

Calcium-sensing receptor signaling: it's all about multiplicity

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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.

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Current Opinion in Physiology 2020, **17**:243–254

This review comes from a themed issue on **Calcium signaling**

Edited by **Indu S Ambudkar** and **Aldebaran M Hofer**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 27th August 2020

<https://doi.org/10.1016/j.cophys.2020.08.014>

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Introduction

One of the most versatile messengers 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 subdomains 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 [6–8]. 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 (AP2 σ ; 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 *et al.* 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 *et al.* [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 overstimulate 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, that is, 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-valent and tri-valent cations, such as Mg^{2+} , Sr^{2+} , Ba^{2+} , Gd^{3+} and Al^{3+} [5,26]; 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 Ref. [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 [44,46,47] 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, that is, 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].

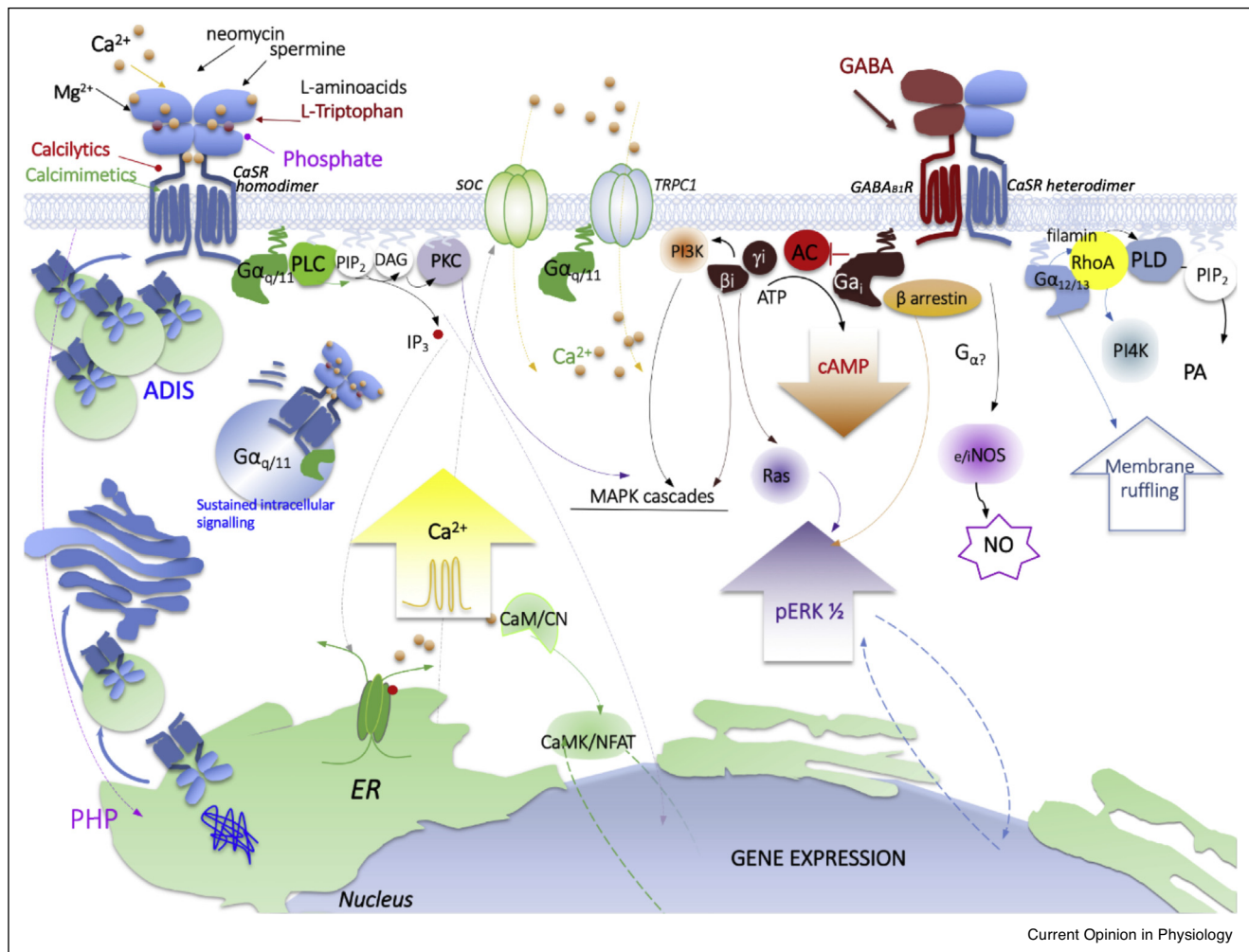
The $G_{i/o}$ pathway by which cAMP levels are reduced [52–54] has also been involved in ERK1/2 phosphorylation/activation via beta gamma-mediated Ras and MAPK activation [55] or G protein-independent β -arrestin recruitment [56*].

The involvement of $G_{q/11}$ 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 have been hotly debated [59].

While this review was being written, a paper from Onopiuk *et al.* 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 $[\text{Ca}^{2+}]_o$, 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+}

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/trafficking 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, extracellular-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.

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 has 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 [65–69]. 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 Ca²⁺ was biased toward cAMP inhibition and IP₃ 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 hypocalcemic hypocalcemia case, which increased the effects of external Ca²⁺ 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 *et al.* demonstrated that germline mutations can alter both the expression and the preference of the CaSR for downstream signaling modes. By using Ca²⁺ mobilization and ERK1/2 phosphorylation as functional read-outs, 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 Ca²⁺ response [72]. Interestingly, it has been shown that also allosteric modulators exhibit stimulus bias, inducing greater activation of intracellular Ca²⁺ 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 past 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 *et al.* 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 Ca²⁺ 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 is 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 clathrin-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 *et al.* [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 clathrin-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’ Gq-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 [89–91], 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/InsP₃ 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 *et al.* [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.

Cyclosporin 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 lipopolysaccharide (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 [101–104], 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, that is, 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 calcilic 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 of the 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 calcilic 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 *et al.* 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 $\text{G}_q/\text{PLC/IP}_3$ pathway.

One year later, Liu *et al.* [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 affect 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, versus rats versus guinea pig). It is clear that adult models possess different characteristics from the neonatal, although each of the models can contribute to the understanding of the pathophysiological 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 bias 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 messenger's 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.

Conflict of interest statement

Nothing declared.

ORCID authorship contribution statement

Isabella Maiellaro: Writing - review & editing. **Roberto Barbaro:** Writing - review & editing. **Rosa Caroppo:** Writing - review & editing. **Matilde Colella:** Conceptualization, Writing - review & editing, Supervision.

Acknowledgements

IM is supported by an Anne McLaren Research Fellowship from the University of Nottingham. We thank Dr. Kevin F. Webb for careful reading of the manuscript.

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