Calcium sensing receptor signaling: it's all about multiplicity

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Abstract

The Calcium Sensing Receptor (CaSR), a G-protein-coupled receptor mainly known for its role in the homeostatic regulation of Ca^{2+} levels in the extracellular fluid, is also expressed in a multiplicity of tissues where it regulates a variety of physiological and pathological processes.

The main features of CaSR are its capacity to activate multiple downstream signaling pathways and its ability to itself be activated by a variety of ligands.

Recent data have demonstrated that these features are actually connected by the concept of biased signaling.

The recent availability of crystal structures of CaSR extracellular domain, and the functional characterization of clinically-relevant mutations, have catalyzed a great step forward in the field of CaSR signaling.

In the past two years, CaSR signaling characteristics have been shown to be even more complicated than expected: heterodimerization, phosphate sensing, and compartment bias are only a fraction of the exciting developments.

This review will focus on some of these topics, and on the debated case of CaSR signaling in cardiomyocytes.

Introduction

One of the most versatile messenger is certainly calcium, but how can a single messenger modulate so many different aspects of cell physiology and pathology simultaneously, thus influencing life and death mechanisms in human cells at the same time? How can calcium dynamics act to trigger fast events such as muscle contraction and exocytosis, and also long-term tasks, such as gene expression? How can calcium convey contrary physiological messages, such as apoptosis and proliferation? We now know that it depends not only on the cell-specific toolkit of calcium sensing and handling proteins they each contain, but also on the kinetics and localization of intracellular calcium events within those cells – down to the (sub)microscopic level [1].

Intracellular calcium microdomains are among the most prominent aspects of this story [2] [3]. Thanks to the coordinated action of calcium channels, transporters, pumps, cytosolic and intraluminal calcium-binding proteins, and whole organelles (ER, Golgi apparatus, endocytotic and exocytotic vesicles, lysosomes), these high-calcium sub-domains are dynamically created within the cell, and induce spatially- and temporally-defined activation of protein signaling cascades [4].

While this part of calcium story happens on the inside of eukaryotic cells, calcium ions also play a fundamental role from the outside. Extracellular free calcium concentration is strictly maintained in the range of 1.1-1.3 mM by the coordinated action of hormonal signaling, parathyroid glands, kidneys, bones and intestines [5]. Despite this global homeostasis, Ca^{2+} levels can substantially fluctuate within the small intercalatory diffusion spaces of a tissue - in the so called **extracellular microdomains,** that mirror the intracellular ones [6] [7] [8]. Here Ca^{2+} changes its identity from second to **first messenger**: Ca^{2+} can in fact directly activate a number of plasma membrane proteins, generically named **extracellular calcium sensors** [9].

Physiological role of CaSR signaling

The existence of a sensor able to monitor extracellular Ca^{2+} in the parathyroid gland was hypothesized in 1966 [10] [11] and finally confirmed in 1993, when Brown and colleagues cloned an "**extracellular Calcium-sensing receptor**"(CaSR), from bovine parathyroid gland [12]. Since then it has become clear that the main physiological role of CaSR is to sense serum Ca^{2+} levels in parathyroid glands and, when it increases above 1.4 mM, to inhibit PTH secretion and increase urinary Ca^{2+} excretion to maintain bodily homeostasis. Reduced PTH levels induce a reduction in both kidney Ca^{2+} reabsorption and Ca^{2+} release from bone. The CaSR also controls calcitonin secretion from thyroidal C-cells [5], while decreasing intestine Ca^{2+} reabsorption via 1,25-dihydroxyvitamin D. Combined, these effectors act to reduce serum Ca^{2+} back to physiological levels. Conversely, when serum Ca^{2+} levels fall, the resulting decreased CaSR activity permits increased PTH synthesis and secretion and the restoration of normal Ca^{2+} levels through the corresponding effects on the kidneys, bone and intestine [13].

Mutations of the CaSR gene, located on chromosome 3q21.1, are responsible for inherited disorders [14]. Familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT) are caused by loss-of-function mutations, while autosomally dominant hypocalcemia (ADH) and Bartter Syndrome type V are produced by gain-of-function mutations of the CaSR. Variant forms of FHH and ADH caused by germline mutations of genes encoding for CaSR-interacting proteins involved in transducing its downstream signaling ($G_{\alpha 11}$; FHH2 and ADH2) and trafficking (AP2σ; FHH3) have also been recently characterized [15] [16].

Besides its role in the above-mentioned calcitropic tissues directly involved in the control of systemic Ca^{2+} homeostasis, the CaSR has been found in an astonishing number of "non calcitropic" tissues - such as pancreas, brain, stomach, liver and heart, just to name a few. In each of these, CaSR has been found to be involved in a

variety of physiological and pathological processes - ranging from secretion to regulation of gene expression, proliferation, differentiation, migration, adhesion, apoptosis and cancer [17].

Molecular features of CaSR

The primary structure of the CaSR, which belongs to the class C (or 3) of the GPCR superfamily, consists of a large *N*-terminal extracellular domain (ECD) characterized by a bilobed, nutrient-binding Venus Flytrap (VFT), a nine cysteine-rich domain (CRD), a seven-transmembrane domain (TMD), and a carboxyterminal intracellular domain (ICD) **[18] [19]**.

Functional receptors localize at the plasma membrane mostly as disulphide homodimers, although heterodimers with other class C receptors have been described (mGluR [20] or $GABA_B$ [21] [22]). A very interesting pathological implication of CaSR heterodimerization has recently been revealed [22]. In particular, CaSR and GABA B heterocomplexes have been proven to be responsible for PTH hypersecretion in hyperparathyroidism. In their elegant work, the authors demonstrate that hyperplastic parathyroid glands of patients with primary and secondary hyperparathyroidism are endowed with an autocrine cycle in which locally-synthesized GABA B binds to CaSR and GABA B heterodimers. This binding blocks the function of CaSR homodimers, and thus induces tonic, detrimental PTH secretion [22].

A milestone in the field of CaSR research was reached by the attainment of the high-resolution crystal structure of the ECD. This result has led to fundamental insights on the mechanism of CaSR activation of the human homodimers **[18] [19]**.

In 2016 J Yang and collaborators reported the first crystal structure of the human ECD bound with Mg^{2+}, clarifying the mechanism for cooperative activation of CaSR by Ca^{2+} and Mg^{2+} ions. Importantly, they first described an additional, orthosteric, binding site for a tryptophan derivative that is so crucial in the stabilization and the activation of the dimer that this aminoacid has been suggested to act as a co-agonist at the CaSR **[18]**.

Interestingly, in a paper of the same year, Geng and colleagues **[19]** found three anion binding sites which were suggested to stabilize the open-inactive state. PO_4^{3-} and SO_4^{2-} were indicated as the most probable physiological ligands.

The physiological significance of such sites has been recently revealed by Ward and collaborators **[23]**. Specifically, one of these anion binding sites seems to have a fundamental role in parathyroid, where CaSR might work also as a "**phosphate sensor**". The results reported in this interesting study offer an explanation to the PO_4^{3-} and concentration-dependent increase in PTH secretion which has been observed since 1996 [24] - PO_4^{3-} acts at the CaSR as a non-competitive agonist. Importantly, this mechanism would also explain how pathophysiological levels of PO_4^{3-}, such as those found in chronic kidney disease (CKD), can over-stimulate PTH secretion and induce secondary hyperparathyroidism.

CaSR promiscuity: orthosteric agonists and allosteric modulators

One of the first signatures of CaSR, as also demonstrated by the above-cited binding sites for tryptophan and anions, is its' promiscuity, i.e. ability to bind a large number of different ligands.

While the Ca^{2+} ion certainly represents the main agonist, which binds to the CaSR with a high positive cooperativity [25], a number of **orthosteric agonists (or type I calcimimetics)** that can activate the receptor in the absence of other ligands, have been identified. Among these are inorganic di- and tri-valent cations, such as Mg^{2+} Sr^{2+}, Ba^{2+}, Gd^{3+}, Al^{3+} [26] [5]; organic polycations, such as polyamines (spermine and spermidine) [27]; some

aminoglycoside antibiotics (neomycin [28] and gentamycin [29]); basic polypeptides, polylysine, polyarginine [30] and amyloid β-peptides [31].

Allosteric modulators are ligands which bind to different sites from those of the orthosteric agonists and modify the receptor conformation, in turn increasing (type II **calcimimetics**, also defined as positive allosteric modulators:**PAMs**) or decreasing (**calcilytics**, also defined as Negative Allosteric Modulators: **NAMs**) the affinity and/or the signaling capacity of the orthosteric agonist [16].

Natural calcimimetics are L-amino acids, especially aromatics [32], some of which have been shown to act as CaSR co-agonists [18] and glutathione analogs [33]. Low extracellular pH [34] and high ionic strength [35] function as physiological calcilytics.

A number of molecules with calcimimetic or calcilytic properties have been synthesized [36]. Among the calcimimetics, there are phenylalkylamine derivatives NPS R-568, NPS R-467 and Cinacalcet [37], which is presently used in patients with end-stage renal disease. The more recent AC-265347, with a benzothiazole structure [38], seems to act more precisely on PTH and calcitonin release in rats [39] [38] via biased allosteric modulation of the CaSR activity [40].

A recent study from Leach and collaborators has clarified the function and binding of four NAMs which may explain their different abilities to inhibit CaSR signaling [41].

Relevant news come from the calcilytics: tested in clinical trials for treating osteoporosis [42], they failed to stimulate new bone formation. Nonetheless, it has been suggested that they might provide an ideal therapy for ADH1 and 2, certain form of hypoparathyroidism, and pulmonary disorders [43]. For a comprehensive review of calcilytics see [16].

Also relevant is the capacity of **CaSR modulators to act as pharmaco-chaperones**, that is to permeate cell membranes and reach a misfolded protein at its intracellular location, stabilize it, and rescue the receptor to the cell membrane surface [44] [45].

Both NPS-R-568 [46] [44] [47] and cinacalcet, [40] **[48]** have been shown to effectively rescue signaling of loss-of-function mutant CaSR proteins. Interestingly, the calcilytic NPS-2143, was able to positively modulate CaSR trafficking to the cell membrane, while negatively modulating CaSR signaling **[48]** [49].

Remarkably the pharmaco-chaperone action of both NPS R-568 [50] and NPS-2143 [46] [50] appears to be mutant-specific, and able to mediate signaling bias (see below).

CaSR pleiotropicity: the many signaling pathways activated by CaSR

Besides its promiscuity, CaSR is also known for its pleiotropicity, i.e. its ability to activate a wide array of intracellular signaling mechanisms [51] (Figure 1).

As predicted by older studies, and according to the recent structural work [18] [19], the signal transduction cascade is initiated when, after Ca^{2+} (or other agonist) binding, each monomer undergoes a rotation - bringing the two lobes of the VFT and the CRD in closer proximity [19]. The consequent conformational change of the TMD, in turn, determines the interaction with G-proteins, thus initiating signal transduction.

The CaSR is known to activate all the four **heterotrimeric G proteins**: **$G_{q/11}$, $G_{i/o}$, $G_{12/13}$ and G_s.**

The most frequent signaling cascades described for CaSR are certainly those which lead to IP_3 accumulation and Ca^{2+} elevation, cAMP inhibition and ERK1/2 phosphorylation which are among the most widely used functional readouts of CaSR activation.

$G_{q/11}$ activation induces **Ca^{2+} mobilization** from the intracellular stores via phospholipase C (PLC)/inositol-1,4,5-trisphosphate ($InsP_3$), and concomitant activation of different isoforms of protein kinase C (PKC) and ERK1/2 [51].

The **$G_{i/o}$** pathway by which **cAMP levels are reduced** [52] [53] [54] has also been involved in ERK1/2 **phosphorylation/activation** via beta gamma-mediated Ras and MAPK activation [55] or G protein-independent β-arrestin recruitment **[56]**.

The involvement of G_{q11} versus $G_{\alpha i/o}$ [57] [58] and the specific identity of downstream proteins involved in CaSR-mediated inhibitory control of PTH secretion from the parathyroids has been hotly debated [59].

While this review was being written, a paper from Onopiuk and colleagues has shed some light on this matter. This study indeed indicates the TRPC1 channel as a fundamental player in this pathway **[60].** It is worth noting that store-operated calcium entry is not involved in such a mechanism, thus excluding both STIM1 and Orai. In particular, the authors show that mice lacking the TRPC1 show a phenotype mimicking human FHH. Next, by straightforward *in vitro* and *ex vivo* approaches, they show that upon CaSR activation with high $[Ca^{2+}]_o$, the $G_{\alpha 11}$ subunit physically interacts and stabilizes TRPC1, causing a fast Ca^{2+} entry which, in turn, suppresses PTH secretion.

A number of CaSR-dependent **$G_{12/13}$**-mediated signaling pathways have also been described [51] and are involved in the appearance of CaSR-mediated intracellular Ca^{2+} oscillations ($G_{12/13}$ /Rho/filamin pathway) [61] or sustained Ca^{2+}signals (G_{12}/phosphatase PP2A-dependent dephosphorylation of CaSR) [62].

CaSR-induced increase of cAMP levels via **G_s** have been identified in human breast cancer [63] and pituitary cells [64] while a CaSR-dependent modulation of cAMP levels independently from G_s or G_i proteins seems to be present in a number of tissues via signaling crosstalk mechanisms [9].

A physiologically-relevant nitric oxide (NO) modulation by the CaSR has been described in the vasculature [65] [66] [67] [68] [69]. A schematic view of CaSR-mediated intracellular pathways is illustrated in Figure 1.

Structure-function relationship: new details on biased signaling at the CaSR

Another striking and quite logical characteristic of CaSR, given its promiscuity and pleiotropicity, is its capability to give rise to so-called **ligand-directed signaling, or "biased agonism"**. As for other GPCRs, CaSR ligands are able to stabilize different conformational states of the receptor, which preferentially direct its signaling towards a specific intracellular pathway [70].

Recently, Bräuner-Osborne's group **[56]** straightforwardly demonstrated, in HEK-293-CaSR cells, that high extracellular Ca^{2+} was biased toward cAMP inhibition and IP_3 accumulation, while spermine showed a significant bias toward ERK1/2 phosphorylation. Fundamental insights within this field come from the study of biased signaling in pathological states, such as germline CaSR mutations. One of the first such examples was found in the effect of an autoantibody characteristic of an acquired hypocalciuric hypocalcemia case, which increased the effects of external Ca^{2+} on G_q signaling while reduced the activation of the G_i-induced signaling pathway [71].

Subsequently, it was demonstrated that some **naturally occurring CaSR mutations have altered signaling bias** [72] [73]**.** By using a rigorous pharmacological approach Leach and collaborators demonstrated that germline mutations can alter both the expression and the preference of the CaSR for downstream signaling modes. By using Ca^{2+} mobilization and ERK1/2 phosphorylation as functional readouts, it was found that FHH1-causing mutations can determine a switch from a preferential coupling to calcium signaling - characteristic of WT CaSR - to a signaling mode in which calcium signaling and MAPK pathways are equally activated or a preferential MAPK signaling is used. Also ADH-1-associated CaSR mutation can alter signaling bias toward a stronger Ca^{2+} response [72]. Interestingly, it has been shown that also **allosteric modulators exhibit stimulus**

bias, inducing greater activation of intracellular Ca^{2+} mobilization relative to ERK1/2 phosphorylation, and a higher affinity of the modulators for the state of CaSR-mediating plasma membrane ruffling **[74]**.

A further step forward in the understanding of the mechanisms underlying signaling was undertaken in the last few years by the group of Thakker **[75]**. **Defined residues have been recognized as critical for CaSR activation and biased signaling.** With the aid of multiple functional assays directed to $G_{q/11}$ $G_{i/o}$ and G-protein-independent β-arrestin activation, homology modeling and site directed mutagenesis, Gorvin and colleagues identified the structural motif responsible for biased signaling of an ADH1-causing mutation. The authors clearly showed that the disruption of a salt-bridge between the transmembrane domain 3 and the extracellular loop 2 of the CaSR induces the CaSR to adopt a conformational state that facilitates the binding of β-arrestin, thus causing a signaling bias toward a β-arrestin-mediated MAPK cascade activation [75]. In addition, a number of mutations have been mapped and revealed to cluster in specific sites relevant for structural integrity. CaSR dimerization and ligand binding, and the corresponding alteration in signaling bias, have been evidenced [15].

By analyzing more than 300 FHH and ADH mutations, Gorvin and colleagues **[76]** identified five "disease switch" residues, to be added to the previously reported four [18] [77], which are the location of both FHH1 and ADH1 associated mutations. The functional studies in HEK-293 cells showed that these disease-switch residue mutations commonly exhibit signaling bias toward either Ca^{2+} or MAPK pathway activation. Structural analysis next demonstrated that these residues are located at sites that appear relevant for the transition of the CaSR from the inactive to the active conformation, such as the extracellular dimer interface and transmembrane domain.

Trafficking/signaling modes of the CaSR

Dimers of glycosylated CaSR proteins are formed intracellularly [78] and stabilized by ligand binding at the cell surface [79]. The net level of dimers expression at the plasma membrane is relevant to both physiology and pathology, since it influences the strength of signaling **[80]** and depends on the dynamic equilibrium between maturation, trafficking from the ER membrane to the Golgi and thence to the plasma membrane, internalization, recycling, and degradation [81]. It's worth noting that allosteric modulators can modulate CaSR trafficking to the cell membrane, acting as pharmaco-chaperones [48].

A peculiar mode of signaling of the CaSR is the so called **Agonist-Driven Insertional Signaling (ADIS) [82]**, which seeks to explain two peculiar features of the CaSR: a minimal functional desensitization and a significant pool of CaSR proteins in intracellular membrane compartments. ADIS regulates the level of CaSR expression at the plasma membrane on the basis of the dynamic equilibrium between the rate of trafficking from a large pool located at the Golgi vesicles and the process of clathrin-mediated endocytosis and retrograde trafficking of cell-surface CaSR receptors.

A number of CaSR-interacting proteins have been shown to play a fundamental role in the complex scenario of CaSR trafficking [83]. A significant part is played by the **sigma subunit of the chlatrin-binding protein AP2 (AP2σ)**, involved in internalization of clathrin-coated vesicles containing the CaSR that fuse with endosomes. Mutations of AP2σ have in fact been linked to a novel form of FHH, named FHH3 [84]. The study of germline mutations of AP2σ has added a new level of complexity to the multifaceted signaling capacity of the CaSR: like other GPCRs [85] CaSR has also been shown to produce **sustained signals after internalization of ligand–receptor complexes in endosomes.**

In fact, a recent work by Gorvin and colleagues **[86]** has elegantly demonstrated that AP2s mutations increasing plasma membrane expression level of CaSR, while reducing its signaling - cause the disruption of a previously-unrecognized **endosomal signaling mode**. The impaired internalization of CaSR by chlatrin-mediated

endocytosis of the analyzed germline mutation produces a "compartmental biased" signaling, comprising a sustained Gq mediated signal most probably located at the endosomes **[86]**. It thus appears that the CaSR has yet another way of signaling: **besides the (ADIS-regulated and biased) "fast" plasma membrane signaling CaSR possesses a "sustained" G_q-specific endosomal pathway.**

The CaSR in cardiomyocyte physiology and pathology: bad guy or good guy?

While the role of CaSR in diseases related to systemic calcium homeostasis has been widely investigated, CaSR role in non-calcitropic tissues has been and remains a matter of debate. In fact, the role of CaSR seems to oscillate between the "good" and the "bad" in, for example, the **cancer field**. On the one hand, it is widely accepted that an aberrant expression or function of CaSR contributes to the pathogenesis of cancer in various tissues (e.g. pancreatic, prostate, breast, colorectal, ovarian, gastric, skin, parathyroid, brain), where mutations of the receptor are implicated in neoplastic progression [87] [88]. On the other hand, depending on the cellular context and type of cancer, CaSR expression is increased or decreased - thus turning the CaSR from putative oncogene to potential tumor suppressor [88].

One of the most intricate and challenging pictures emerges from the cardiovascular field.

In particular, the role of CaSR in cardiomyocytes is far from being widely accepted. Assessing the function of CaSR in cardiomyocytes is particularly intriguing because these cells undergo through functionally essential intracellular Ca^{2+} oscillations, which might be easily modulated by the action of a "complex" GPCR receptor such as the CaSR. Also, the fast intracellular Ca^{2+} oscillations can be mirrored by extracellular Ca^{2+} changes [89] [90] [91], which may directly activate and/or modulate CaSR signaling capacity

A role for CaSR in the heart was suggested since 2003, when Wu and collaborators found CaSR transcript and protein both in atria and ventricles of adult rats [92]. In that work the authors suggested, for the first time, a prominent activation of the PLC/$InsP_3$ signaling pathway upon CaSR stimulation with high extracellular Ca^{2+}, spermine and Gd^{3+}. Calcium dynamics, and changes of Inositol Phosphate levels (IPs) were used as readout of CaSR activation. Next, Tfelt-Hansen and collaborators [93] clearly demonstrated that the calcimimetic AMG 073 was able to cause both IPs accumulation and ERK1/2 activation in neonatal rat ventricular myocytes (NRVMs). The specificity of the response was straightforwardly demonstrated by the significant inhibition of Ca^{2+}-induced IPs accumulation after expression of the dominant-negative CaSR R185Q.

A **detrimental role** of CaSR activation in the heart was described already in 2003 by Wang and colleagues [92].Specifically, a **pro-apoptotic action** of Gd^{3+}, used as single CaSR agonist, was claimed in NRVMs. A few years later it was suggested that this action was mediated by the **mitogen-activated protein kinases (MAPK) and caspase 9** [94]. In subsequent studies it was suggested that Gd^{3+} was able to increase CaSR, TRPC6 [95] and TRPC3 expression levels [96].

The involvement of CaSR in the **effects of known cardiotoxic substances** has been also investigated.

Ciclosporin A (CsA) was described to induce cell apoptosis and to increase CaSR expression both in NRVMs [97], Wistar rats [98] and H9c2 cardiomyoblasts [99]. Gd^{3+} was found to exacerbate CsA-induced effects, while NPS2390 appeared somehow protective. A similar mechanism, albeit with some differences in the intracellular cascades activated, was described for lipopolysaccaride (LPS)-induced apoptosis [100].

The pro-apoptotic action of increased CaSR expression levels and signaling pathways have been also analyzed in the context of **ischemia/reperfusion** (I/R) or **hypoxia/reoxygenation** protocols, both *in vivo* and *in vitro.* CaSR pro-apoptotic effect was studied in NRVMs [101] [102] [103] [104], adult rat cardiomyocytes [105], and adult rat hearts [102] subjected to ischemia/reperfusion.

In summary, the **pro-apoptotic effect of CaSR activation, often exacerbated by increased CaSR expression due to I/R, has been attributed to the activation of the Ca^{2+} signaling pathways** with subsequent effects on the Bcl-2, Fas/FasL death receptor pathway [101], cytochrome C/caspase 3 axis [105], Bac/Bax mitochondrial translocation [103], ER stress [102], and PKC delta activation [106].

Along a similar trajectory are the results on the anti-apoptotic effect of hepatocyte growth factor (HGF) in NRVMs subjected to simulated I/R: here a downregulation of CaSR expression and signaling was observed [107].

Taken together, these results suggest that **I/R increases CaSR expression, thus inducing a CaSR-mediated Ca^{2+} overload - which exacerbates the effects of the pro-apoptotic machinery**.

In line with the above results, there are reports which identify CaSR as one of the GPCRs inhibited by protective **post-conditioning** mechanisms, i.e. by PKCε-mediated negative feedback [108] [109] or downregulation of CaSR expression levels [110].

Contrary to the studies described above, a **cardioprotective role** was proposed by Sun and Murphy [111] in **ischemic pre-conditioning (IPC).** In fact, these authors reported that the calcilitic NPS 2143 was able to attenuate cardioprotection of IPC, and proposed that changes in pH and ionic strength at the caveolae may activate the CaSR, which would thus mediate IPC. A protective role for CaSR was also suggested by Bai and colleagues [112]. On the basis the of observed reduction in CaSR expression in diabetic rat hearts, it was suggested that the subsequent impaired Ca^{2+} signaling could contribute to the progress of **diabetic cardiomyopathy** in adult rat cardiac cells.

Both the primary observation and its interpretation of data were to the contrary in the work by Qi and colleagues [113]. CaSR expression was found to be augmented in diabetic rat hearts and, similarly to observations in I/R work above it was hypothesized that CaSR signaling could contribute to apoptosis via Ca^{2+} overload, reduction of the Bcl2/Bax ratio, and modulation of the MAP kinase cascade. The apparent contrast with the results by Bai et al was explained by invoking a difference in the experimental setups - representative of type 2 diabetes in the first work [112] and type 1 diabetes in the latter [113].

CaSR role in **cardiac hypertrophy** is similarly still controversial**.**

In 2006, a protective role for CaSR against cardiac hypertrophy was hypothesized in NRVMs, where CaSR induced decrease in DNA synthesis [93]. In conflict with this idea, an increased expression of CaSR, coupled to worsening effects of Gd^{3+} on hypertrophic markers, was later demonstrated in NRVMs subjected to *in vitro* hypertrophy [114]. The activation of the Ca^{2+} -sensitive Calcineurin/NFAT pathway [115] was indicated as one of the molecular determinants [116]. The role of the CaSR in cardiac hypertrophy and heart failure was further investigated *in vivo* in isoproterenol-treated Wistar rats and mice subjected to thoracic aorta constriction (TAC) [117]. CaSR expression was found to be significantly increased in isoproterenol-treated rats, Calindol was found to induce a significant increase of cardiac hypertrophy, while the calcyilitic Calhex 231 was shown to significantly reduce the cross-sectional diameter of hypertrophic cardiomyocytes [117]. ER stress, Ca^{2+} overload of mitochondria, and subsequent initiation of apoptosis were suggested to cause cardiac hypertrophy and failure. More recently, Calhex 231 was proposed to ameliorate isoproterenol-induced cardiac hypertrophy both *in vivo* and *in vitro* [118], via inhibition of autophagy and the CaMK-AMPK-mTOR signaling pathway. A role for the CaSR in promoting cardiac fibrosis has also been suggested [119].

The CaSR in cardiac physiology: the importance of experimental models and multiple approaches

In contrast to the abundance of papers on the role of the CaSR in heart pathophysiology, only a few reports have been produced on its role in heart normophysiology.

First, a multifaceted study was performed by Schreckenberg and colleagues on adult male rats [120]. The effects of acute CaSR stimulation or inhibition and downregulation by siRNA were assessed on the contractile response of ventricular cardiomyocytes, Ca^{2+} dynamics, and cardiac performance [120]. In this study the authors provided the first evidence that CaSR action is relevant for basal cell shortening of ventricular cardiomyocytes, and demonstrated that activation of the CaSR augments cell shortening and relaxation rate - most probably via the G_q/PLC/IP_3 pathway.

One year later, Liu and colleagues [121] suggested that CaSR could be involved into the stabilization of the resting membrane potential of guinea pig cardiomyocytes.

CaSR activation by NPSR568 was found to activate both the PLC and phosphatidylinositol-4 kinase (PI_4) pathways. The prevailing effect of PI_4 Kinase was a significant increase in currents through K_{ir} channels due to PIP_2 elevation at the plasma membrane. In the same year, in a paper by Riccardi's group [122], it was suggested that CaSR deletion from heart could directly affects chronotropy *via* decreased pacemaker activity.

These results draw an intricate picture in which, while it is clear that the CaSR play a significant role in the heart, the mechanism and timing remain open questions, lacking significant and important detail.

One of the main reasons for apparent inconsistency of some results certainly relates to the heterogeneity of the experimental models used (neonatal versus adult ventricular myocytes, whole heart, mice, vs rats vs guinea pig). It is clear that adult models possess different characteristic from the neonatal, although each of the models can contribute to the understanding of the physio-pathological role of the CaSR if the right questions are asked and the results are discussed in the correct context. Also, given the promiscuity and the pleiotropicity of the CaSR, and its biased and compartmentalized signaling, it is not surprising to find contradictory results -especially in older reports, where single and poorly-specific CaSR agonists and single functional readouts were available to researchers.

A very interesting and well-designed study has recently addressed this problem by using two CaSR agonists and novel functional readouts for G_i and G_q activation in adult rat atrial myocytes [123]. In particular, the authors took advantage of the known dependence of G-protein-activated K^+ channels (GIRK) by the $\beta\gamma$ subunits of G_i and PIP_2 levels to quantify, by whole cell patch clamping, a putative biased signaling at the CaSR, activated by high $[Ca^{2+}]_o$ and spermine. The authors used FRET-based biosensors transfected into CaSR-expressing HEK293 cells in the presence or absence of the other molecular players involved in the process (CaSR, GIRK channels subunits), and compared the results obtained in the two experimental models. Interestingly, while CaSR did show biased signaling in HEK293-CaSR cells, this signaling mode was absent in atrial myocytes - where only the G_q pathway was activated by high $[Ca^{2+}]_o$, with subsequent inhibition of pre-activated GIRK current via PIP_2 depletion and PKC activation. Notably, G_i activation of GIRK channels was not detectable, and modulation of GIRK channel activity by spermine was found to be negligible - and thus physiologically irrelevant. The results were interpreted in the light of a possible contribution of CaSR to atrial fibrillation [123].

Conclusions and future perspective

On the basis of the data available on CaSR structure, and the novel insights on ligand- and compartment bias, it is predictable that an in-depth investigation on germline CaSR mutations will reveal more details on the intricate signaling mode of the CaSR. The accurate pharmacological assessment of the effects exerted by diverse CaSR agonists and modulators on different signaling pathways in *in vitro* models of germline mutations, together with homology modeling and mutagenesis studies, will certainly uncover new molecular players. The ultimate delineation of the entire CaSR structure in the active and inactive conformation will certainly be of paramount importance to resolving remaining conflicts in the field.

In parallel, it would be highly advisable to proceed with studies on physiologically relevant experimental models. $[Ca^{2+}]_i$ oscillations, cAMP changes, and MAP kinases activation are indeed not separated pathways. On the contrary, they can impinge one on the other, changing the kinetics of second messengers dynamics, thus determining the identity of the final molecular target of the extracellular signal. The exploitation of the extraordinary vast pool of fluorescent biosensors [124], in parallel to classical electrophysiological technique in physiologically relevant models, would help us to understand what does happen in real cells, in real time. If the single-cell and organ-derived approaches remain the elective technique to explore fine details of CaSR signaling, as far as the pharmacological branch is concerned, the use of easily-accessed and-manipulated cardiac cells (such as the NRVMs or iPSC) in high-throughput screenings for multiple readouts is highly advisable.

The therapeutic implication of such studies is enormous: knowing the details of CaSR structure and function relationship, as well as the mechanisms of CaSR activation and biased signaling, we can improve the design of targeted drugs able to activate the desired signaling pathway in a targeted manner, while avoiding effects on the many other tissues in which CaSR exert a functional role.

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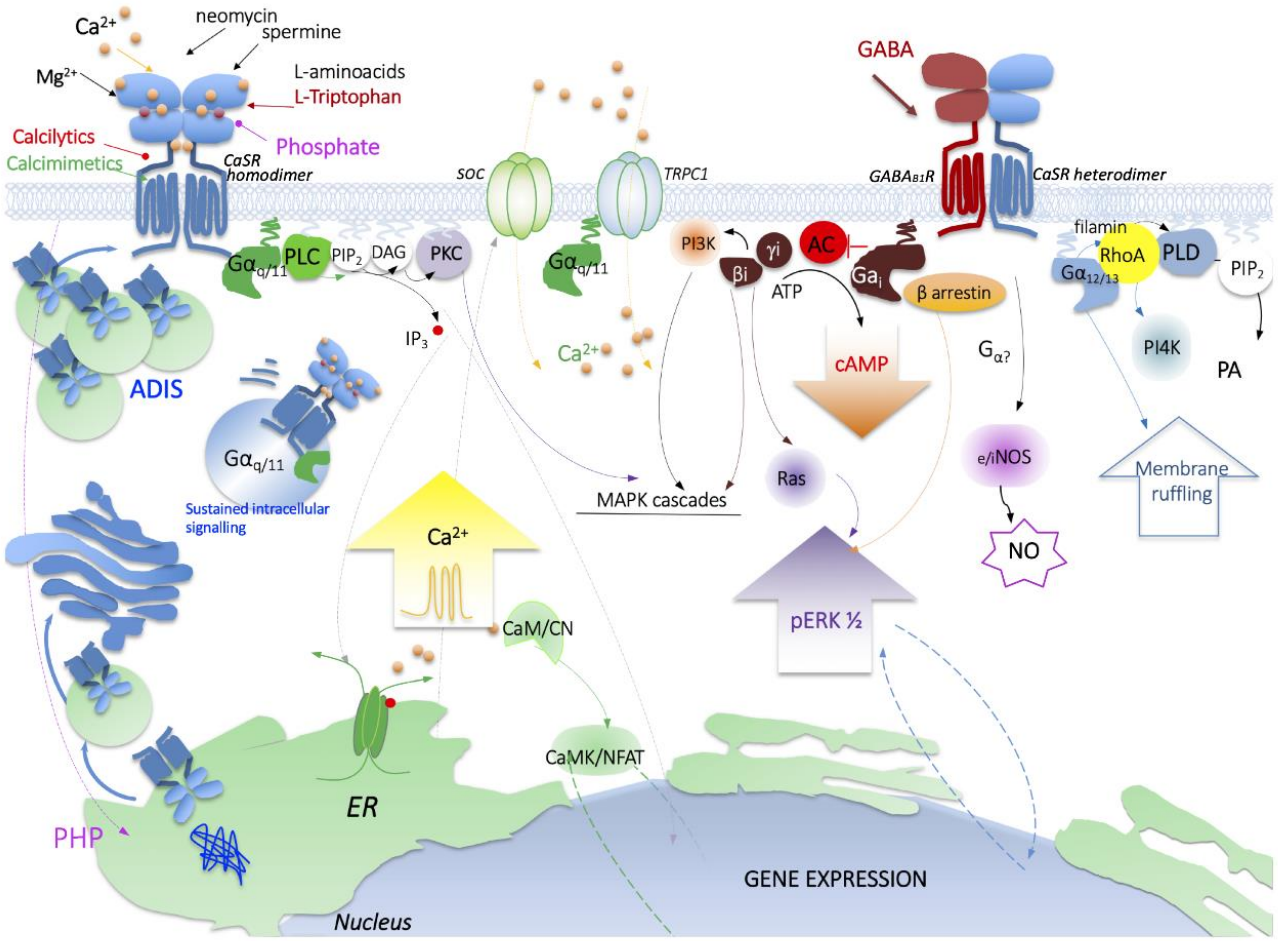

Figure 1. Simplified view of the intracellular signaling pathways activated by CaSR.

Schematic of plasma membrane dimers, agonists/modulators and intracellular signaling/trafficking of CaSR. The two examples of CaSR dimers (homodimeric and heterodimeric) are not exhaustive; also the vicinity to a particular intracellular signaling pathway is not meant to imply that the dimers or ligands depicted are linked preferentially to the closest signaling pathway.

Abbreviations: AA, arachidonic acid; AC,adenylate cyclase; Akt, protein kinase B; ATP, adenosine triphosphate; CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; cAMP, cyclic AMP; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK1/2, extracellullar-signal regulated kinase; $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha 12/13}$, α subunits of the s-, i-, q-, and 12/13-type heterotrimeric G-proteins, respectively; iNOS, inducible nitric oxide synthase; IP_3, inositol-1,4,5-trisphosphate; JNK, Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NO, nitric oxide; p38, p38 mitogen-activated protein kinase; PA, phosphatidic acid; PHP, pharmacoperones; PI_3K, phosphatidylinositol 3-kinase; PI_4K, phosphatidylinositol 4-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; RhoA, Ras homolog gene family, member A; SOC, store-operated Ca^{2+} channel.

References

1. Berridge MJ, Lipp P, Bootman MD: **The versatility and universality of calcium signalling.** *Nature Reviews Molecular Cell Biology* (2000) **1**(1):11-21.

2. Rizzuto R, Pozzan T: **Microdomains of intracellular ca2+: Molecular determinants and functional consequences.** *Physiological reviews* (2006) **86**(1):369-408.

3. Berridge MJ: **Calcium microdomains: Organization and function.** *Cell calcium* (2006) **40**(5-6):405-412.

4. Hofer AM, Lefkimmiatis K: **Extracellular calcium and camp: Second messengers as "third messengers"?** *Physiology (Bethesda)* (2007) **22**(320-327.

5. Brown EM, MacLeod RJ: **Extracellular calcium sensing and extracellular calcium signaling.** *Physiological reviews* (2001) **81**(1):239-297.

6. Ashby MC, Tepikin AV: **Polarized calcium and calmodulin signaling in secretory epithelia.** *Physiological reviews* (2002) **82**(3):701-734.

7. Hofer AM: **Another dimension to calcium signaling: A look at extracellular calcium.** *J Cell Sci* (2005) **118**(Pt 5):855-862.

8. Gerbino A, Maiellaro I, Carmone C, Caroppo R, Debellis L, Barile M, Busco G, Colella M: **Glucose increases extracellular [ca2+] in rat insulinoma (ins-1e) pseudoislets as measured with ca2+-sensitive microelectrodes.** *Cell calcium* (2012) **51**(5):393-401.

9. Gerbino A, Colella M: **The different facets of extracellular calcium sensors: Old and new concepts in calcium-sensing receptor signalling and pharmacology.** *Int J Mol Sci* (2018) **19**(4).

10. Care AD, Sherwood LM, Potts JT, Jr., Aurbach GD: **Perfusion of the isolated parathyroid gland of the goat and sheep.** *Nature* (1966) **209**(5018):55-57.

11. Sherwood LM, Potts JT, Jr., Care AD, Mayer GP, Aurbach GD: **Evaluation by radioimmunoassay of factors controlling the secretion of parathyroid hormone.** *Nature* (1966) **209**(5018):52-55.

12. Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, Hebert SC: **Cloning and characterization of an extracellular ca(2+)-sensing receptor from bovine parathyroid.** *Nature* (1993) **366**(6455):575-580.

13. Hofer AM, Brown EM: **Extracellular calcium sensing and signalling.** *Nat Rev Mol Cell Biol* (2003) **4**(7):530-538.

14. Brown EM: **Mutations in the calcium-sensing receptor and their clinical implications.** *Horm Res* (1997) **48**(5):199-208.

15. Gorvin CM: **Molecular and clinical insights from studies of calcium-sensing receptor mutations.** *J Mol Endocrinol* (2019) **63**(2):R1-R16.

16. Hannan FM, Olesen MK, Thakker RV: **Calcimimetic and calcilytic therapies for inherited disorders of the calcium-sensing receptor signalling pathway.** *Br J Pharmacol* (2018).

17. Hannan FM, Kallay E, Chang W, Brandi ML, Thakker RV: **The calcium-sensing receptor in physiology and in calcitropic and noncalcitropic diseases.** *Nat Rev Endocrinol* (2019) **15**(1):33-51.

18. Zhang C, Zhang T, Zou J, Miller CL, Gorkhali R, Yang JY, Schilmiller A, Wang S, Huang K, Brown EM, Moremen KW *et al*: **Structural basis for regulation of human calcium-sensing receptor by magnesium ions and an unexpected tryptophan derivative co-agonist.** *Sci Adv* (2016) **2**(5):e1600241.

Here the authors report the first crystal structure of the extracellular domain (ECD) of the human CaSR bound with Mg^{2+}.The finding of a tryptophan derivative bound in the hinge region between the two ECD indicates that this aminoacid can be proposed as a novel and high-affinity co-agonist of the CaSR. This work, together with the one by Geng et al, represents a milestone in the understanding of CaSR's structure-function relationship

19. Geng Y, Mosyak L, Kurinov I, Zuo H, Sturchler E, Cheng TC, Subramanyam P, Brown AP, Brennan SC, Mun HC, Bush M *et al*: **Structural mechanism of ligand activation in human calcium-sensing receptor.** *Elife* (2016) **5**(

The authors report the crystal structures of the entire extracellular domain of CaSR in the resting and active conformations, confirming the presence of L-Tryptophan bound at at the interdomain cleft, and defining it as essential for receptor activation. They also find multiple binding sites for calcium and phosphate ions, which may stabilize the active state or the inactive conformation, respectively.
This work, together with the one by Zhang et al, represents a milestone in the understanding of CaSR's structure-function relationship.

20. Gama L, Wilt SG, Breitwieser GE: **Heterodimerization of calcium sensing receptors with metabotropic glutamate receptors in neurons.** *J Biol Chem* (2001) **276**(42):39053-39059.

21. Chang W, Tu C, Cheng Z, Rodriguez L, Chen TH, Gassmann M, Bettler B, Margeta M, Jan LY, Shoback D: **Complex formation with the type b gamma-aminobutyric acid receptor affects the expression and signal transduction of the extracellular calcium-sensing receptor. Studies with hek-293 cells and neurons.** *J Biol Chem* (2007) **282**(34):25030-25040.

22. Chang W, Tu C-L, Jean-Alphonse FG, Herberger A, Cheng Z, Hwong J, Ho H, Li A, Wang D, Liu H, White AD *et al*: **Pth hypersecretion triggered by a gabab1 and ca2+-sensing receptor heterocomplex in hyperparathyroidism.** *Nature Metabolism* (2020) **2**(3):243-255.

23. Centeno PP, Herberger A, Mun HC, Tu C, Nemeth EF, Chang W, Conigrave AD, Ward DT: **Phosphate acts directly on the calcium-sensing receptor to stimulate parathyroid hormone secretion.** *Nat Commun* (2019) **10**(1):4693.

The authors reveal the mechanism by which phosphate ions increase PTH secretion and in pathological conditions, such as in chronic kidney disease (CKD), can induce secondary hyperparathyroidism. Phosphate acts at CaSR as a non-competitive agonist and thus the CaSR can be considerate also a "phosphate sensor".

24. Nielsen PK, Feldt-Rasmussen U, Olgaard K: **A direct effect in vitro of phosphate on pth release from bovine parathyroid tissue slices but not from dispersed parathyroid cells.** *Nephrol Dial Transplant* (1996) **11**(9):1762-1768.

25. Zhang C, Miller CL, Gorkhali R, Zou J, Huang K, Brown EM, Yang JJ: **Molecular basis of the extracellular ligands mediated signaling by the calcium sensing receptor.** *Front Physiol* (2016) **7**(441.

26. Brown EM, Fuleihan Ge-H, Chen CJ, Kifor O: **A comparison of the effects of divalent and trivalent cations on parathyroid hormone release, 3',5'-cyclic-adenosine monophosphate accumulation, and the levels of inositol phosphates in bovine parathyroid cells.** *Endocrinology* (1990) **127**(3):1064-1071.

27. Quinn SJ, Ye CP, Diaz R, Kifor O, Bai M, Vassilev P, Brown E: **The ca2+-sensing receptor: A target for polyamines.** *Am J Physiol* (1997) **273**(4 Pt 1):C1315-1323.

28. Brown EM, Butters R, Katz C, Kifor O: **Neomycin mimics the effects of high extracellular calcium concentrations on parathyroid function in dispersed bovine parathyroid cells.** *Endocrinology* (1991) **128**(6):3047-3054.

29. Molostvov G, James S, Fletcher S, Bennett J, Lehnert H, Bland R, Zehnder D: **Extracellular calcium-sensing receptor is functionally expressed in human artery.** (2007) **293**(3):F946-F955.

30. Brown EM, Katz C, Butters R, Kifor O: **Polyarginine, polylysine, and protamine mimic the effects of high extracellular calcium concentrations on dispersed bovine parathyroid cells.** *J Bone Miner Res* (1991) **6**(11):1217-1225.

31. Ye C, Ho-Pao CL, Kanazirska M, Quinn S, Rogers K, Seidman CE, Seidman JG, Brown EM, Vassilev PM: **Amyloid-beta proteins activate ca(2+)-permeable channels through calcium-sensing receptors.** *J Neurosci Res* (1997) **47**(5):547-554.

32. Conigrave AD, Quinn SJ, Brown EM: **L-amino acid sensing by the extracellular ca2+-sensing receptor.** *Proc Natl Acad Sci U S A* (2000) **97**(9):4814-4819.

33. Broadhead GK, Mun HC, Avlani VA, Jourdon O, Church WB, Christopoulos A, Delbridge L, Conigrave AD: **Allosteric modulation of the calcium-sensing receptor by gamma-glutamyl peptides: Inhibition of pth secretion, suppression of intracellular camp levels, and a common mechanism of action with l-amino acids.** *J Biol Chem* (2011) **286**(11):8786-8797.

34. Quinn SJ, Bai M, Brown EM: **Ph sensing by the calcium-sensing receptor.** *J Biol Chem* (2004) **279**(36):37241-37249.

35. Quinn SJ, Kifor O, Trivedi S, Diaz R, Vassilev P, Brown E: **Sodium and ionic strength sensing by the calcium receptor.** *J Biol Chem* (1998) **273**(31):19579-19586.

36. Nemeth EF, Goodman WG: **Calcimimetic and calcilytic drugs: Feats, flops, and futures.** *Calcif Tissue Int* (2016) **98**(4):341-358.

37. Nemeth EF: **Calcimimetic and calcilytic drugs: Just for parathyroid cells?** *Cell calcium* (2004) **35**(3):283-289.

38. Ma JN, Owens M, Gustafsson M, Jensen J, Tabatabaei A, Schmelzer K, Olsson R, Burstein ES: **Characterization of highly efficacious allosteric agonists of the human calcium-sensing receptor.** *J Pharmacol Exp Ther* (2011) **337**(1):275-284.

39. Henley C, 3rd, Yang Y, Davis J, Lu JY, Morony S, Fan W, Florio M, Sun B, Shatzen E, Pretorius JK, Richards WG *et al*: **Discovery of a calcimimetic with differential effects on parathyroid hormone and calcitonin secretion.** *J Pharmacol Exp Ther* (2011) **337**(3):681-691.

40. Cook AE, Mistry SN, Gregory KJ, Furness SG, Sexton PM, Scammells PJ, Conigrave AD, Christopoulos A, Leach K: **Biased allosteric modulation at the cas receptor engendered by structurally diverse calcimimetics.** *Br J Pharmacol* (2015) **172**(1):185-200.

41. Josephs TM, Keller AN, Khajehali E, DeBono A, Langmead CJ, Conigrave AD, Capuano B, Kufareva I, Gregory KJ, Leach K: **Negative allosteric modulators of the human calcium-sensing receptor bind to overlapping and distinct sites within the 7-transmembrane domain.** *Br J Pharmacol* (2020) **177**(8):1917-1930.

42. Nemeth EF: **The search for calcium receptor antagonists (calcilytics).** *J Mol Endocrinol* (2002) **29**(1):15-21.

43. Cipriani C, Nemeth EF, Bilezikian JP: **Chapter 71 - drugs acting on the calcium receptor: Calcimimetics and calcilytics.** In: *Principles of bone biology (fourth edition).* Bilezikian JP, Martin TJ, Clemens TL, Rosen CJ (Eds), Academic Press, (2020):1657-1670.

44. White E, McKenna J, Cavanaugh A, Breitwieser GE: **Pharmacochaperone-mediated rescue of calcium-sensing receptor loss-of-function mutants.** *Mol Endocrinol* (2009) **23**(7):1115-1123.

45. Colella M, Gerbino A, Hofer AM, Curci S: **Recent advances in understanding the extracellular calcium-sensing receptor.** *F1000Res* (2016) **5**

46. Huang Y, Breitwieser GE: **Rescue of calcium-sensing receptor mutants by allosteric modulators reveals a conformational checkpoint in receptor biogenesis.** *J Biol Chem* (2007) **282**(13):9517-9525.

47. Rus R, Haag C, Bumke-Vogt C, Bahr V, Mayr B, Mohlig M, Schulze E, Frank-Raue K, Raue F, Schofl C: **Novel inactivating mutations of the calcium-sensing receptor: The calcimimetic nps r-568 improves signal transduction of mutant receptors.** *J Clin Endocrinol Metab* (2008) **93**(12):4797-4803.

48. Leach K, Wen A, Cook AE, Sexton PM, Conigrave AD, Christopoulos A: **Impact of clinically relevant mutations on the pharmacoregulation and signaling bias of the calcium-sensing receptor by positive and negative allosteric modulators.** *Endocrinology* (2013) **154**(3):1105-1116.

By characterizing the effects of 21 clinically relevant CaSR mutations in respect to their expression levels at the plasma membrane, intracellular Ca^{2+} mobilization and ERK1/2 phosphorylation, the authors identify CaSR biased signaling in response to different CaSR mutations.

49. Leach K, Sexton PM, Christopoulos A, Conigrave AD: **Engendering biased signalling from the calcium-sensing receptor for the pharmacotherapy of diverse disorders.** *Br J Pharmacol* (2014) **171**(5):1142-1155.

50. Nakamura A, Hotsubo T, Kobayashi K, Mochizuki H, Ishizu K, Tajima T: **Loss-of-function and gain-of-function mutations of calcium-sensing receptor: Functional analysis and the effect of allosteric modulators nps r-568 and nps 2143.** *J Clin Endocrinol Metab* (2013) **98**(10):E1692-1701.

51. Conigrave AD, Ward DT: **Calcium-sensing receptor (casr): Pharmacological properties and signaling pathways.** *Best Pract Res Clin Endocrinol Metab* (2013) **27**(3):315-331.

52. Caroppo R, Gerbino A, Fistetto G, Colella M, Debellis L, Hofer AM, Curci S: **Extracellular calcium acts as a "third messenger" to regulate enzyme and alkaline secretion.** *J Cell Biol* (2004) **166**(1):111-119.

53. Gerbino A, Ruder WC, Curci S, Pozzan T, Zaccolo M, Hofer AM: **Termination of camp signals by ca2+ and g(alpha)i via extracellular ca2+ sensors: A link to intracellular ca2+ oscillations.** *J Cell Biol* (2005) **171**(2):303-312.

54. Gerbino A, Fistetto G, Colella M, Hofer AM, Debellis L, Caroppo R, Curci S: **Real time measurements of water flow in amphibian gastric glands: Modulation via the extracellular ca2+-sensing receptor.** *J Biol Chem* (2007) **282**(18):13477-13486.

55. Kifor O, MacLeod RJ, Diaz R, Bai M, Yamaguchi T, Yao T, Kifor I, Brown EM: **Regulation of map kinase by calcium-sensing receptor in bovine parathyroid and car-transfected hek293 cells.** *Am J Physiol Renal Physiol* (2001) **280**(2):F291-302.

56. Thomsen AR, Hvidtfeldt M, Brauner-Osborne H: **Biased agonism of the calcium-sensing receptor.** *Cell calcium* (2012) **51**(2):107-116.

By using functional readouts for Gq/11, Gi/o and extracellular signal-regulated kinases 1 and 2 (ERK1/2), the authors analyze the signaling pathways of 12 orthosteric CaSR agonists. They show for the first time that ERK1/2 can be activated also through recruitment of β-arrestins. The study clearly demonstrate that In CaSR transfected HEK293 cells, CaSR exists in diverse active conformations, each stabilized by a specific ligand, which thus bias CaSR-mediated intracellular signaling.

57. Fitzpatrick LA, Brandi ML, Aurbach GD: **Prostaglandin f2 alpha and alpha-adrenergic agonists regulate parathyroid cell function via the inhibitory guanine nucleotide regulatory protein.** *Endocrinology* (1986) **118**(5):2115-2119.

58. Chen CJ, Barnett JV, Congo DA, Brown EM: **Divalent cations suppress 3',5'-adenosine monophosphate accumulation by stimulating a pertussis toxin-sensitive guanine nucleotide-binding protein in cultured bovine parathyroid cells.** *Endocrinology* (1989) **124**(1):233-239.

59. Conigrave AD: **The calcium-sensing receptor and the parathyroid: Past, present, future.** (2016) **7**(563).

60. Onopiuk M, Eby B, Nesin V, Ngo P, Lerner M, Gorvin CM, Stokes VJ, Thakker RV, Brandi ML, Chang W, Humphrey MB *et al*: **Control of pth secretion by the trpc1 ion channel.** *JCI Insight* (2020) **5**(8).

The authors demonstrate that mice lacking TRPC1 show a phenotype that mimics human FHH, thus representing a suitable model for FHH. By using multiple approaches, the researchers demonstrate that upon CaSR activation with high extracellular Ca2+, the Gα11 subunit physically interacts and stabilizes TRPC1 causing a fast and store-independent Ca^{2+} entry which in turn suppresses PTH secretion.

61. Rey O, Young SH, Jacamo R, Moyer MP, Rozengurt E: **Extracellular calcium sensing receptor stimulation in human colonic epithelial cells induces intracellular calcium oscillations and proliferation inhibition.** *J Cell Physiol* (2010) **225**(1):73-83.

62. McCormick WD, Atkinson-Dell R, Campion KL, Mun HC, Conigrave AD, Ward DT: **Increased receptor stimulation elicits differential calcium-sensing receptor(t888) dephosphorylation.** *J Biol Chem* (2010) **285**(19):14170-14177.

63. Mamillapalli R, VanHouten J, Zawalich W, Wysolmerski J: **Switching of g-protein usage by the calcium-sensing receptor reverses its effect on parathyroid hormone-related protein secretion in normal versus malignant breast cells.** *J Biol Chem* (2008) **283**(36):24435-24447.

64. Mamillapalli R, Wysolmerski J: **The calcium-sensing receptor couples to galpha(s) and regulates pthrp and acth secretion in pituitary cells.** *J Endocrinol* (2010) **204**(3):287-297.

65. Ziegelstein RC, Xiong Y, He C, Hu Q: **Expression of a functional extracellular calcium-sensing receptor in human aortic endothelial cells.** *Biochem Biophys Res Commun* (2006) **342**(1):153-163.

66. Smajilovic S, Sheykhzade M, Holmegard HN, Haunso S, Tfelt-Hansen J: **Calcimimetic, amg 073, induces relaxation on isolated rat aorta.** *Vascul Pharmacol* (2007) **47**(4):222-228.

67. Greenberg HZ, Shi J, Jahan KS, Martinucci MC, Gilbert SJ, Vanessa Ho WS, Albert AP: **Stimulation of calcium-sensing receptors induces endothelium-dependent vasorelaxations via nitric oxide production and activation of ikca channels.** *Vascul Pharmacol* (2016) **80**(75-84.

68. Qu YY, Wang LM, Zhong H, Liu YM, Tang N, Zhu LP, He F, Hu QH: **Trpc1 stimulates calciumsensing receptorinduced storeoperated ca2+ entry and nitric oxide production in endothelial cells.** *Mol Med Rep* (2017) **16**(4):4613-4619.

69. Greenberg HZE, Carlton-Carew SRE, Khan DM, Zargaran AK, Jahan KS, Vanessa Ho WS, Albert AP: **Heteromeric trpv4/trpc1 channels mediate calcium-sensing receptor-induced nitric oxide production and vasorelaxation in rabbit mesenteric arteries.** *Vascul Pharmacol* (2017) **96-98**(53-62.

70. Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, Violin JD, Lefkowitz RJ: **Quantifying ligand bias at seven-transmembrane receptors.** *Mol Pharmacol* (2011) **80**(3):367-377.

71. Makita N, Sato J, Manaka K, Shoji Y, Oishi A, Hashimoto M, Fujita T, Iiri T: **An acquired hypocalciuric hypercalcemia autoantibody induces allosteric transition among active human ca-sensing receptor conformations.** *Proc Natl Acad Sci U S A* (2007) **104**(13):5443-5448.

72. Leach K, Wen A, Davey AE, Sexton PM, Conigrave AD, Christopoulos A: **Identification of molecular phenotypes and biased signaling induced by naturally occurring mutations of the human calcium-sensing receptor.** *Endocrinology* (2012) **153**(9):4304-4316.

73. Leach K, Gregory KJ, Kufareva I, Khajehali E, Cook AE, Abagyan R, Conigrave AD, Sexton PM, Christopoulos A: **Towards a structural understanding of allosteric drugs at the human calcium-sensing receptor.** *Cell Res* (2016) **26**(5):574-592.

74. Davey AE, Leach K, Valant C, Conigrave AD, Sexton PM, Christopoulos A: **Positive and negative allosteric modulators promote biased signaling at the calcium-sensing receptor.** *Endocrinology* (2012) **153**(3):1232-1241.

By using a novel analytical model on stably transfected HEK293-TREx c-myc-CaSR cell line, the authors straightforwardly demonstrate that positive and negative allosteric modulators of the CaSR exhibit biased signaling.

75. Gorvin CM, Babinsky VN, Malinauskas T, Nissen PH, Schou AJ, Hanyaloglu AC, Siebold C, Jones EY, Hannan FM, Thakker RV: **A calcium-sensing receptor mutation causing hypocalcemia disrupts a transmembrane salt bridge to activate beta-arrestin-biased signaling.** *Sci Signal* (2018) **11**(518).

The authors show that the disruption of a salt-bridge between two residues at the CaSR induces a conformational change that eases the binding of β-arrestin. Thus, they uncover mechanistic details underlying a signaling bias toward a β-arrestin-mediated MAPK cascade activation.

76. Gorvin CM, Frost M, Malinauskas T, Cranston T, Boon H, Siebold C, Jones EY, Hannan FM, Thakker RV: **Calcium-sensing receptor residues with loss- and gain-of-function mutations are located in regions of conformational change and cause signalling bias.** *Hum Mol Genet* (2018) **27**(21):3720-3733.

The authors identify five "disease switch" residues, which are the location of both FHH1 and ADH1 germline mutations of the CaSR. They show that these mutations commonly exhibit signaling bias. The study demonstrate that these disease switch residues are mostly located at sites important for the switch of the CaSR protein from the inactive to the active conformation

77. Hannan FM, Nesbit MA, Zhang C, Cranston T, Curley AJ, Harding B, Fratter C, Rust N, Christie PT, Turner JJ, Lemos MC *et al*: **Identification of 70 calcium-sensing receptor mutations in hyper- and hypo-calcaemic patients: Evidence for clustering of extracellular domain mutations at calcium-binding sites.** *Hum Mol Genet* (2012) **21**(12):2768-2778.

78. Pidasheva S, Grant M, Canaff L, Ercan O, Kumar U, Hendy GN: **Calcium-sensing receptor dimerizes in the endoplasmic reticulum: Biochemical and biophysical characterization of casr mutants retained intracellularly.** *Hum Mol Genet* (2006) **15**(14):2200-2209.

79. Ward DT, Brown EM, Harris HW: **Disulfide bonds in the extracellular calcium-polyvalent cation-sensing receptor correlate with dimer formation and its response to divalent cations in vitro.** *J Biol Chem* (1998) **273**(23):14476-14483.

80. Brennan SC, Mun HC, Leach K, Kuchel PW, Christopoulos A, Conigrave AD: **Receptor expression modulates calcium-sensing receptor mediated intracellular ca2+ mobilization.** *Endocrinology* (2016) **156**(4):1330-1342.

The authors show CaSR-mediated calcium responses are modulated by receptor expression. The crucial implication of these findings is that CaSR-mediated signaling responses would be controlled by hormones, cytokines, and pathological conditions that modulate its expression in the presence of constant ligand concentrations.

81. Breitwieser GE: **Minireview: The intimate link between calcium sensing receptor trafficking and signaling: Implications for disorders of calcium homeostasis.** *Mol Endocrinol* (2012) **26**(9):1482-1495.

82. Grant MP, Stepanchick A, Cavanaugh A, Breitwieser GE: **Agonist-driven maturation and plasma membrane insertion of calcium-sensing receptors dynamically control signal amplitude.** *Sci Signal* (2011) **4**(200):ra78.

The authors describe a new signaling mode, named Agonist-driven insertional Signallig (ADIS), by which activation of the CaSR induces the insertion into the cell surface of new receptors from a near plasma membrane pool in dependence of the ligand concentration. The authors suggest that this peculiar type of signaling may explain the high cooperativity and the minimal desensitization that characterize the CaSR.

83. Breitwieser GE: **The calcium sensing receptor life cycle: Trafficking, cell surface expression, and degradation.** *Best Pract Res Clin Endocrinol Metab* (2013) **27**(3):303-313.

84. Nesbit MA, Hannan FM, Howles SA, Babinsky VN, Head RA, Cranston T, Rust N, Hobbs MR, Heath H, 3rd, Thakker RV: **Mutations affecting g-protein subunit alpha11 in hypercalcemia and hypocalcemia.** *N Engl J Med* (2013) **368**(26):2476-2486.

85. Irannejad R, von Zastrow M: **Gpcr signaling along the endocytic pathway.** *Current opinion in cell biology* (2014) **27**(109-116.

86. Gorvin CM, Rogers A, Hastoy B, Tarasov AI, Frost M, Sposini S, Inoue A, Whyte MP, Rorsman P, Hanyaloglu AC, Breitwieser GE *et al*: **Ap2sigma mutations impair calcium-sensing receptor trafficking and signaling, and show an endosomal pathway to spatially direct g-protein selectivity.** *Cell Rep* (2018) **22**(4):1054-1066.

The authors demonstrate that the impaired internalization of CaSR by chlatrin-mediated endocytosis of the analyzed germline mutation, affects a "compartmental biased" signaling which is a sustained Gq mediated signal most probably located at the endosomes.

87. Tennakoon S, Aggarwal A, Kallay E: **The calcium-sensing receptor and the hallmarks of cancer.** *Biochim Biophys Acta* (2016).

88. Saidak Z, Mentaverri R, Brown EM: **The role of the calcium-sensing receptor in the development and progression of cancer.** *Endocr Rev* (2009) **30**(2):178-195.

89. Cleemann L, Pizarro G, Morad M: **Optical measurements of extracellular calcium depletion during a single heartbeat.** *Science* (1984) **226**(4671):174-177.

90. Hilgemann DW, Langer GA: **Transsarcolemmal calcium movements in arterially perfused rabbit right ventricle measured with extracellular calcium-sensitive dyes.** *Circ Res* (1984) **54**(4):461-467.

91. Hilgemann DW: **Extracellular calcium transients at single excitations in rabbit atrium measured with tetramethylmurexide.** *J Gen Physiol* (1986) **87**(5):707-735.

92. Wang R, Xu C, Zhao W, Zhang J, Cao K, Yang B, Wu L: **Calcium and polyamine regulated calcium-sensing receptors in cardiac tissues.** *Eur J Biochem* (2003) **270**(12):2680-2688.

93. Tfelt-Hansen J, Hansen JL, Smajilovic S, Terwilliger EF, Haunso S, Sheikh SP: **Calcium receptor is functionally expressed in rat neonatal ventricular cardiomyocytes.** *Am J Physiol Heart Circ Physiol* (2006) **290**(3):H1165-1171.

94. Sun YH, Liu MN, Li H, Shi S, Zhao YJ, Wang R, Xu CQ: **Calcium-sensing receptor induces rat neonatal ventricular cardiomyocyte apoptosis.** *Biochem Biophys Res Commun* (2006) **350**(4):942-948.

95. Sun YH, Li YQ, Feng SL, Li BX, Pan ZW, Xu CQ, Li TT, Yang BF: **Calcium-sensing receptor activation contributed to apoptosis stimulates trpc6 channel in rat neonatal ventricular myocytes.** *Biochem Biophys Res Commun* (2010) **394**(4):955-961.

96. Feng SL, Sun MR, Li TT, Yin X, Xu CQ, Sun YH: **Activation of calcium-sensing receptor increases trpc3 expression in rat cardiomyocytes.** *Biochem Biophys Res Commun* (2011) **406**(2):278-284.

97. Tang J, Wang G, Liu Y, Fu Y, Chi J, Zhu Y, Zhao Y, Yin X: **Cyclosporin a induces cardiomyocyte injury through calcium-sensing receptor-mediated calcium overload.** *Pharmazie* (2011) **66**(1):52-57.

98. Zhao Y, Hou G, Zhang Y, Chi J, Zhang L, Zou X, Tang J, Liu Y, Fu Y, Yin X: **Involvement of the calcium-sensing receptor in cyclosporin a-induced cardiomyocyte apoptosis in rats.** *Pharmazie* (2011) **66**(12):968-974.

99. Chi J, Zhu Y, Fu Y, Liu Y, Zhang X, Han L, Yin X, Zhao D: **Cyclosporin a induces apoptosis in h9c2 cardiomyoblast cells through calcium-sensing receptor-mediated activation of the erk mapk and p38 mapk pathways.** *Mol Cell Biochem* (2013) **367**(1-2):227-236.

100. Wang HY, Liu XY, Han G, Wang ZY, Li XX, Jiang ZM, Jiang CM: **Lps induces cardiomyocyte injury through calcium-sensing receptor.** *Mol Cell Biochem* (2013) **379**(1-2):153-159.

101. Jiang CM, Han LP, Li HZ, Qu YB, Zhang ZR, Wang R, Xu CQ, Li WM: **Calcium-sensing receptors induce apoptosis in cultured neonatal rat ventricular cardiomyocytes during simulated ischemia/reperfusion.** *Cell Biol Int* (2008) **32**(7):792-800.

102. Lu F, Tian Z, Zhang W, Zhao Y, Bai S, Ren H, Chen H, Yu X, Wang J, Wang L, Li H *et al*: **Calcium-sensing receptors induce apoptosis in rat cardiomyocytes via the endo(sarco)plasmic reticulum pathway during hypoxia/reoxygenation.** *Basic Clin Pharmacol Toxicol* (2010) **106**(5):396-405.

103. Lu FH, Tian Z, Zhang WH, Zhao YJ, Li HL, Ren H, Zheng HS, Liu C, Hu GX, Tian Y, Yang BF *et al*: **Calcium-sensing receptors regulate cardiomyocyte ca2+ signaling via the sarcoplasmic reticulum-mitochondrion interface during hypoxia/reoxygenation.** *J Biomed Sci* (2010) **17**(50.

104. Guo J, Li HZ, Zhang WH, Wang LC, Wang LN, Zhang L, Li GW, Li HX, Yang BF, Wu L, Wang R *et al*: **Increased expression of calcium-sensing receptors induced by ox-ldl amplifies apoptosis of cardiomyocytes during simulated ischaemia-reperfusion.** *Clin Exp Pharmacol Physiol* (2010) **37**(3):e128-135.

105. Zhang WH, Fu SB, Lu FH, Wu B, Gong DM, Pan ZW, Lv YJ, Zhao YJ, Li QF, Wang R, Yang BF *et al*: **Involvement of calcium-sensing receptor in ischemia/reperfusion-induced apoptosis in rat cardiomyocytes.** *Biochem Biophys Res Commun* (2006) **347**(4):872-881.

106. Zheng H, Liu J, Liu C, Lu F, Zhao Y, Jin Z, Ren H, Leng X, Jia J, Hu G, Dong S *et al*: **Calcium-sensing receptor activating phosphorylation of pkcdelta translocation on mitochondria to induce cardiomyocyte apoptosis during ischemia/reperfusion.** *Mol Cell Biochem* (2011) **358**(1-2):335-343.

107. Yan L, Zhu TB, Wang LS, Pan SY, Tao ZX, Yang Z, Cao K, Huang J: **Inhibitory effect of hepatocyte growth factor on cardiomyocytes apoptosis is partly related to reduced calcium sensing receptor expression during a model of simulated ischemia/reperfusion.** *Mol Biol Rep* (2011) **38**(4):2695-2701.

108. Zhang WH, Lu FH, Zhao YJ, Wang LN, Tian Y, Pan ZW, Lv YJ, Wang YL, Du LJ, Sun ZR, Yang BF *et al*: **Post-conditioning protects rat cardiomyocytes via pkcepsilon-mediated calcium-sensing receptors.** *Biochem Biophys Res Commun* (2007) **361**(3):659-664.

109. Dong S, Teng Z, Lu FH, Zhao YJ, Li H, Ren H, Chen H, Pan ZW, Lv YJ, Yang BF, Tian Y *et al*: **Post-conditioning protects cardiomyocytes from apoptosis via pkc(epsilon)-interacting with calcium-sensing receptors to inhibit endo(sarco)plasmic reticulum-mitochondria crosstalk.** *Mol Cell Biochem* (2010) **341**(1-2):195-206.

110. Zhang L, Cao S, Deng S, Yao G, Yu T: **Ischemic postconditioning and pinacidil suppress calcium overload in anoxia-reoxygenation cardiomyocytes via down-regulation of the calcium-sensing receptor.** *PeerJ* (2016) **4**(e2612.

111. Sun J, Murphy E: **Calcium-sensing receptor: A sensor and mediator of ischemic preconditioning in the heart.** *Am J Physiol Heart Circ Physiol* (2010) **299**(5):H1309-1317.

112. Bai SZ, Sun J, Wu H, Zhang N, Li HX, Li GW, Li HZ, He W, Zhang WH, Zhao YJ, Wang LN *et al*: **Decrease in calcium-sensing receptor in the progress of diabetic cardiomyopathy.** *Diabetes Res Clin Pract* (2012) **95**(3):378-385.

113. Qi H, Cao Y, Huang W, Liu Y, Wang Y, Li L, Liu L, Ji Z, Sun H: **Crucial role of calcium-sensing receptor activation in cardiac injury of diabetic rats.** *PLoS One* (2013) **8**(5):e65147.

114. Wang LN, Wang C, Lin Y, Xi YH, Zhang WH, Zhao YJ, Li HZ, Tian Y, Lv YJ, Yang BF, Xu CQ: **Involvement of calcium-sensing receptor in cardiac hypertrophy-induced by angiotensinii through calcineurin pathway in cultured neonatal rat cardiomyocytes.** *Biochem Biophys Res Commun* (2008) **369**(2):584-589.

115. Colella M, Pozzan T: **Cardiac cell hypertrophy in vitro: Role of calcineurin/nfat as ca2+ signal integrators.** *Ann N Y Acad Sci* (2008) **1123**(64-68.

116. Zhong X, Liu J, Lu F, Wang Y, Zhao Y, Dong S, Leng X, Jia J, Ren H, Xu C, Zhang W: **Calcium sensing receptor regulates cardiomyocyte function through nuclear calcium.** *Cell Biol Int* (2012) **36**(10):937-943.

117. Lu FH, Fu SB, Leng X, Zhang X, Dong S, Zhao YJ, Ren H, Li H, Zhong X, Xu CQ, Zhang WH: **Role of the calcium-sensing receptor in cardiomyocyte apoptosis via the sarcoplasmic reticulum and mitochondrial death pathway in cardiac hypertrophy and heart failure.** *Cell Physiol Biochem* (2013) **31**(4-5):728-743.

118. Liu L, Wang C, Sun D, Jiang S, Li H, Zhang W, Zhao Y, Xi Y, Shi S, Lu F, Tian Y *et al*: **Calhex(2)(3)(1) ameliorates cardiac hypertrophy by inhibiting cellular autophagy in vivo and in vitro.** *Cell Physiol Biochem* (2016) **36**(4):1597-1612.

119. Zhang X, Zhang T, Wu J, Yu X, Zheng D, Yang F, Li T, Wang L, Zhao Y, Dong S, Zhong X *et al*: **Calcium sensing receptor promotes cardiac fibroblast proliferation and extracellular matrix secretion.** *Cell Physiol Biochem* (2014) **33**(3):557-568.

120. Schreckenberg R, Dyukova E, Sitdikova G, Abdallah Y, Schluter KD: **Mechanisms by which calcium receptor stimulation modifies electromechanical coupling in isolated ventricular cardiomyocytes.** *Pflugers Arch* (2015) **467**(2):379-388.

121. Liu CH, Chang HK, Lee SP, Shieh RC: **Activation of the ca(2+)-sensing receptors increases currents through inward rectifier k(+) channels via activation of phosphatidylinositol 4-kinase.** *Pflugers Arch* (2016) **468**(11-12):1931-1943.

122. Schepelmann M, Yarova PL, Lopez-Fernandez I, Davies TS, Brennan SC, Edwards PJ, Aggarwal A, Graca J, Rietdorf K, Matchkov V, Fenton RA *et al*: **The vascular ca2+-sensing receptor regulates blood vessel tone and blood pressure.** *Am J Physiol Cell Physiol* (2016) **310**(3):C193-204.

123. Kienitz M-C, Niemeyer A, König GM, Kostenis E, Pott L, Rinne A: **Biased signaling of ca2+-sensing receptors in cardiac myocytes regulates girk channel activity.** *Journal of Molecular and Cellular Cardiology* (2019) **130**(107-121.

124. Zhou X, Mehta S, Zhang J: **Genetically encodable fluorescent and bioluminescent biosensors light up signaling networks.** *Trends in Biochemical Sciences*.