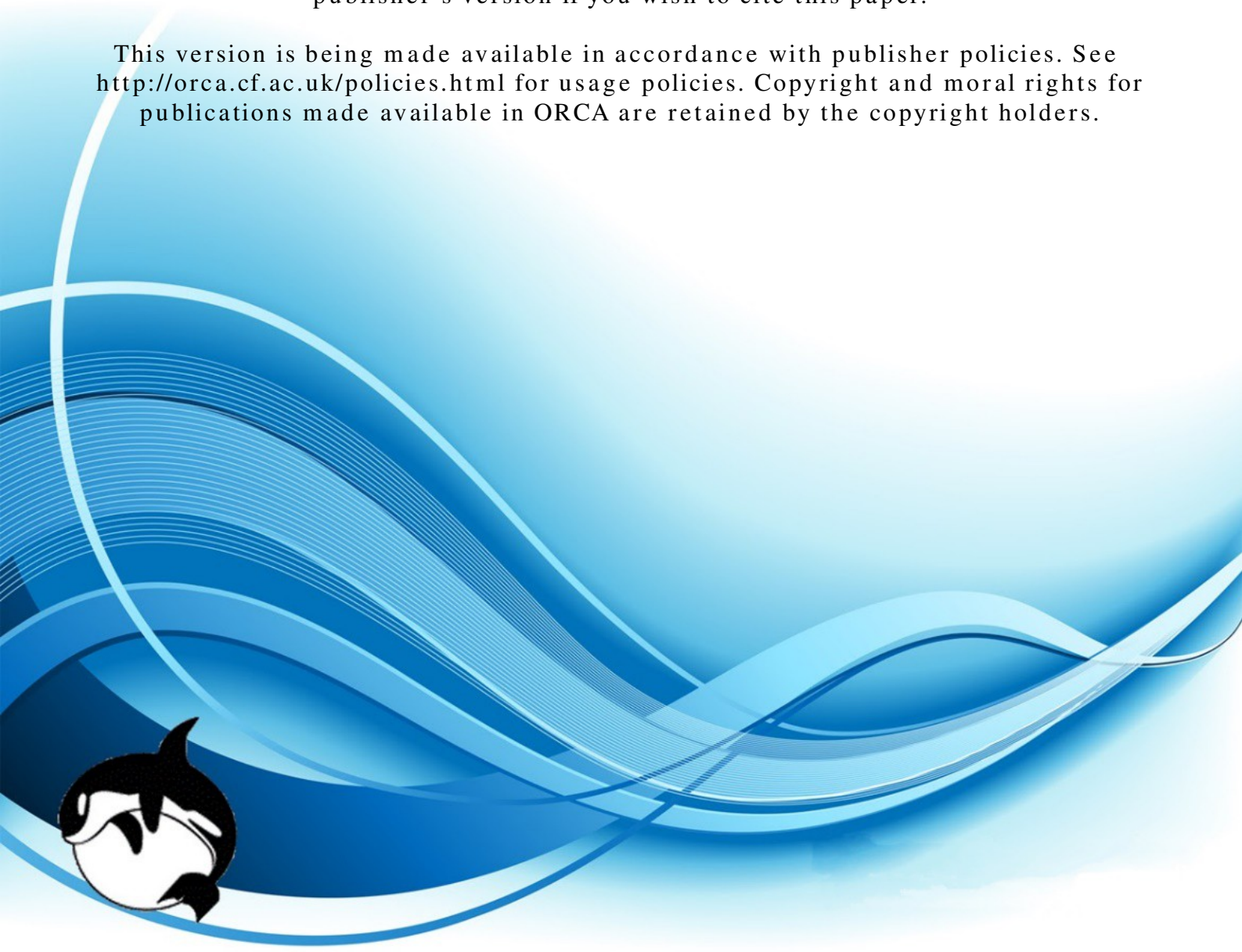

The calcium-sensing receptor: just one-of-a-kind

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New Findings

What is the topic of this review?

The extracellular calcium-sensing receptor, CaSR, ensures whole body Ca^{2+} homeostasis. Recent developments highlight the importance of the CaSR beyond mineral ion metabolism. This review will focus on novel roles and the use of CaSR-based therapeutics within the vasculature, the gut and the lung.

What advances does it highlight?

The ability of the CaSR to act as a multimodal chemosensor has led to the identification of CaSR signalling pathways that are both ligand- and cellular context-dependent. Development of cell-specific CaSR modulators is now being harnessed to rescue aberrant CaSR expression or function beyond impaired mineral ion metabolism.
outside the extracellular Ca^{2+} homeostatic system.

Abstract
The extracellular calcium-sensing receptor, CaSR, is the first G protein-coupled receptor that has an inorganic ion, calcium (Ca^{2+}), as its physiological agonist. It is highly expressed in all organs involved in the regulation of mineral ion metabolism, namely the parathyroid, the kidney and the bone. The CaSR the master controller of extracellular Ca^{2+} concentration, as highlighted by the evidence that both inherited and acquired mutations in the *CASR* gene cause disturbances in mineral ion metabolism. CaSR positive allosteric modulators have been successfully employed in the clinic for over a decade to restore CaSR function, which is reduced in hyperparathyroidism secondary to kidney failure while negative allosteric modulators are currently being tested in patients with hypocalcemia with hypercalciuria due to gain-of-function CaSR mutations. In addition to its expression within the bone-kidney-parathyroid axis, the CaSR can be found in other tissues, although not limited to, the gut, the vasculature and the lung. Here, the CaSR acts as a chemosensor, integrating signals deriving from nutrient availability, salinity, acidification and the presence of ubiquitous polyamines. Knowledge of what these stimuli are, and of the cell-specific signalling responses they evoke, is crucial to our understanding of the non-calciotropic roles of the CaSR in physiology, and how these are affected in disease states.

List of abbreviations

Ca^{2+}	Free ionised calcium
Ca^{2+}_o	Free ionised extracellular calcium
Ca^{2+}_i	Free ionised intracellular calcium
CaSR	Extracellular calcium-sensing receptor
FHH1-3	Familial hypocalciuric hypercalcemia variant 1-3
GPCR	G protein-coupled receptor
PTH	Parathyroid hormone

Unique features of the CaSR

The CaSR is the first guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) to have been identified that has an ion, Ca^{2+}, as its physiological ligand (Brown *et al.*, 1993). It belongs to family C of GPCRs, with whom it shares a large amino-terminal nutrient-binding domain shaped like a Venus flytrap (Khan & Conigrave, 2010). However, several unique features set the CaSR aside from other GPCRs. The main role of the CaSR is to monitor extracellular free ionised Ca^{2+} (Ca^{2+}_o), allowing very little fluctuation either side of the narrow physiological range (1.1-1.3 mM)(Brown, 1991). As such, the CaSR is constantly exposed to half-maximally activating Ca^{2+}_o concentrations. Therefore, unlike many other GPCRs which are transiently exposed to their agonists, after which they are rapidly inactivated, the CaSR appears to be refractory to desensitization (Breitwieser, 2013). Recent studies suggest that this is due to a phenomenon known as agonist-driven insertional signalling whereby CaSR signalling directly drives biosynthesis and insertion of new receptor molecules while endocytosis remains constitutively active (Grant *et al.*, 2011). In addition, the relatively hypercalcaemic environment (~1.6-1.7 mM in late gestational stages compared to the adult) which is present in the prenatal developing lung and peripheral nervous system, and where the CaSR is also expressed (Finney *et al.*, 2008; Vizard *et al.*, 2008), would lead to constitutive CaSR activation. Here, receptor expression is developmentally regulated, suggesting that in the fetus, CaSR-mediated events are largely achieved by controlling receptor expression levels, rather than by changes in agonist concentrations.

In vast majority of the circumstances the CaSR couples preferentially to $G_{q/11}$, G_i, and $G_{12/13}$ subunits of the G proteins, leading to an increase in intracellular Ca^{2+} (Ca^{2+}_i) concentration, released from inositol 1,4,5 trisphosphate-sensitive stores. CaSR molecule density is known to regulate its own signalling and, potentially, also that of other Gq-coupled GPCRs (Brennan & Conigrave, 2009; Brennan *et al.*, 2015). In addition, the type of ligand, along with the duration and the intensity of the signal, can promote activation of differential intracellular pathways, leading to agonist-dependent biological responses, through the formation of signalling scaffolding complexes, a process called "ligand-directed targeting of receptor stimulus" (Conigrave & Ward, 2013). It has been postulated that an alteration in the CaSR-specific signalling machinery underpins certain types of malignancies, as it appears to be the case in the mammary glands, where CaSR in normal epithelial cells is preferentially coupled to $G\alpha_{i/o}$ while it switches to $G\alpha_s$ (with attendant increases in intracellular cyclic adenosine monophosphate levels) in breast cancer cells (Mamillapalli *et al.*, 2008).

CaSR , Ca^{2+}_o homeostasis and CaSR-based therapeutics

The CaSR is encoded by a single copy gene and its transcription is regulated by both vitamin D and by certain pro-inflammatory cytokines (Hendy *et al.*, 2013). Genetic disorders of mineral ion metabolism arise as a consequence of CaSR mutations or, as recently demonstrated, in its associated proteins. Familial hypocalciuric hypercalcemia, FHH, is due to inactivating mutations in the *CASR* gene (FHH1), in one of its G protein partners $G\alpha_{11}$ (*GNA11*, FHH2) or in adaptor-related protein complex 2, sigma 1 subunit (FHH3), which is involved in receptor endocytosis. Gain-of-function mutations in the *CASR* or *GNA11* genes lead to

autosomal dominant hypocalcemia with hypercalciuria type 1 and 2, respectively (Nesbit *et al.*, 2013).

Consistent with the direct involvement of the CaSR in mineral ion homeostasis, receptor mRNA and protein are found in the parathyroid, kidney and bone where tissue-specific gene ablation studies show increased parathyroid gland mass and hyperparathyroidism (Chang *et al.*, 2008), defective calcium reabsorption by the kidney thick ascending limb (Toka *et al.*, 2012) and impaired skeletal development and demineralization (Chang *et al.*, 2008).

Acquired disorders of mineral ion metabolism also arise as a consequence of abnormal CaSR expression. Under normal conditions, the CaSR suppresses PTH release in the parathyroid glands. However, in chronic kidney disease there is a progressively exaggerated secretion of parathyroid hormone, driven by an elevation in circulating levels of Pi, due to the inability of the failing kidney to eliminate its excess (Hruska & Mathew, 2011). Thus, the CaSR became a drug target even before its molecular identification (Nemeth *et al.*, 1996). The low affinity of the Ca^{2+} binding sites on the receptor meant that the discovery of traditional orthosteric activators did not yield successful results. However, an entirely novel approach led to the identification of positive allosteric modulators, termed calcimimetics, which have been on the market since 2004 for the treatment of hyperparathyroidism secondary to advanced chronic kidney disease (Block *et al.*, 2004). The key advantages of allosteric modulators over traditional orthosteric agonists are that they sensitise the CaSR to its physiological ligand, Ca^{2+}_o, within its physiological range (Christopoulos & Kenakin, 2002). In addition, owing to the ability to evoke endogenous pulses of plasma PTH (a known bone anabolic stimulus), negative allosteric CaSR modulators, termed calcilytics, were initially developed for the treatment of age-related osteoporosis. Although preclinical studies showed promising results, their development was halted due to lack of efficacy in clinical settings (Caltabiano *et al.*, 2013). More recently, oral calcilytics are being repurposed for the treatment of autosomal dominant hypocalcemia with hypercalciuria due to activating CaSR mutations (Nemeth & Shoback, 2013).

<u>CaSR outside the Ca^{2+}_o homeostatic system</u>

Following the identification of the CaSR in the bone-kidney-parathyroid axis, the receptor was also found, albeit to a much lesser extent, in many other tissues, which are outside the Ca^{2+}_o homeostatic system, such as the intestine, the vasculature and the lung. While it has long been unclear what the role of the CaSR might be in these non-calciotropic tissues, it is well accepted that cations other than Ca^{2+}, as well as amino acids, can activate the receptor (Brown & MacLeod, 2001) and could, too, activate the CaSR in these contexts. For instance, the CaSR is expressed all along the gastrointestinal tract where accumulating evidence indicates that the CaSR senses dietary nutrients and acts as a taste receptor for Ca^{2+} and proteins within taste cells while contributing to hormone release in the intestine (Brennan *et al.*, 2014). CaSR also modulates fluid and electrolyte movement across the mammalian colon (Geibel & Hebert, 2009). Furthermore, it has long been known from epidemiology studies that dietary calcium intake reduces the incidence of colon cancer (Garland *et al.*, 1985) and hypertension (Hatton & McCarron, 1994) , but whether the CaSR played any role in mediating these effects was unknown. In the colon, the CaSR regulates the

switch between proliferation and differentiation, with greatest expression levels at the apex of the crypts. CaSR expression levels correlate with the differentiation status of colonic tumours with receptor protein being almost completely absent in undifferentiated tumours (Chakrabarty *et al.*, 2003). Thus, the CaSR in the colon acts as a tumour suppressor gene, an effect which is potentiated by the active vitamin D metabolite, calcitriol (Aggarwal *et al.*, 2015). Because the CaSR transcription is upregulated by vitamin D, a Ca^{2+}-containing diet could be protective against colon cancer partly via upregulation of CaSR expression.

The CaSR is expressed in the vasculature, in the smooth muscle, the endothelium and the perivascular nerve (Smajilovic *et al.*, 2011). Increase dietary calcium intake leads to a reduction in blood pressure (Hatton & McCarron, 1994) and studies suggest that, in isolated aortic segments, Ca^{2+}_o induces to nitric oxide—dependent relaxation, which was blocked by calcilytic treatment (Loot *et al.*, 2013). Thus, dietary calcium intake could elicit a hypotensive effect by acting via the endothelial CaSR. Intriguingly, mice with targeted CaSR ablation from vascular smooth muscle cells exhibit hypotension due to impaired vascular resistance (Schepelmann *et al.*, 2013). Together, these observations suggest a dual role for the vascular CaSR, with the receptor having pro-relaxing effects in the endothelium (Lopez-Fernandez et al, unpublished observations) and pro-contractile actions in the vascular smooth muscle (Schepelmann *et al.*, 2013). While in physiological conditions these two actions are balanced, given also the multimodal actions of the receptor one can envisage pathological conditions that might selectively affect one receptor over the other and therefore lead to altered blood pressure control. Furthermore, CaSR expression is lost during vascular calcification (Alam *et al.*, 2009), which often occurs as a consequence of chronic kidney disease and results in severely increased risks of cardiovascular morbidity and mortality (London *et al.*, 2005). Calcimimetics have been used in the clinic since 2004 to rectify the hyperparathyroidism secondary to kidney failure and recent studies suggest that, in elderly haemodialysis patients, they reduce the risk of cardiovascular events, when compared to placebo-controlled patients (Parfrey *et al.*, 2015). *In vitro* studies show that calcimimetic treatment reduces vascular calcification (Alam *et al.*, 2009) and increases CaSR expression levels in human primary vascular smooth muscle cells (Henaut *et al.*, 2014). Furthermore, mouse vascular smooth muscle cells lacking the CaSR exhibit greater calcification *in vitro* than do cells from wild-type animals (Schepelmann *et al.*, 2013). Thus, some of the beneficial effects of calcimimetics in patients with advanced chronic kidney disease could be directly ascribed to the ability of calcimimetics to restore CaSR expression levels in the vasculature.

CaSR expression is also found in the human and mouse airways, both epithelium and smooth muscle (Yarova *et al.*, 2015). It had long been known that serum and sputum of asthmatic subjects contains many cationic proteins, particularly eosinophil cationic proteins, major basic proteins and polyamines (Kurosawa *et al.*, 1992), whose expression levels correlate with disease severity. While their mechanism of action was unknown, exposure to polycations is associated with increases in Ca^{2+}_i concentration in airway smooth muscle cells, and mimicking this effect by knocking down the sarco/endoplasmic reticulum Ca^{2+} ATPase

expression recapitulates many of the phenotypic hallmarks of asthma (Mahn *et al.*, 2009). Recently we have shown that CaSR expression is increased in asthmatic patients and in mouse models of allergic asthma, compared to their non-asthmatic counterparts, and that CaSR activation by Ca^{2+}_o or polycations leads to airway hyperresponsiveness and bronchoconstriction, an effect which could not be seen in mice with CaSR ablation from the airway smooth muscle. Polycations also induced airway hyperresponsiveness, bronchoconstriction and inflammation in murine asthma models *in vivo*. Excitingly, all of these effects could be prevented by nebulised calcilytics (Yarova *et al.*, 2015). Together, these results suggest that the airway CaSR represents a novel target for asthma treatment in humans.

Therapeutic applications of CaSR allosteric modulators

The first calcimimetic developed, Cinacalcet, is in clinical use for the treatment of secondary hyperparathyroidism in dialysis patients and in certain forms of primary hyperparathyroidism. Calcilytics, initially developed as an anti-osteoporosis treatment, are now being repurposed for the treatment of certain hypocalcaemic and hypercalciuric disorders (Nemeth & Shoback, 2013). However, given the widespread distribution of the CaSR throughout the body and its potential implications in many physiopathological conditions, the use of CaSR allosteric modulators has also been proposed for other disorders where the CaSR is potentially involved.

For instance, in the brain amyloid β peptides induce a surge in cytosolic Ca^{2+}, which has been suggested to be at the basis of the neurotoxicity during Alzheimer's disease (Brorson *et al.*, 1995). Since amyloid β peptides can activate the CaSR *in vitro* CaSR (Ye *et al.*, 1997), recent studies have suggested the potential therapeutic application of calcilytics in delaying disease progression (Armato *et al.*, 2013). Furthermore, in the gut, CaSR modulators have been proposed for the treatment of secretory diarrhoea, metabolic acidosis and colon cancer. Finally, calcilytics could provide a novel therapeutic avenue for the treatment of asthma and potentially other inflammatory lung disorders. It is unlikely that a systemic route of administration of CaSR modulators is a viable option to target CaSR functions in these tissues because of off-target effects on the parathyroid and kidney CaSR. However, the local delivery of these drugs, together with ligand bias signalling properties of the receptor, could potentially achieve the specificity and selectivity required to target CaSR functions in non-calciotropic tissues.

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