2009; Leach *et al.*, 2013). However, due to the positive modulation of CaSR signalling, CaSR activity is also significantly enhanced in response to calcimimetics and can readily exceed that of the WT CaSR (Figure 5). Therefore, drug-induced increases in the cell surface expression and signalling of mutant receptors may drive a gain-of-function phenotype with enhanced sensitivity to Ca^{2+}_o and attendant hypocalcaemia. An ideal drug in this instance would positively modulate cell surface expression of the mutant receptor but have neutral cooperativity on signalling responses. The CaSR is localized to both the cell membrane and to intracellular compartments in recombinant and native CaSR-expressing cells (Rodriguez *et al.*, 2005; Pidasheva *et al.*, 2006; Bonomini *et al.*, 2012). CaSR agonists and positive allosteric modulators promote the forward trafficking and attendant

glycosylation of intracellular receptors to the plasma membrane (McCormick *et al.*, 2010; Grant *et al.*, 2011), a phenomenon that has been termed agonist-driven insertional signalling (ADIS). This is directly linked to membrane-localized receptor signalling events, as evidenced by attenuation of ADIS by inactivating CaSR mutations (Grant *et al.*, 2011; 2012). Strikingly, however, the small-molecule negative modulator (calcilytic) NPS 2143 also rescued cell surface expression of loss-of-expression mutants in HEK293 cells (Leach *et al.*, 2013). This is a remarkable example of biased allosteric modulation, with a complete reversal in cooperativity (positive vs. negative) between pathways (trafficking vs. acute signalling). Thus, NPS 2143 negatively modulates CaSR-mediated signalling and positively modulates receptor trafficking to the cell surface.

The use of allosteric modulators of the CaSR that bias receptor trafficking to the exclusion of signalling may be extended to disease states in which aberrant CaSR expression contributes to the pathogenesis. Thus, up-regulated CaSR expression upon exposure to calcimimetics may provide an effective approach to the prevention and/or treatment of colon cancer.

However, positive modulation of CaSR signalling in other cell types in the gastrointestinal tract may induce unwanted side effects, for example, arising from enhanced release of gut hormones including gastrin and CCK (Buchan *et al.*, 2001; Liou *et al.*, 2011; Wang *et al.*, 2011). For example, oral administration of the calcimimetic KRN568 (NPS R568) caused gastrin release in human subjects (Igarashi *et al.*, 2000). Interestingly, Ca^{2+}_o-induced gastrin secretion from human G-cells occurs independently of cAMP inhibition, ERK1/2 or PLA_2 signalling (Buchan *et al.*, 2001), so that a CaSR-acting agent that promotes CaSR trafficking and selectively modulates signalling towards ERK1/2 may be effective in the treatment of colon cancer and exhibit a favourable side effect profile. Alternatively, a positive modulator of trafficking with neutral effects on receptor signalling that possesses a rapid receptor off rate might be ideal for mediating the cell surface chaperoning effect while minimizing interference with receptor activation once at the plasma membrane.

Some proof of concept for targeting CaSR expression has been provided by treatment of nephrectomized rats (an animal model of secondary hyperparathyroidism) with the novel calcimimetic, AMG641, which up-regulates parathyroid CaSR protein and mRNA expression and inhibits parathyroid gland hyperplasia (Mendoza *et al.*, 2009). Furthermore, attenuation of CaSR expression has been linked to calcification of vascular smooth muscle cells (Alam *et al.*, 2009) and expression is increased in the aortic intima of uraemic rats treated with the calcimimetic, NPS-R568. These rats show diminished calcification and reduced proliferation of vascular smooth muscle and endothelial cells in comparison with vehicle-treated controls (Koleganova *et al.*, 2009). These examples provide direct evidence that up-regulation of CaSR expression may be effective in treating certain disease states.

Summary

The CaSR is expressed widely in tissues throughout the body where it serves a multitude of functions through selective activation of distinct signalling pathways in response to diverse ligands. We now know that endogenous orthosteric and allosteric ligands as well as synthetic small-molecule allosteric drugs engender significant stimulus bias and provide the theoretical basis for the development of novel, highly selective therapeutics. Thus, drugs that activate a discrete 'signature' subset of receptor-mediated signalling pathways may be useful in the treatment of disorders such as osteoporosis, in which the selective recruitment and/or activation of osteoblasts would be beneficial. However, drugs that selectively promote receptor expression without disturbing the balance of downstream signalling pathways may be beneficial in disease states arising from defective CaSR expression (e.g. primary and secondary hyperparathyroidism, colon cancer and some forms of cardiovascular disease). Profiling of candidate calcimimetics, calcilytics and neutral modulators that up- or down-regulate CaSR expression across multiple signalling pathways will identify drugs that selectively promote CaSR trafficking and/or signalling and may provide new therapeutic options for various disorders.

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Conflict of interest

KL, PMS and ADC have nothing to declare. AC has previously published work on the CaSR in collaboration with researchers from Amgen.

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