REVIEW

Behind the curtain: cellular mechanisms for allosteric modulation of calcium-sensing receptors

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Calcium-sensing receptors (CaSR) are integral to regulation of systemic Ca²⁺ homeostasis. Altered expression levels or mutations in CaSR cause Ca²⁺ handling diseases. CaSR is regulated by both endogenous allosteric modulators and allosteric drugs, including the first Food and Drug Administration-approved allosteric agonist, Cinacalcet HCl (Sensipar®). Recent studies suggest that allosteric modulators not only alter function of plasma membrane-localized CaSR, but regulate CaSR stability at the endoplasmic reticulum. This brief review summarizes our current understanding of the role of membrane-permeant allosteric agonists in cotranslational stabilization of CaSR, and highlights additional, indirect, signalling-dependent role(s) for membrane-impermeant allosteric drugs. Overall, these studies suggest that allosteric drugs act at multiple cellular organelles to control receptor abundance and hence function, and that drug hydrophobicity can bias the relative contributions of plasma membrane and intracellular organelles to CaSR abundance and signalling.

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Abbreviations

CaSR, calcium-sensing receptor; ERK1/2, extracellular signal-regulated kinase 1 and 2; GABA_B receptor, γ -aminoisobutyric acid receptor; GPCR, G protein-coupled receptor; mGlu, metabotropic glutamate receptor; PTH, parathyroid hormone

Introduction

G protein-coupled receptors (GPCRs) represent a significant fraction of all drug targets for disease prevention and treatment, and remain a major focus for drug development by the pharmaceutical industry. Aside from delivery and clearance issues, the ideal drug would target a specific GPCR in the tissue of interest, with minimal off-target effects. Unfortunately, many GPCRs belong to multi-gene families having the same agonist but diverse tissue distributions and/or signalling outputs. Drugs which effectively interact with the orthosteric site often lack subtype specificity and contribute to unwanted side effects in diverse tissues. Orthosteric drugs may also interact with other cell proteins involved in transport and/or synthesis of the orthosteric ligand. Allosteric drugs, which modulate the effects of the orthosteric ligand, often bind to sites which are less conserved within a GPCR gene family, and therefore provide enhanced specificity and selectivity for activation of specific receptor subtypes and/or biased activation of signalling pathway(s) (reviewed in Smith and Milligan, 2010; Keov *et al.*, 2011). The development and characterization of allosteric modulators for GPCRs has been accelerating in recent years, culminating in the first Food and Drug Administration (FDA)-approved allosteric modulator, Cinacalcet HCl (Sensipar®), which enhances Ca²⁺ activation of calcium-sensing receptor (CaSR) (Brown, 2010). These studies, however, have at their foundation, a plasma membrane-centric view of the cell, and define the role(s) of allosteric modulators strictly in terms of acute regulation of the conformation and hence activation state, of plasma membrane-localized GPCRs. In this brief review, we pull back the curtain, that is, the plasma membrane, and look into the depths of the cell at ways in which allosteric modulators regulate net CaSR signalling at intracellular site(s). Such a holistic view of the cell requires integration of multiple organelle-focused experimental approaches, but is critically

vation of specific receptor subtypes and/or biased activation of signalling pathway(s) (reviewed in Smith and Milligan, 2010; Keov *et al.*, 2011). The development and characterization of allosteric modulators for GPCRs has been accelerating in recent years, culminating in the first Food and Drug Administration (FDA)-approved allosteric modulator, Cinacalcet HCl (Sensipar®), which enhances Ca²⁺ activation of calcium-sensing receptor (CaSR) (Brown, 2010). These studies, however, have at their foundation, a plasma membrane-centric view of the cell, and define the role(s) of allosteric modulators strictly in terms of acute regulation of the conformation and hence activation state, of plasma membrane-localized GPCRs. In this brief review, we pull back the curtain, that is, the plasma membrane, and look into the depths of the cell at ways in which allosteric modulators regulate net CaSR signalling at intracellular site(s). Such a holistic view of the cell requires integration of multiple organelle-focused experimental approaches, but is critically

important for the development of an understanding of potential drug responses *in vivo*, where prolonged exposure to drugs is the norm.

CaSR are allosterically regulated *in vivo*

Endogenous allosteric modulators have been identified for a number of GPCRs, including the muscarinic (Hu *et al.*, 1992; Burgmer *et al.*, 1998), adenosine (Lane *et al.*, 2009) and glucagon-like peptide-1 receptors (Wootten *et al.*, 2011), and most notably, CaSR (reviewed in Saidak *et al.*, 2009a). CaSR is a family 3/C GPCR with structural homology to metabotropic glutamate receptor (mGlu) and GABA_B receptors (Brown and MacLeod, 2001). In common with mGlu receptors, CaSR has a large extracellular domain containing venus flytrap and cysteine-rich modules which bind the orthosteric ligand, and transduce conformational changes to the transmembrane heptahelical domain respectively. CaSR is activated at the orthosteric site within the venus flytrap by Ca²⁺, other di- and trivalent cations (e.g. Mg²⁺, Sr²⁺, Gd³⁺) and polyamines (spermine > spermidine > putrescine) (Quinn *et al.*, 1997), and by Ca²⁺ at a transmembrane domain site (Ray and Northup, 2002; Mun *et al.*, 2004). Allosteric interactions with endogenous modulators (amino acids, protons, glutathione) or drugs (calcimimetics or calcilytics) can occur at sites within the venus flytrap or transmembrane domains (Hu *et al.*, 2000; Miedlich *et al.*, 2004; Mun *et al.*, 2005). While the orthosteric ligand Ca²⁺ is the dominant regulator of CaSR signalling in organs involved in systemic Ca²⁺ homeostasis, including the parathyroid, kidney and bone, multiple studies suggest that allosteric activation may dominate in certain tissues and/or physiological states. Amino acids play a clear role in activation of CaSR in the gastrointestinal tract (Conigrave and Brown, 2006; Feng *et al.*, 2010), while altered polyamines in cancer (Casero and Marton, 2007; Saidak *et al.*, 2009b) or cardiovascular disease (Kawata *et al.*, 2008; Han *et al.*, 2009; Sun and Murphy, 2010) may regulate CaSR expression and/or function. Glutathione contributes to umami taste, transduced by CaSR in taste buds (Ohsu *et al.*, 2010), and can undergo regulated efflux from cells (D'Angelo *et al.*, 2010). Indeed, glutathione and related analogs activate CaSR and suppress parathyroid hormone (PTH) secretion from human parathyroid cells (Broadhead *et al.*, 2011). These examples are by no means exhaustive; readers are directed to some excellent reviews (e.g. Conigrave *et al.*, 2008; Wellendorph *et al.*, 2009). It is clear, however, that CaSR must properly be considered a metabolic sensor which translates the net input of orthosteric and allosteric modulators into tissue- and cell-appropriate responses, and that as a corollary, endogenous allosteric modulators may contribute to regulation of cellular CaSR.

Development of allosteric modulators for CaSR

Acute regulation of CaSR by small molecule allosteric drugs, either agonists (calcimimetics) or antagonists (calcilytics), has been proposed as treatment for a variety of Ca²⁺ handling

disorders. Calcimimetics, which reduce PTH secretion by the parathyroid, have been used in treatment of primary hyperparathyroidism and parathyroid carcinoma (Marcocci *et al.*, 2009; Padhi and Harris, 2009), and secondary hyperparathyroidism in end stage renal disease (Hebert, 2006; Nagano, 2006). Calcilytics have been proposed as an oral treatment for osteoporosis (Nemeth *et al.*, 2001; Hebert, 2006; Marie, 2010). The drive for development of allosteric drugs to modulate CaSR function arises not from the need for specificity among family members, as CaSR has no known isoforms, but rather from the generally low affinity of CaSR for its orthosteric activators. The broad expression profile and lack of CaSR isoforms creates a second problem, that is, the need for specific modulation of particular signalling pathways dominant in the cells or tissues contributing to pathology without collateral activation of CaSR signalling in other cells/tissues, for example, suppression of PTH secretion without the concomitant increases in calcitonin secretion and resulting hypocalcaemia seen in a fraction of patients treated with cinacalcet (Torres, 2006). Development of biased allosteric modulators able to selectively activate a subset of CaSR signalling pathways represents a potential solution to this problem. Finally, reduced expression of CaSR contributes to some Ca²⁺ handling disease phenotypes, for example, parathyroid adenomas (Corbetta *et al.*, 2000), and allosteric modulators which act as pharmacologic chaperones to increase expression of CaSR may be useful in normalizing Ca²⁺ control of PTH secretion.

The first potent calcimimetics able to reduce PTH secretion were the fendilin-based phenylalkylamines, NPS 467 and NPS 568 (Nemeth *et al.*, 1998). These compounds led the way in the dissection of CaSR contributions to signalling responses to Ca²⁺ in various tissues, and provided a framework for development of more bioavailable drugs, culminating in cinacalcet HCl (Sensipar®). Calcimimetic development continues, with most drugs containing α -methyl benzylamines and linked aryl rings (reviewed in Harrington and Fotsch, 2007; Urwyler, 2011). At present, none of the next-generation calcimimetics have been thoroughly investigated for their abilities to bias CaSR signalling, or for their potential role(s) as pharmacologic chaperones. These drugs therefore represent untapped potential for selective modulation of CaSR function and/or signalling.

Osteoporosis can be effectively treated with injections of PTH(1–84) (Pietrogrande, 2010), but the search for effective oral medications continues. Calcilytics, which can 'fool' the parathyroid gland into sensing an apparent reduction in serum Ca²⁺ concentrations, lead to an increase in PTH secretion which is sufficient for generation of anabolic effects on bone in rats (Gowen *et al.*, 2000). The first described calcilytic, NPS 2143, attenuates Ca²⁺-induced signalling in HEK293 cells, increases PTH secretion from acutely isolated bovine parathyroid cells and increases plasma PTH levels when infused into rats (Nemeth *et al.*, 2001). However, the pharmacokinetic profile of NPS 2143 was not compatible with anabolic effects in bone, and has led to successive generations of analogs with more rapid onset and decline of elevated serum PTH in animal models (Arey *et al.*, 2005; Widler *et al.*, 2010). Clinical utility of calcilytics may extend to patients harbouring CaSR gain-of-function mutations (Letz *et al.*, 2010) and diseases exacerbated by increased

expression of CaSR, for example, breast cancer (Saidak *et al.*, 2009b).

Acute allosteric regulation of CaSR signalling at the plasma membrane

First and foremost, allosteric modulators regulate signalling by plasma membrane-localized CaSR. Initial studies on the calcimimetics NPS 467 and NPS R-568 clearly demonstrated potentiation of Ca^{2+} -mediated CaSR activation, leading to elevated intracellular Ca^{2+} in HEK293 cells, and enhanced suppression of PTH secretion by bovine parathyroid cells (Nemeth *et al.*, 1998). Likewise, the calcilytic NPS 2143 antagonizes these same responses (Nemeth *et al.*, 2001). Calcimimetics have been shown to acutely potentiate the whole range of CaSR-mediated signalling pathways *in vitro*, including activation of phospholipases, leading to increased intracellular Ca^{2+} and/or Ca^{2+} oscillations, secretion and activation of protein kinase cascades (reviewed in Brauner-Osborne *et al.*, 2007; Brown, 2010). Allosteric models for the interplay between orthosteric and allosteric sites have been developed (Lu *et al.*, 2009). What is currently lacking is a systematic investigation of the potential for calcimimetic-mediated bias of CaSR signalling, which may provide another layer of specificity for treatment of particular Ca^{2+} handling disorders.

Conformational bias induced by allosteric modulators regulates CaSR stability

Pharmacologic chaperones have been described for a number of GPCRs (reviewed in Conn and Ulloa-Aguirre, 2009), including opioid (Petaja-Repo *et al.*, 2002), vasopressin (Morello *et al.*, 2000) and glycoprotein hormone (Janovick *et al.*, 2009) receptors. Both membrane-permeant, that is, hydrophobic, allosteric agonists and antagonists are effective at stabilizing newly synthesized GPCRs, likely by promoting proper packing of the heptahelical bundle (reviewed in Bernier *et al.*, 2004). Drug contacts with side chains on multiple helices are likely to favour specific packing schemes, allowing the physiological inter-helical contacts to stabilize the native conformation. Treatment of cells with pharmacologic chaperones increases net cellular levels of GPCRs, thus increasing signalling. Such models for pharmacologic chaperones require that the drug be present during biosynthesis of the receptor, so that the earliest forms of competent helical bundles can bind and be stabilized by drug–side chain interactions. A second mode of pharmacologic chaperone action has been described for vasopressin receptors, which can be ‘rescued’ after initial biosynthesis (Wuller *et al.*, 2004; Robben *et al.*, 2007). Receptors which are ‘trapped’ in the ER post-synthesis can be rescued to the plasma membrane by treatment with membrane-permeant allosteric modulators. This mode of post-synthetic rescue may result from drug-induced changes in GPCR conformation that foster forward trafficking through the secretory pathway, analogous to ionotropic

channels which must bind agonist at the ER, that is, forward trafficking to the plasma membrane is predicated upon the ability to achieve a range of conformations consistent with normal function (Penn *et al.*, 2008).

Calcium-sensing receptor is exposed to its orthosteric agonist throughout intracellular organelles and at the plasma membrane. The endoplasmic reticulum is a rich intracellular store of Ca^{2+} required for release during intracellular Ca^{2+} signalling and for proper functioning of luminal chaperones. Estimates of steady-state luminal ER Ca^{2+} are dependent upon the Ca^{2+} indicator used for measurement, but are generally reported in the range of 1–400 μM (Michalak *et al.*, 2002), although additional Ca^{2+} is likely bound to buffers, including calnexin and calreticulin. The ER luminal space also contains glutathione, which participates in redox signalling and disulfide bond formation during biosynthesis (Chakravarthi *et al.*, 2006). Both of these key activators of CaSR also serve critical roles in maintenance of the general ‘health’ of the ER. Indeed, treatment of cells with thapsigargin to decrease ER Ca^{2+} content or redox reagents to alter glutathione levels elicits ER stress and the unfolded protein response, which alters protein translation rates and up-regulates molecular chaperones (Pereira *et al.*, 2010). Thus, studying CaSR biosynthesis by modulating either ER Ca^{2+} or glutathione levels would significantly perturb the biosynthetic landscape, making the resulting alterations in net CaSR difficult to interpret. The binding site(s) within the transmembrane domain of CaSR for allosteric modulators contain hydrophobic side chains (Miedlich *et al.*, 2004), and many drugs participate in π – π interactions with aromatic side chains within the binding pocket (Miedlich *et al.*, 2004; Bu *et al.*, 2008). The general hydrophobic character of CaSR allosteric modulators suggested the possibility for an intracellular role(s) as pharmacologic chaperones, which would be predicted to potentiate the effects of ER Ca^{2+} and glutathione on CaSR conformation during or immediately after biosynthesis. We therefore tested the effects of the calcimimetic NPS R-568 or calcilytic NPS 2143 on net CaSR biosynthesis at normal cellular levels of ER Ca^{2+} and glutathione (Huang and Breitwieser, 2007). Both NPS drugs are predicted to share a subset of side chain contacts in the heptahelical domain (Miedlich *et al.*, 2004), we therefore assumed that each should increase the proportion of properly folded CaSR. As expected for a pharmacologic chaperone, NPS R-568 stabilized CaSR, proportionally increasing both net and plasma membrane levels of receptor. In contrast, NPS 2143 caused a significant reduction in net CaSR. These experiments required incubation of CaSR-expressing cells with allosteric modulators for extended periods, leading to net changes in CaSR quantifiable on Western blots. The results imply that the conformational bias imposed by chronic exposure to either positive or negative allosteric modulators altered the stability of the cellular pool of CaSR, and led directly to the hypothesis of a conformational checkpoint in CaSR biosynthesis. In principle, such a checkpoint would eliminate receptors with a reduced ability to respond to Ca^{2+} and/or glutathione. Because CaSR functions as an obligate disulfide-linked dimer which forms in the ER, early elimination of misfolded CaSR monomers incapable of achieving the active conformation would reduce formation of dysfunctional dimers which might be released to the plasma membrane.

Cotranslational regulation of CaSR by membrane permeant allosteric modulators

Initial evidence for a conformational checkpoint was derived from steady-state measures of net cellular CaSR in response to chronic exposure to allosteric modulators (Huang and Breitwieser, 2007). Unfortunately, such effects might be mediated at diverse stages in the CaSR life cycle, that is, at the ER through an increased success rate during biosynthesis, by stabilizing CaSR at the plasma membrane, or by increased recycling rather than targeting to the lysosome. To define the molecular mechanism(s) which contribute to allosteric modulation of net cellular CaSR, we used [35 S]cysteine pulse labelling methods to mark and track newly synthesized CaSRs throughout their life cycle (Cavanaugh *et al.*, 2010). We reasoned that if initial folding and ER quality control events could be influenced by the presence of the calcimimetic NPS R-568, we should observe an increased rate of [35 S]CaSR generation, monitored by variable length exposures to [35 S]cysteine. Rates of [35 S]CaSR appearance were doubled in the presence of 10 μ M NPS R-568 compared with the DMSO control, strongly suggesting that newly synthesized [35 S]CaSR interacted with and was stabilized by NPS R-568. Comparable results were obtained when we analysed wild-type (wt) CaSR and gain- and loss-of-function mutants having higher and lower sensitivities to Ca^{2+} respectively. The rates of [35 S]cysteine incorporation varied over a tenfold range, with loss-of-function < wt < gain-of-function mutants (Cavanaugh *et al.*, 2010). Thus, bias towards the active conformation, induced either by a calcimimetic acting on wt CaSR or by gain-of-function mutants, increases the cotranslational stability of CaSR during or immediately after biosynthesis. Attesting to the ER-localized role for NPS R-568 in this context, we performed similar experiments using a highly cationic agonist of CaSR, neomycin sulphate, which had no significant effects on the rate of [35 S]CaSR biosynthesis.

The calcimimetic NPS R-568 might also increase plasma membrane CaSR abundance by facilitating exit from the ER, in a manner analogous to ionotropic receptors for glutamate (Penn *et al.*, 2008; Coleman *et al.*, 2009; Kenny *et al.*, 2009). To address this possibility, [35 S]CaSR was followed during a 24 hr chase period in the presence of DMSO or NPS R-568. While the rate of maturation of [35 S]CaSR was slow, that is, <50% maturation after 24 h chase, NPS R-568 significantly increased the rate of appearance of maturely glycosylated CaSR, an effect not seen with neomycin sulphate or elevated extracellular Ca^{2+} . Overall, these results argue for distinct intracellular roles for membrane-permeant calcimimetics as both cotranslational stabilizers to increase net cellular CaSR, and as facilitators of entry into the secretory pathway for delivery to the plasma membrane. The rapidity with which membrane-permeant calcimimetics act to increase cellular CaSR argues that the distinctions between 'acute' effects on signalling and 'long-term' effects on biosynthesis and/or trafficking must be reconsidered.

Allosteric modulators have indisputable effects on plasma membrane-localized CaSR signalling, as reviewed in a previous section. An additional effect of allosteric modulators on net cellular CaSR may therefore derive from plasma

membrane-initiated signalling. Circumstantial evidence for such an effect is seen in chase time courses for [35 S]CaSR in the presence of NPS R-568. Accelerated rates of [35 S]CaSR maturation in the presence of NPS R-568 are observed after ~36 h of transient transfection, coincident with the arrival of CaSR at the plasma membrane (Cavanaugh *et al.*, 2010). In addition, neomycin sulphate is effective at increasing [35 S]CaSR abundance during [35 S]cysteine pulses only in the presence of plasma membrane-localized CaSR, that is, at 48 h after transfection. To directly address the potential for plasma membrane G_q -coupled signalling as a contributor to CaSR biosynthesis, we tested whether the orthosteric agonist Ca^{2+} had similar effects, and further, whether other G_q -coupled GPCRs endogenously expressed in HEK293 cells could increase CaSR abundance. Figure 1 illustrates the experimental approach and results. HEK293 cells expressing wt human FLAG-CaSR were labelled with [35 S]cysteine for 60 min under control conditions, in the presence of CaSR agonists (300 $\mu\text{g}\cdot\text{mL}^{-1}$ neomycin sulphate or 5 mM total Ca^{2+}) or GPCR agonists (100 μM carbachol, histamine or ATP). [35 S]CaSR generated during the pulse was imaged, and the same blots were probed for both total CaSR and GAPDH, for normalization. A representative experiment is illustrated, and data from three to seven independent experiments were quantified and plotted. The CaSR agonists increased [35 S]CaSR accumulation by >50%, and activation of other G_q -coupled GPCRs also significantly increased synthesis of [35 S]CaSR. The relative enhancement of [35 S]CaSR synthesis in response to agonists of other GPCRs may reflect different expression levels of the various GPCRs, and/or differences in their abilities to activate the signalling pathway(s) which control CaSR synthesis. Overall, these results argue for signalling-dependent feedback on CaSR biosynthesis and/or cotranslational stability, via a mechanism distinct from the pharmacologic chaperone-dependent effects observed for membrane-permeant calcimimetics such as NPS R-568. A working model incorporating both types of regulation is illustrated in Figure 2. Further, these results hint at the potential for coupling between signalling and GPCR biosynthesis which likely extends beyond CaSR.

Signalling-mediated regulation of protein synthesis has been extensively characterized (reviewed in Proud, 2007; Musnier *et al.*, 2010). Distinct signalling pathways activated by G_q -coupled GPCRs lead to phosphorylation cascades which ultimately impinge upon phosphoinositide 3 kinase, extracellular signal-regulated kinase 1 and 2 (ERK1/2) and mammalian target of rapamycin, leading to enhanced translation initiation (Musnier *et al.*, 2010). Arrestins also participate in GPCR signalling-regulated increases in translation rates by promoting activation of ERK1/2, Mnk1 and eIF4E (DeWire *et al.*, 2008). G protein-mediated signalling pathway activation increases the complement of active cellular protein kinases which may phosphorylate newly synthesized CaSR, fostering protein interaction(s) which may protect against interactions with ubiquitin ligases. Alternatively, phosphorylation of key residues may itself be sufficient to block interaction with ubiquitin ligases. Such cotranslational phosphorylation stabilizes multi-subunit protein kinase complexes in yeast (Farrell and Morgan, 2000), and promotes proper folding and prevents Akt ubiquitination during synthesis (Oh *et al.*, 2010). While the key signalling events which acutely up-regulate CaSR are not yet defined, it is likely that regulation of both translation

Figure 1

Activation of plasma membrane-localized G_q -coupled GPCRs increases CaSR biosynthesis. Activation of plasma membrane GPCRs, including CaSR, muscarinic, histamine or purinergic receptors, which couple to G_q , increases [35 S]cysteine incorporation into [35 S]CaSR during a 60 min labelling period. HEK293 cells were transfected with FLAG-CaSR for 48 h prior to starvation and labelling with [35 S]cysteine as previously described (Cavanaugh *et al.*, 2010). After immunoprecipitation of both FLAG-CaSR and GAPDH (for normalization), blots were exposed to measure [35 S]CaSR, then probed with anti-CaSR and anti-GAPDH antibodies. Plot indicates average of three to seven independent experiments, normalized to control conditions (no additions) of each experiment. Cells were treated with a total of 5 mM Ca^{2+} , 300 μ g·mL $^{-1}$ neomycin sulphate, or 100 μ M of either carbachol, histamine or ATP, added acutely at the time of initiation of [35 S]cysteine labelling period. Data were compared with one-way ANOVA; Ca^{2+} , carbachol and ATP treatments ($n = 7$ each) showed significance at $P = 0.034$. Data for neomycin and histamine ($n = 3$ each) showed a similar trend but did not reach significance. CaSR, calcium sensing receptor.

Figure 2

Model for multiple sites for modulation of CaSR protein stability and function. Membrane-permeant allosteric drugs can influence CaSR abundance at the ER by biasing conformations, leading to either increased (calcimimetics) or decreased (calcilytics) stabilization of newly synthesized CaSR. In cells expressing CaSR at the plasma membrane, these drugs can also influence CaSR biosynthesis by regulation of translation initiation and/or post-translational modifications, in addition to directly regulating function of plasma membrane-localized CaSR. GPCRs which activate G_q -dependent signalling can also evoke indirect, signalling-dependent modulation of CaSR biosynthesis, by regulating either translation initiation and/or post-translational modifications affecting stability. CaSR, calcium sensing receptor; GPCR, G protein-coupled receptor.

initiation rates and post-translational quality control mechanisms may be altered by plasma membrane GPCR signalling, and that such mechanisms will be generalizable to biosynthetic regulation of most GPCRs.

Allosteric modulators regulate wt and mutant CaSR abundance at the plasma membrane

Calcium handling diseases result from either CaSR mutations or alterations in expression levels of wt CaSR (reviewed in Tfelt-Hansen and Brown, 2005). Familial hypocalcaemic hypercalcaemia, FHH (OMIM 14598), an autosomal dominant disorder resulting from a loss-of-function mutation in a single allele of CaSR, causes mild to moderate hypercalcaemia with hypocalcaemia due to enhanced renal Ca^{2+} resorption. Homozygous or distinct heterozygous loss-of-function CaSR mutations cause neonatal severe primary hyperparathyroidism, NSHPT (OMIM 239200), with severe hypercalcaemia, multiple fractures, respiratory difficulties, dehydration and hypotonia. Activating mutations of CaSR result in autosomal dominant hypocalcaemia (OMIM 601298) or Bartter's syndrome type V (OMIM 601199.0035), characterized by hypocalcaemia, low serum PTH levels and hypercalcaemia which may lead to nephrolithiasis. FHH can also be caused by autoantibodies against the extracellular domain of CaSR (Brown, 2009). The most common polymorphisms of CaSR (A986S, R990G, Q1011E) are clustered in a 25 amino acid stretch of the carboxyl terminus, and can predispose individuals to a variety of complex diseases dependent on Ca^{2+} metabolism (Yun *et al.*, 2007), and may alter patient sensitivity to calcimimetic therapy (Rothe and Mayer, 2006). Development of specific treatments to alleviate symptoms resulting from CaSR mutations or altered expression has progressed slowly, from the modulation of symptoms with general treatments to regulate

serum Ca^{2+} , to specific use of CaSR allosteric modulators to rescue expression levels or function of CaSR mutants.

Enhanced functional activity (IP generation, increases in intracellular Ca^{2+}) in the presence of NPS R-568 has been demonstrated for wt CaSR and gain-of-function mutations localized to the extracellular domain (Hauache *et al.*, 2000), and for loss-of-function mutations throughout the CaSR primary sequence (Huang and Breitwieser, 2007; Rus *et al.*, 2008; Lu *et al.*, 2009; White *et al.*, 2009; Cavanaugh *et al.*, 2010). Conversely, the calcilytic NPS 2143 normalized the activities of gain-of-function mutants (Letz *et al.*, 2010). Discrimination of direct effects of calcimimetics/calcilytics on plasma membrane-localized CaSR from cellular effects on CaSR stability and/or trafficking have been documented in only a few studies (Timmers *et al.*, 2006; Huang and Breitwieser, 2007; White *et al.*, 2009; Cavanaugh *et al.*, 2010; Reh *et al.*, 2011). Prolonged incubation of cells with calcimimetics in experiments designed to monitor IP metabolism result in increased maximal responses for a subset of loss-of-function mutants (Lu *et al.*, 2009). Such increases may be attributable to allosteric agonism as modelled by Lu *et al.* (2009), that is, potentiation of Ca^{2+} efficacy at low receptor reserve, but may also contain a component related to cellular CaSR stability and/or trafficking. The good news from these studies is that both wt CaSR and a large proportion of gain- and loss-of-function mutations identified in patients can be 'normalized' by treatment with the appropriate calcimimetic or calcilytic. Further dissection of the mechanism(s) contributing to rescue of individual CaSR mutant phenotypes, and continued development of a range of CaSR allosteric modulators with signalling and/or permeation biases, will ultimately permit a pharmacogenomic approach to treatment of CaSR-initiated Ca^{2+} handling diseases.

Prospects for development of biased allosteric drugs for CaSR

Calcium-sensing receptor presents significant challenges as a drug development target. First and foremost, a single gene encodes this widely expressed GPCR, and only one splice variant in the coding region (deletion of exon 5) has been described (Yun *et al.*, 2007). Thus, tissue and/or cell specificity of drugs will be difficult to achieve. Second, the orthosteric and allosteric endogenous agonists are generally of low affinity, and most cells expressing CaSR are exposed to agonist(s) in a chronic manner. Finally, the binding pockets within the heptahelical domain which have emerged as the primary focus for drug development contain hydrophobic side chains which limit either affinity of the more hydrophilic candidates or solubility of the more hydrophobic ones. Despite these limitations, cinacalcet HCl became the first FDA-approved allosteric drug targeting a GPCR, and development of calcilytics effective in treatment of osteoporosis is underway. The current review highlights some additional difficulties (or opportunities) which present themselves in terms of drug development. The growing emphasis in drug development on conformational bias which favours specific signalling pathways may be applicable to CaSR, particularly as amino acids have been shown to induce intracellular Ca^{2+} oscillations having distinct phenotypes and underlying signalling pathways (Young and

Rozengurt, 2002; Rey *et al.*, 2005). We would here argue that bias of another type might be introduced by appropriate phenotypic screening, that is, the relative hydrophobicity of drug candidates will likely influence the relative importance of plasma membrane versus intracellular organellar contributions to net cellular CaSR and hence function.

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Conflicts of interest

None.

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