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Review

Therapeutic Opportunities of Targeting Allosteric Binding Sites on the Calcium-Sensing Receptor

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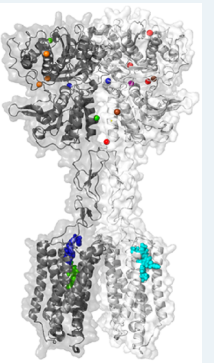
ABSTRACT: The CaSR is a class C G protein-coupled receptor (GPCR) that acts as a multimodal chemosensor to maintain diverse homeostatic functions. The CaSR is a clinical therapeutic target in hyperparathyroidism and has emerged as a putative target in several other diseases. These include hyper- and hypocalcaemia caused either by mutations in the *CASR* gene or in genes that regulate CaSR signaling and expression, and more recently in asthma. The development of CaSR-targeting drugs is complicated by the fact that the CaSR possesses many different binding sites for endogenous and exogenous agonists and allosteric modulators. Binding sites for endogenous and exogenous ligands are located throughout the large CaSR protein and are interconnected in ways that we do not yet fully understand. This review summarizes our current understanding of CaSR physiology, signaling, and structure and how the many different binding sites of the CaSR may be targeted to treat disease.



KEYWORDS: *CaSR, hyperparathyroidism, asthma, CASR gene, FHH, ADH, osteoporosis, allosteric modulator*

The CaSR is ubiquitously expressed in the human body but is found abundantly in the parathyroid glands and kidney. In these organs, the CaSR is responsible for exquisite control of extracellular Ca^{2+} (Ca^{2+}_o) to maintain systemic ionized Ca^{2+}_o concentrations within 1.2−1.4 mM (reviewed in ref 1). The CaSR negatively regulates parathyroid hormone (PTH) secretion in response to elevated Ca^{2+}_o. When Ca^{2+}_o concentrations rise, CaSR-mediated suppression of PTH synthesis and secretion decreases Ca^{2+} resorption from bone and Ca^{2+} reabsorption in the renal thick ascending limb of the loop of Henle. Within the kidneys, the CaSR responds to elevated Ca^{2+}_o independently of PTH to further decrease Ca^{2+} reabsorption.[2] Elevated Ca^{2+}_o concentrations are thus reduced. The CaSR is expressed in additional tissues involved in Ca^{2+}_o homeostasis. In the thyroid, elevated Ca^{2+}_o stimulates calcitonin release via the CaSR,[2] leading to Ca^{2+} uptake into bone. The CaSR also promotes differentiation and proliferation of bone-forming osteoblasts,[3] inhibits osteoclast-mediated bone resorption,[4] and facilitates chondrocyte-mediated skeletal growth and development.[5] In mammary epithelial cells, the CaSR mediates Ca^{2+} transport into milk[6] and suppresses mammary gland release of parathyroid hormone-related protein to reduce osteoclast-mediated release of Ca^{2+} from bone.[7]

The pivotal role of the CaSR in Ca^{2+}_o homeostasis is well-established; however, the CaSR also responds to additional stimuli to mediate a number of noncalciotropic functions. In taste buds, the CaSR responds to food-derived γ-glutamyl peptides to enhance certain tastes.[8] The CaSR is expressed along the entire gastrointestinal (GI) tract, where it senses amino acids to regulate inflammatory responses[9] as well as nutrient intake and digestion via the release of satiety and pancreatic hormones.[10,11] CaSRs in the pancreas also contribute to glucose-mediated insulin secretion, thus helping to maintain blood glucose levels.[12] In the vasculature, CaSR activation on vascular smooth muscle leads to vasodilatation, thus contributing to blood pressure control.[13] In the skin, the CaSR promotes keratinocyte differentiation, barrier function, and wound healing.[14] CaSRs located in the lungs detect fluctuations in local polyamines to mediate airway defense mechanisms such as airway contraction and inflammation.[15] In addition to responding to different stimuli in a tissue specific manner, CaSR expression in both calciotropic and non-calciotropic tissues is controlled in a tissue- and environment-specific manner. *CASR* gene promoters contain response elements for 1,25-dihydroxyvitamin D, pro-inflammatory cytokines, and the parathyroid cell-specific transcription factor, glial cells missing-2. Consequently, CaSR expression may be

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increased in inflammation and decreased in states of vitamin D deficiency.[16,17] The many diverse CaSR functions and changes in CaSR expression during pathophysiological situations highlight that the CaSR is a multifunctional chemosensor in human (patho)physiology. This review will focus on how its many allosteric ligands bind to the CaSR and how allosteric binding sites have been targeted to manipulate CaSR activity in disease.

CASR SIGNALING

The CaSR couples primarily to $G_{i/o}$ and $G_{q/11}$ G proteins to reduce cyclic adenosine monophosphate (cAMP) levels[18] and trigger the release of intracellular Ca^{2+} (Ca^{2+}_i) from stores, respectively (Figure 1). The CaSR also increases Ca^{2+}_i via

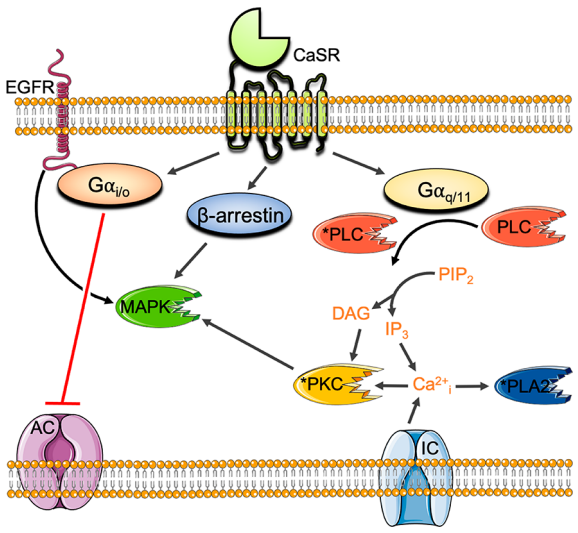

Figure 1. Principal CaSR signaling pathways. CaSR activation of $G_{i/o}$ inhibits adenylyl cyclase (AC) to reduce cAMP levels. CaSR coupling to $G_{q/11}$ activates phosphatidylinositol-specific phospholipase C (PI–PLC) to increase inositol triphosphate (IP_3) and diacyl glycerol (DAG) and trigger the release of Ca^{2+}_i from stores. Ca^{2+}_i activates phospholipase A_2 (PLA_2) and PKC. The CaSR also increases Ca^{2+}_i via influx through L-type voltage-gated and transient receptor potential ion channels (IC), in part via PKC. The CaSR activates MAPK signaling cascades via $G_{q/11}$-mediated PKC, $G_{i/o}$-mediated activation of epidermal growth factor receptor (EGFR), and β arrestin.

influx through L-type voltage-gated and transient receptor potential ion channels on the plasma membrane,[19−21] in part via a PKC-dependent mechanism.[20]

In addition to canonical G protein coupling, the CaSR stimulates mitogen activated protein kinases (MAPK) downstream from $G_{q/11}$, $G_{i/o}$, and β arrestin.[22−25] In some cell types, the CaSR couples to $G_{12/13}$,[26] although the (patho)-physiological relevance is unknown. The CaSR also activates G_s proteins in immortalized or malignant breast cells[27] and in murine pituitary corticotroph-derived AtT-20 cells,[28] resulting in increased cAMP production in these cell types. Thus, the CaSR is promiscuously coupled to several different G protein families, adding even greater pharmacological complexity.

Due to its promiscuous G protein coupling preferences, the CaSR is subject to biased agonism whereby distinct ligands activate or inhibit a subset of possible signaling pathways linked to the CaSR to the relative exclusion of others.[25,29,30] While the physiological relevance of biased CaSR agonism is not known, it likely plays an important role in regulating CaSR activation by its many varied endogenous ligands. Small molecule allosteric ligands also engender biased modulation at the CaSR (discussed in more detail below), demonstrating that CaSR function may be fine-tuned with allosteric drugs.

STRUCTURE

The CaSR protein is encoded by 7 exons and is expressed as a 1078 amino acid polypeptide. Exons 2−6 encode the large extracellular domain (ECD), while exon 7 encodes the 7 transmembrane (7TM)-spanning region and C-terminal tail. The CaSR forms a homodimer mediated by covalent and noncovalent interactions.[31,32] The ECD contains a bilobed "venus flytrap" (VFT) domain, so-called because the two lobes (named LB1 and LB2) open and close around a ligand-binding cleft[31,33] much like the VFT plant closes around its prey (Figure 2).

The VFT is linked via a cysteine-rich domain (CRD) to the 7TM and its connecting intracellular and extracellular loops (ICL and ECL, respectively). The structural integrity of the 7TM is in part maintained by a disulfide bond between C677 in ECL1 and C765 in ECL2.[34] The 7TM is proceeded by a large C-terminal tail that contributes to cell surface expression, signaling, and binding to accessory proteins.[35−37] Further, C-terminal tail residues (S875 and T888) are predicted to be key protein kinase C (PKC) phosphorylation sites, which serve to negatively regulate CaSR activity.[38,39]

ENDOGENOUS CASR LIGANDS AND THEIR BINDING SITES

The primary physiological ligand of the CaSR is Ca^{2+}. X-ray crystallography combined with anomalous scattering analysis suggest Ca^{2+} binds to four sites within the VFT domain (Figure 2)[31] in a cooperative manner, such that binding to one site positively modulates Ca^{2+} binding to the other sites.[40] A loss in Ca^{2+}_o agonism was observed upon mutation of residues located in the four Ca^{2+} sites, although the effects of mutations on receptor expression, Ca^{2+}_o affinity, and Ca^{2+}_o efficacy were not delineated. Consequently, it is unclear to what extent these mutations alter Ca^{2+}_o binding to the CaSR and therefore whether these sites are physiologically relevant or simply an artifact of the crystallization conditions. Ca^{2+} also binds to at least one site in the 7TM or ECLs, evidenced by the fact that Ca^{2+}_o retains agonist activity at a CaSR lacking its entire ECD.[41] Thus, while Ca^{2+}_o is considered the orthosteric agonist, strictly speaking Ca^{2+}_o is an allosteric modulator of itself. In addition to Ca^{2+}_o, the CaSR responds to additional divalent cations, including Mg^{2+}, as well as trivalent cations such as Gd^{3+}.[42,43] Structural studies suggest a Mg^{2+} binding site that overlaps with a Ca^{2+} binding site in the VFT, as well as other distinct binding sites for Mg^{2+} and Gd^{3+}[33] (Figure 2). However, anomalous scattering analysis was not used to assign Mg^{2+} or Gd^{3+} in the CaSR VFT crystal structure and can therefore be made with less confidence. Importantly, all six cation binding sites are topographically distinct from the VFT cleft between LB1 and LB2, which is the orthosteric agonist binding site in all other class C GPCRs.

The CaSR VFT cleft is the binding site for endogenous positive allosteric modulators (PAMs), which include aromatic L-amino acids[44] and γ-glutamyl peptides.[45] Larger aliphatic and aromatic L-amino acids, in particular L-Phe and L-Trp, are the most potent PAMs at the CaSR.[44] CaSR VFT domain structures solved by X-ray crystallography suggest the fully

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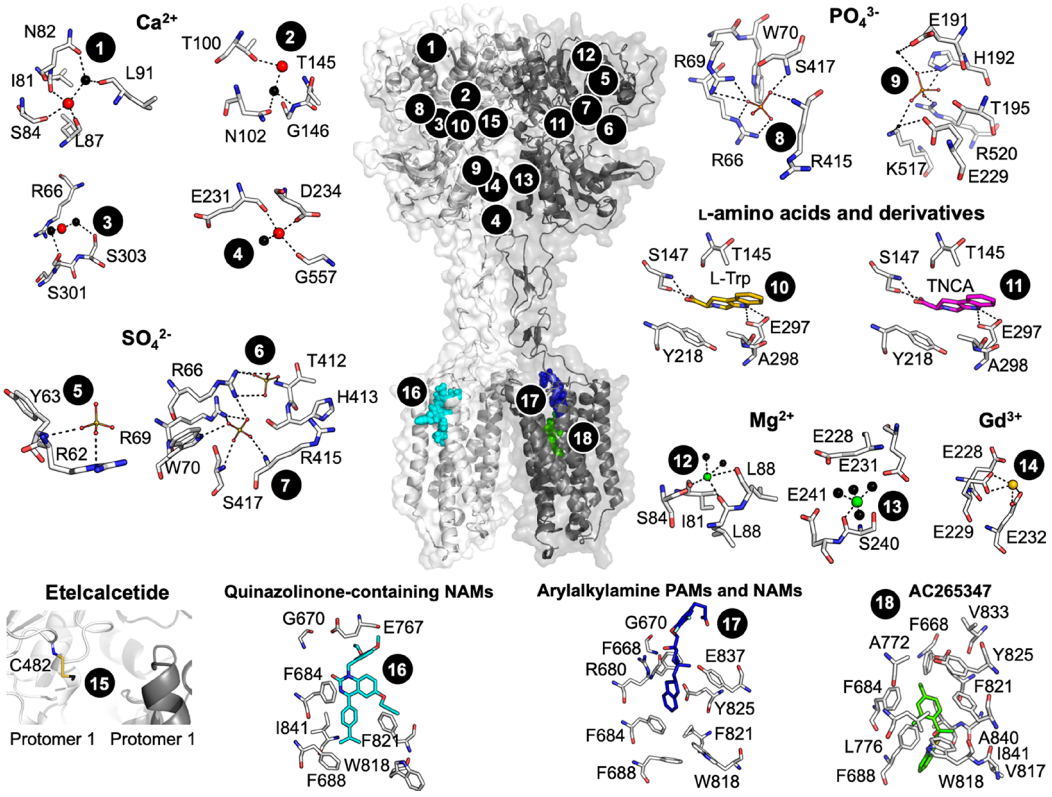

Figure 2. CaSR model and predicted ligand binding sites. The published crystal structure of the CaSR ECD (PDB 5K5S) was superimposed onto a published model of the CaSR 7TM, ECLs, and ICLs[71] based on homology with the $mGlu_5$ crystal structure (PDB 6N51). Numbers correspond to ligand binding sites predicted as follows: Ca^{2+} (sites 1−4) by anomalous scattering analysis and SO_4^{2-} (5−7), PO_4^{3-} (8−9), and L-Trp (10) by electron density distribution analysis of the crystallized ECD (PDBs 5K5S and 5K5T);[31] TNCA (11), Mg^{2+} (12−13), and Gd^{3+} (13) by electron density distribution analysis of the crystallized VFT (PDBs 5FBK and 5FBH);[33] etelcalcetide (15) from mutagenesis and mass spectrometry, where the yellow stick represents a putative disulfide bond as a rough depiction of where etelcalcetide is predicted to bind;[66] quinazolinone-containing NAMs (16), arylalkylamine PAMs and NAMs (17), and AC265347 (18) from mutagenesis combined with homology modeling and computational docking.[41,71]

active (closed) VFT conformation only exists when the cleft is occupied by an L-amino acid or similar entity.[31,33] The CaSR also responds to other positively charged endogenous ligands that interact with allosteric sites. These include polyamines such as spermine, spermidine, and putrescine.[46] Polyamines activate the CaSR in the absence of Ca^{2+}_o and are therefore agonists, but they may also act as PAMs.[46] The binding site for polyamines has not been elucidated, but it is located somewhere in the 7TM, ECLs, or ICLs.[47]

In addition to numerous endogenous activators, anions such as phosphate and sulfate, protons, or elevated osmolarity all serve to inhibit CaSR activity.[31,48−50] While anions bind in the CaSR VFT,[31] the site of action of protons is unknown.[49] These findings suggest potential clinical implications for changes in the CaSR's environment, which may occur in pathophysiological states such as alkalosis, elevated serum phosphate observed in chronic kidney disease, or increased osmolarity as occurs during dehydration. Altered levels of endogenous CaSR activators or inhibitors in different physiological or disease states may also impact discovery and validation of CaSR small molecule allosteric ligands.

EXOGENOUS ALLOSTERIC LIGANDS AND THEIR BINDING SITES

Small Molecule PAMs. Given that the endogenous ligands of the CaSR bind to a number of distinct sites in a cooperative manner, it is unsurprising that the CaSR possesses several distinct allosteric sites for exogenous molecules. The first CaSR-targeting small molecules discovered were the calcium channel blockers fendiline and prenylamine (Figure 3), which gave rise to the arylalkylamine CaSR PAMs, NPS R-467, NPS R-568, and cinacalcet (Figure 3, reviewed in ref 51). Several structurally related CaSR PAMs have since been identified, including evocalcet, calindol, and variants thereof (Figure 3). Arylalkylamine PAMs potentiate CaSR-mediated Ca^{2+}_i mobilization in recombinant cells or suppress PTH secretion from parathyroid cells in culture in the presence of physiological Ca^{2+}_o concentrations with reduced potency or affinity in the absence of Ca^{2+}_o.[52−54] However, high arylalkylamine concentrations (>1 μM) activate the CaSR in the absence of cations,[51] indicating arylalkylamines are most accurately referred to as PAM agonists. The fact that the CaSR functions in the absence of Ca^{2+}_o raises the question of whether endogenous agonists can activate the CaSR by binding to the small molecule allosteric binding site.

In the absence of a CaSR structure with a bound allosteric modulator, mutagenesis studies combined with computational docking have predicted the binding site of arylalkylamine PAMs.[41,53,55−58] Due to a lack of radiolabeled ligands, early drug discovery campaigns did not determine the affinity of CaSR PAMs; therefore, initial binding site predictions were based on the effect of mutations on PAM potency for potentiation of a single Ca^{2+}_o concentration.[55−58] However, potency changes do not discern individual effects of mutations

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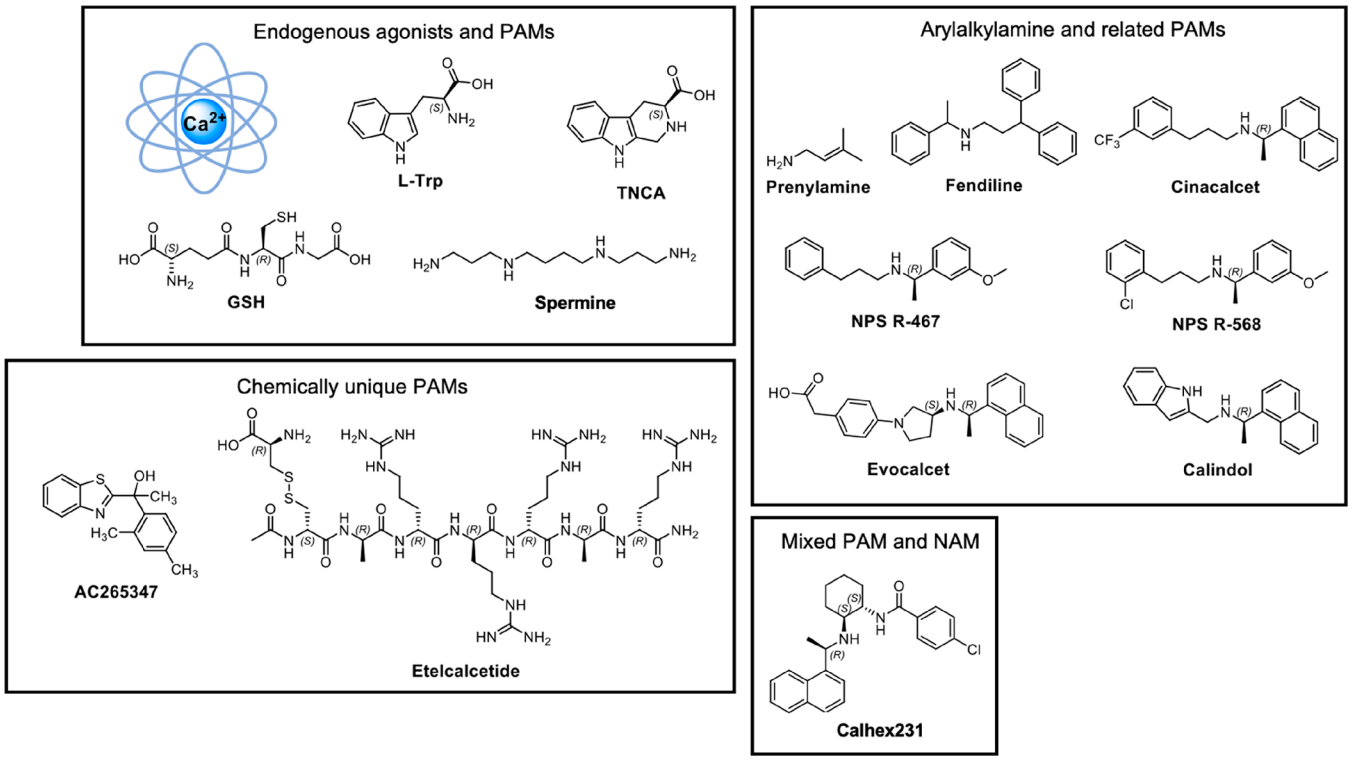

Figure 3. CaSR agonists and PAMs and their structures.

on affinity, cooperativity or efficacy. More recent studies have quantified the effect of amino acid substitutions using an operational model of allosterism[59,60] (Figure 4). Key amino

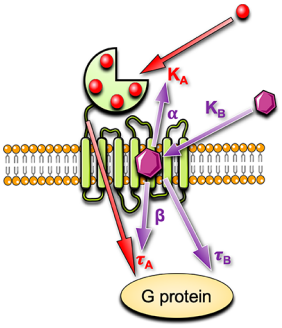

Figure 4. An operational model of allosterism to quantify CaSR agonist, PAM, and NAM actions. Endogenous CaSR agonists such as Ca^{2+}_o bind with a mean equilibrium dissociation constant, K_A, to multiple sites. When the receptor is occupied by an agonist, the agonist stimulates a response, depicted by an operational measure of efficacy, τ_A. Allosteric modulators may alter the equilibrium dissociation constant of the agonist via a cooperativity factor, α, or alter the efficacy of the orthosteric agonist via a scaling factor, β. Allosteric modulators may also have their own efficacy, τ_B.

acid residues that contribute to the affinity of arylalkylamine PAMs are located in TMs 2, 3, 5, 6, and 7 as well as ECLs 2 and 3, where numbering in superscript throughout this manuscript denotes residue positions relative to the most highly conserved residue in each TM domain across the class C GPCRs;[61] $F668^{2.56}$, $F684^{3.36}$, $F688^{3.40}$, $A772^{5.39}$, $W818^{6.50}$, $F821^{6.53}$, $Y825^{6.57}$, $E837^{7.32}$, $A840^{7.35}$, $I841^{7.36}$, $E767^{ECL2}$, and $V833^{ECL3}$.[41,53] Mapping these residues onto a homology model of the CaSR based on the metabotropic glutamate receptor subtypes 1 and 5 ($mGlu_1$ and $mGlu_5$) crystal structures revealed a large cavity that spans from the top to the middle of the 7TMs (Figure 2).[41] The arylalkylamine PAM secondary amine is protonated at physiological pH to form an ammonium salt that facilitates PAM binding by hydrogen bonding with $E837^{7.32}$ in addition to forming a strong electrostatic interaction.[41,53,55−58] The predicted 7TM binding pocket of CaSR arylalkylamine PAMs is commensurate with that observed for the binding site of a NAM cocrystallized with $mGlu_1$[62] and common across the class C GPCR family.

In 2010, Acadia Pharmaceuticals discovered benzothiazole-containing CaSR PAMs that were structurally and chemically distinct from the arylalkylamine PAMs, leading to the identification of AC265347 (Figure 3).[63,64] Like the arylalkylamines, AC265347 demonstrates agonist activity in the absence of cations, but it is a more potent agonist when compared to arylalkylamine PAMs.[54] AC265347 is also a biased CaSR allosteric modulator that preferentially enhances CaSR-mediated phosphorylation of ERK1/2 (pERK1/2) versus Ca^{2+}_i mobilization.[54] In contrast, phenylalkylamine PAMs show the reverse biased modulatory profile.[54] While AC265347 is predicted to bind within the 7TM cavity, it is unaffected by many of the mutations that reduce arylalkylamine affinity.[41,53] Importantly, AC265347 lacks an ionizable nitrogen and is therefore not predicted to form an ionic interaction with $E837^{7.32}$. Computational docking studies supported by mutagenesis suggest that AC265347 sits deeper in the 7TM bundle in comparison to the arylalkylamine PAMs[41] (Figure 2). By binding deeper within the 7TM bundle, AC265347 may stabilize distinct receptor states relative to arylalkylamine PAMs, engendering biased CaSR signaling.

The Peptide PAM, Etelcalcetide. In addition to small molecule PAMs, etelcalcetide was identified as a unique CaSR PAM, being an octapeptide comprising a linear chain of seven

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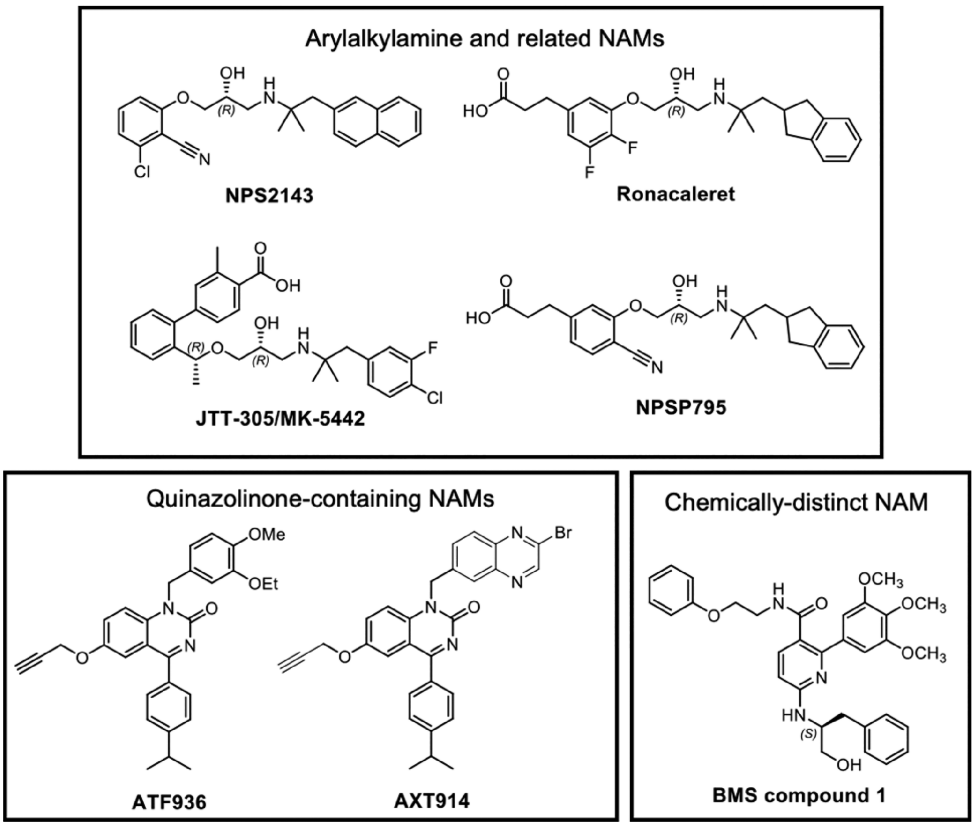

Figure 5. CaSR NAMs and their structures.

D-amino acids linked to a L-cysteine via a disulfide bond.[65] Both the C- and N-terminus are capped and the D-amino acid backbone is attached to four D-Arg residues. Etelcalcetide is predicted to bind to the CaSR VFT by forming a disulfide bond with C482 located near a "hinge" region in VFT LB1 that mediates VFT closure.[66] In the absence of Ca^{2+}_o, etelcalcetide activity in HEK293 cells is significantly decreased.[65] Nonetheless, etelcalcetide retains agonist activity in the absence of Ca^{2+}_o; therefore, it is a PAM agonist.[65] Given that etelcalcetide interacts with a unique site relative to the small molecule PAMs, etelcalcetide is likely to stabilize a distinct receptor conformation and therefore has the potential to engender biased CaSR agonism or modulation, although this remains to be determined.

CaSR NAMs. A high throughput screen and subsequent medicinal chemistry effort at NPS Pharmaceuticals and SmithKline Beecham led to the discovery of the first CaSR NAM, NPS2143 (Figure 5), which has an arylalkylamine scaffold.[67] Subsequent efforts to progress arylalkylamine NAMs clinically led to the development of several NAMs with structural and chemical similarity to NPS2143, including ronacaleret,[67,68] JTT305 (otherwise known as MK3552),[69] and NPSP795[70] (Figure 5). Arylalkylamine NAMs are predicted to bind within the same 7TM cavity as arylalkylamine PAMs, with the NAM secondary amine predicted to interact with $E837^{7.32}$ in a manner akin to the arylalkylamine PAMs.[41,71] In fact, the predicted binding pose for the arylalkylamine NAMs is very similar to the predicted pose for arylalkylamine PAMs, making it difficult to discern from computational modeling how these structurally similar PAMs and NAMs could have opposing effects on CaSR signaling. Future structural elucidation of the CaSR 7TM bound to PAMs and NAMs is needed to fully appreciate how these small molecules differentially alter CaSR structure and function.

In addition to the arylalkylamine NAMs, a screening program at Novartis identified quinazolinone-containing compounds as CaSR NAMs, which were advanced to yield ATF936 and AXT914 (Figure 5).[72,73] ATF936 has greater negative cooperativity in comparison to NPS2143, meaning that it is better at blocking CaSR activity.[71] Mutagenesis and docking studies predict the quinazolinone-containing NAMs bind in the 7TM allosteric cavity but in a distinct manner to the arylalkylamine PAMs and NAMs.[71] For instance, some mutations that reduce NPS2143 affinity have no effect on the affinity of ATF936 affinity.[71] A more detailed structural understanding of the binding of quinazolinone-containing NAMs may afford the opportunity to design NAMs with even greater affinity or cooperativity.

Intriguingly, a structurally and chemically distinct CaSR NAM, known as BMS compound 1, is predicted to bind to a second as yet unidentified allosteric site in the 7TM, ECLs, or ICLs of the CaSR.[71] Multiple allosteric binding sites within the 7TM of class C GPCRs is not unprecedented. For $mGlu_5$, several PAM chemotypes are thought to bind outside the common allosteric pocket.[74,75] Structural resolution of the BMS compound 1 binding site could provide opportunities to identify novel allosteric modulators that target this site and that may possess biased modulatory properties.

Calhex231: A Mode-Switching Allosteric Modulator. Calhex231 is structurally and chemically related to cinacalcet and the other arylalkylamine PAMs, and indeed it was discovered from an SAR study based on the PAM calindol.[76] Surprisingly, calhex231 was reported to be a NAM because it inhibited a maximally effective concentration of Ca^{2+}_o in an IP

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accumulation assay.[76] Recent work, however, has revealed that calhex231 is both a PAM and a NAM depending on whether it occupies a single protomer in the CaSR dimer or both protomers.[77] The binding of calhex231 to one protomer inhibits the binding of calhex231 to the second protomer. Using an allosteric quaternary complex model, it was shown that calhex231 switches to a NAM because its negative cooperativity with itself is greater in the presence of an agonist.[77] The calhex231 binding site overlaps with the binding site for cinacalcet, NPS2143 and other arylalkylamine PAMs and NAMs.[77] The ability of calhex231, but not other PAMs and NAMs, to mode-switch is predicted to be due to a disubstituted cyclohexane ring in calhex231, which may offer more flexibility when bound to the CaSR and thus allow calhex231 to adopt at least two distinct binding poses.[77]

CLINICAL UTILITY OF CASR ALLOSTERIC MODULATORS

CaSR PAMs for Hyperparathyroidism. Given the pivotal role of the CaSR in negatively regulating PTH secretion, three CaSR PAMs are currently on the market to treat hyperparathyroidism. Hyperparathyroidism is typically caused by parathyroid adenoma or carcinoma, resulting in primary hyperparathyroidism (PHPT). Alternatively, it is secondary to chronic kidney disease, where impaired phosphate excretion and renal 1,25-dihydroxyvitamin D3 synthesis leads to decreased Ca^{2+}_o and a consequent increase in PTH synthesis and secretion as well as parathyroid hyperplasia. Cinacalcet (Sensipar) was the first CaSR-targeting drug to gain FDA approval in 2004 for hemodialysis patients with secondary hyperparathyroidism (SHPT) caused by chronic kidney disease. Cinacalcet was also the first FDA-approved GPCR allosteric modulator to reach the market. Cinacalcet has since been approved to treat hypercalcemia in adults with parathyroid carcinoma or who cannot undergo parathyroidectomy.

Cinacalcet is generally safe and well tolerated, although GI adverse events including nausea, vomiting, or loss of appetite occur in approximately 30% of patients.[78] Cinacalcet can also cause transient episodes of hypocalcaemia in some patients. Further, there is some variability in the degree to which patients respond to cinacalcet.[79] While cinacalcet responsiveness can depend on the severity of SHPT, CaSR single nucleotide polymorphisms (SNP) may influence cinacalcet efficacy. For instance, SHPT patients with an R990G SNP demonstrate higher sensitivity to cinacalcet, with a larger proportion of G990 carriers experiencing a cinacalcet-mediated suppression in PTH compared to patients with the predominant R990 allele.[80] These findings suggest that a personalized medicines approach may need to be considered when treating patients with CaSR-targeting therapies.

In 2017, etelcalcetide (Parsabiv) was approved by the FDA as an intravenous CaSR PAM for the treatment of SHPT in adults. The intravenous administration of etelcalcetide is advantageous because it can be delivered at the end of a hemodialysis session, thus ensuring patient compliance. While intravenous etelcalcetide was expected to induce fewer GI adverse events compared to oral cinacalcet, self-reported symptoms of nausea and vomiting were not significantly different between SHPT patients given etelcalcetide or cinacalcet.[81] Nonetheless, a one-year safety and efficacy trial of intravenous etelcalcetide administration revealed no major safety concerns,[82] although, like cinacalcet, etelcalcetide can cause hypocalcaemia.[83]

Recently, evocalcet (alternative names MT-4580 and KHK7580) was approved for the management of SHPT in Japanese patients that remain refractory to cinacalcet treatment because adverse GI events prevent cinacalcet dose escalation. Evocalcet has higher bioavailability in comparison to cinacalcet, and lower doses are therefore required to suppress serum PTH levels.[84] In rats, evocalcet suppresses PTH secretion while having no significant effect on gastric emptying, which is delayed in cinacalcet-treated rats and patients.[84] Evocalcet also had a reduced incidence of vomiting in marmosets.[84,85] In humans, evocalcet offers good short-term tolerability in terms of upper GI symptoms while still providing therapeutic efficacy similarly to cinacalcet.[86] However, while the severity of GI side effects is reduced compared to cinacalcet,[83] approximately 19% of evocalcet-treated patients still experience nausea and vomiting compared to 33% of patients treated with cinacalcet.[78] Evocalcet also causes hypocalcaemia in some patients.[83] Thus, while all three clinically approved CaSR PAMs are effective at reducing PTH levels in hyperparathyroidism, the risk of hypocalcaemia and incidence of GI side effects limits their use in the clinic.[83] There is still therefore a need for novel PAMs with reduced adverse effects.

PAMs for Hypercalcaemia. The importance of the CaSR in Ca^{2+}_o homeostasis is highlighted by the many naturally occurring mutations in the *CASR* gene or in genes encoding $G\alpha_{11}$ (*GNA11*), which mediates CaSR signal transduction, or adapter protein 2 sigma subunit 1 (*AP2S1*), which regulates CaSR cell surface expression. Inactivating mutations in these proteins cause familial hypocalciuric hypercalcaemia types 1 to 3 (FHH1−3) or neonatal severe primary hyperparathyroidism (NSHPT).

FHH1 (the most common form of FHH at 1 per 1350 people[87]) and NSHPT are caused by inactivating *CASR* mutations. These mutations reduce CaSR sensitivity to Ca^{2+}_o or impair the biosynthesis and post-translational processing of the CaSR within the endoplasmic reticulum or Golgi apparatus, leading to CaSR misfolding and impaired cell surface expression.[88,89] Furthermore, some mutations alter CaSR coupling to signaling pathways to the relative exclusion of others.[90] FHH1 is characterized by mild or moderate elevations of serum calcium and magnesium with mildly elevated or normal PTH levels. While FHH1 patients are often asymptomatic, up to 30% of patients experience symptomatic hypercalcaemia, whereas others develop chondrocalcinosis, acute pancreatitis, and gallstones.[91] Importantly, FHH1-causing mutations increase the risk of numerous diseases, most notably cardiovascular, neurodegenerative, and psychiatric diseases.[87] These findings suggest that it may be appropriate to treat FHH1 even in asymptomatic patients. Further, the much rarer but more severe disorder, NSHPT, is characterized by life-threatening hypercalcaemia, skeletal under-mineralization and deformities, and death if left untreated.[91] It is therefore essential that infants diagnosed with NSHPT are treated. Increasingly, cinacalcet has shown some success in treating NHSPT in addition to complications related to FHH1 in patients harboring loss-of-function or loss-of-expression CaSR mutations.[92−98] There are, however, increasing reports of NSHPT patients who do not respond adequately to cinacalcet, in some cases due to homozygous mutations that result in truncation of the CaSR before the 7TM cinacalcet binding site.[99,100] Cinacalcet-unresponsive patients may also harbor missense mutations or in-frame

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deletions that result in expression of a full-length CaSR with single amino acid mutations or a shortened CaSR in which exon 5, encoding amino acids 476−536 in the ECD, is deleted.[101−103] In these instances, cinacalcet may be ineffective because the mutation may reduce cinacalcet affinity or its ability to potentiate Ca^{2+}_o by decreasing allosteric cooperativity. In cases of severe mutation-induced receptor impairment, the mutation may render cinacalcet unable to sufficiently restore receptor function even if affinity or cooperativity are unaffected.[104] Interestingly, compared to cinacalcet, AC265347 was more effective at potentiating Ca^{2+}_o-mediated signaling responses at some FHH1/NSHPT-causing CaSR mutants, suggesting that alternative PAMs may be better than cinacalcet at rescuing inactivating CaSR mutants.[64] However, as AC265347 is not approved clinically, total parathyroidectomy is currently required to normalize serum PTH levels in patients who do not respond to current pharmacological interventions.

Four *GNA11* mutations have been identified in FHH2-affected individuals (FHH2 is the least common form of FHH), which are predicted to impair guanine nucleotide binding or disrupt G protein activation of intracellular signaling proteins such as PLC.[91,105] FHH2 patients typically have mild hypercalcaemia and normal serum concentrations of PTH.[105] In recombinant cells expressing FHH2-causing *GNA11* mutations, cinacalcet restored impaired CaSR signaling.[106] Similarly, in mice with a germline loss-of-function *GNA11* mutation, cinacalcet corrected hypercalcaemia and reduced elevated serum PTH concentrations.[107] Cinacalcet also normalized serum calcium concentrations in a FHH2 patient with hypercalcaemia.[108] These studies suggest that cinacalcet stabilizes a CaSR conformation that couples more favorably to G_{11}, thus overcoming mutations that impair G_{11} function. However, given the typically asymptomatic nature of FHH2, there is no clear benefit in treating most FHH2 patients with pharmacological interventions.

Four missense *AP2S1* mutations that cause FHH3 have been identified.[109−111] FHH3-causing mutations disrupt AP2σ-mediated CaSR endocytosis and consequently impair CaSR signaling from endosomes.[105,112] FHH3 is the most severe form of FHH and is more commonly characterized by symptomatic hypercalcaemia.[109,113] FHH3 may also be associated with recurrent pancreatitis and cognitive dysfunction.[114] Cinacalcet corrected impaired CaSR signaling resulting from FHH3-causing *AP2S1* mutations and rectified symptomatic hypercalcaemia in three FHH3 patients.[115] The molecular mechanisms by which cinacalcet corrects mutation-induced impairments in AP2σ-mediated CaSR internalization are not known, but there are several possibilities. Cinacalcet may simply stabilize a CaSR conformation that interacts more favorably with AP2σ, thus restoring CaSR internalization and trafficking to endosomes. Alternatively, by crossing the cell membrane, cinacalcet may potentiate the activity of CaSRs already localized to endosomes. Regardless, these findings demonstrate that CaSR PAMs such as cinacalcet may be useful in the management of FHH3.

NAMs for Osteoporosis. The first manifestation of osteoporosis is typically a fracture.[116] Therefore, treatments are aimed at preventing further bone loss, such as with the use of bisphosphonates (e.g., alendronate), or restoring bone mass and density with recombinant human PTH(1−34) (rhPTH(1−34) or rhPTH(1−84)), which have anabolic actions by increasing the number of bone-forming osteoblasts.[117] However, rhPTH(1−34) has received a black box warning label in the United States because high doses induced osteosarcoma in long-term carcinogenicity studies in rats.[118] Further, rhPTH requires daily subcutaneous administration, and an orally active anabolic compound therefore continues to be of interest. Small molecule CaSR NAMs were consequently developed as potential orally available therapeutics for osteoporosis because they stimulate the release of endogenous PTH by mimicking a drop in Ca^{2+}_o levels.

NPS2143 was the first CaSR NAM to be evaluated in an ovariectomized rat model of postmenopausal osteoporosis. However, following 5 weeks of daily NPS2143 administration, no net increase in bone mass and density was observed.[67,119,120] The high volume of NPS2143 distribution resulted in prolonged NPS2143 exposure and sustained elevations in PTH levels, in contrast to plasma levels of rhPTH(1−34), which reached a comparable maximum concentration but returned to baseline much more rapidly. It was soon realized that CaSR NAMs would best exert an anabolic effect if they had a short half-life to ensure transient stimulation of PTH release that promptly returned to basal levels. This is because prolonged exposure to PTH mimics hyperparathyroidism, thus stimulating bone resorption at the expense of bone formation.[121]

Ronacaleret was the second CaSR NAM to be evaluated in osteoporosis. While ronacaleret is structurally similar to NPS2143, it is more metabolically labile.[122,123] However, a clinical trial in postmenopausal women given ronacaleret for 12 months demonstrated only a modest increase in bone mass and density of the lumbar spine compared to large increases seen in patients receiving rhPTH(1−34) or alendronate, while hip, femoral neck, and trochanter bone mass and density was decreased in the ronacaleret-treated group.[124] Similarly, in a phase 2 clinical trial in postmenopausal women treated with JTT305 for 6 months, no significant increase in bone mass and density was observed over placebo, despite evidence of an increase in markers of bone formation.[125] A clinical trial of AXT914 was also terminated early due to a lack of effect of AXT914 on bone formation markers and a dose-limiting increase in serum calcium after four weeks of treatment.[126]

The reasons why CaSR NAMs do not stimulate bone formation are not fully understood but may be linked to on-target CaSR effects in cells and tissues outside the parathyroid gland. For instance, CaSR NAMs may inhibit the important function of the CaSR in bone-forming osteoblasts, thus counteracting the effects of transient PTH release. Further, while the pharmacokinetic profiles of ronacaleret, JTT305, and AXT914 were more favorable than NPS2143, NAM-mediated elevations in serum PTH concentrations remained above baseline in humans for more than 3.5 h,[124−126] whereas levels of rhPTH return to baseline rapidly following rhPTH injection.[127] Prolonged PTH release was more apparent at higher NAM doses that were cleared less rapidly. The design of NAMs that can be administered at lower doses, such as those with greater affinity or cooperativity, could help to overcome this issue. Regardless of the reasons for CaSR NAM failures in the clinic, the development of NAMs for osteoporosis has been discontinued, and efforts have instead focused on repurposing CaSR NAMs for alternative disorders.

NAMs for ADH and Bartter Syndrome V. Recent interest has been garnered in repurposing CaSR NAMs for heterozygous activating mutations in the *CASR* or *GNA11* genes, which cause autosomal dominant hypocalcaemia type 1 (ADH1; caused by *CASR* mutations), Bartter syndrome V

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(*CASR* mutations), or ADH2 (*GNA11* mutations). ADH is characterized by a mild or moderate decrease in serum calcium and PTH concentrations.[91] Many ADH sufferers experience symptomatic hypocalcaemia, which may include tingling and painful muscular spasms in the hands and feet as well as seizures. Some ADH1 patients also suffer from calcifications in the kidneys and basal ganglia or elevated bone mineral density.[91] In more severe cases, gain-of-function *CASR* mutations promote renal loss of sodium, potassium, magnesium, and chloride ions and consequent hypokalaemic alkalosis and hyperreninaemic hyperaldosteronism, a condition called Bartter syndrome V.[91] The prevalence of ADH1 is approximately 1 per 25 000.[87] Currently, over 90 different CaSR mutations have been linked to ADH1. Among them, over 95% are missense mutations, with the remaining 5% represented by frameshift or in-frame insertion and deletion mutations.[128] ADH2 is rarer and has been associated with six different activating missense *GNA11* mutations. ADH2-causing mutations are located at the interface between the helical and GTPase domains of the $G\alpha_{11}$ protein and involved in GDP-GTP exchange or located at the $G\alpha_{11}$ carboxyl terminal, which is involved in receptor coupling.[129,130] CaSR NAMs are a viable therapeutic strategy for reducing hyper-function caused by gain-of-function mutations in both the CaSR and $G\alpha_{11}$.

Promisingly, NPS2143 can normalize signaling responses associated with ADH-causing *CASR* and *GNA11* mutations *in vitro*[104,106,131,132] as well as increase $Ca^{2+}{}_o$ and PTH concentrations in ADH1 and ADH2 mouse models[131,133,134] and prevent nephrocalcinosis in an ADH1 mouse model.[135] However, NPS2143 is less effective, at least *in vitro*, at rectifying gain-of-function *CASR* mutations that cause Bartter syndrome V.[104,132] In contrast, quinazolinone-derived NAMs (e.g., AXT914 and ATF936) can better rectify Bartter syndrome V mutations *in vitro*[136] and may represent a class of NAMs with lower propensity to be affected by pharmacogenetic effects compared to arylalkylamine-derived NAMs like NPS2143.

Although originally developed for osteoporosis, the arylalkylamine-derived NAM, NPSP795, entered phase II clinical trials for the treatment of ADH1. NPSP795 robustly increased PTH in 3 out of 5 ADH1 trial patients and caused a small reduction in renal Ca^{2+} excretion. However, NPSP795 had no significant effect on serum $Ca^{2+}{}_o$ levels and had variable effects on PTH.[137] The high variability in NPSP795 efficacy may in part be attributable to the underlying disease-causing mutations. Some ADH1 mutations are in close proximity to the common 7TM allosteric binding site, or NPSP795 may not have sufficient affinity or cooperativity to overcome some mutation-induced enhancement in CaSR signaling.[41,104] However, pharmacogenetic effects do not completely explain interpatient variation in the efficacy of NPSP795. For instance, two patients in the study carried the same mutation (A840 V). A840 V faces into the 7TM binding cavity and contributes to the binding of the arylalkylamine NAM, NPS2143.[41] However, while serum PTH levels were robustly increased in one A840 V-harboring patient, NPSP795 had only a modest effect on PTH levels in the other, despite similar NPSP795 concentrations being reached in both patients. It therefore remains to be determined why some ADH1 patients may respond to CaSR NAMs, while others do not.

NAMs for Asthma. Asthma affects ∼340 million people worldwide, posing significant health risks particularly to approximately 10% of asthmatics whose asthma is poorly controlled with current drugs. The efficacy of asthma medications, which include β_2 adrenergic receptor agonists and corticosteroids, is further limited by acute exacerbations typically caused by respiratory virus infections or environmental pollutants.[138] Identifying novel treatments for poorly controlled asthma is therefore a key health priority.

Recently, the CaSR was identified as a putative therapeutic target in asthma. The CaSR is expressed in bronchial smooth muscle and the epithelium.[15] CaSR expression is upregulated in human bronchial biopsies from asthmatics, in murine asthma models, and in human airway smooth muscle cells exposed to asthma-associated cytokines,[15] presumably via STAT and κB response elements in the *CASR* gene promoters. Further, CaSR agonists such as polyamines are established mediators of airway inflammation, remodelling, and constriction;[139−146] polyamine concentrations are elevated in the sputum or blood of human asthmatics and in murine models of allergic airways disease.[141,143,147] While spermine potentiated airway smooth muscle contraction induced by acetylcholine, this effect was diminished in mice with selective CaSR ablation in airway smooth muscle cells.[15] These findings were consistent with observations that the CaSR NAM, NPS2143, attenuated $Ca^{2+}{}_i$ release in human airway smooth muscle cells in response to acetylcholine or histamine,[15] suggesting potential benefit in opposing CaSR signaling in asthma.

In murine models of allergic airways disease, chronic treatment with CaSR NAMs attenuated airway inflammation, fibrosis, and airway hyper-responsiveness (AHR) to the muscarinic acetylcholine receptor agonist, methacholine.[15,148,149] The CaSR NAM, NPS2143, also decreased immune cell counts in mouse bronchoalveolar lavage fluid (BALF) following allergen challenge[15] or lipopolysaccharide (LPS)-induced lung injury,[148] and suppressed serum and BALF cytokine levels.[148] The latter effects of *in vivo* treatment with NPS2143 are consistent with the established role of the CaSR in promoting pro-inflammatory cytokine release from T cells,[150] macrophages,[151,152] and airway epithelial cells *in vitro*.[148] More recent findings demonstrated that the NAMs ronacaleret, JTT-305, NPSP795, and AXT914 all reduced airway inflammation and prevented goblet cell hyperplasia in a chronic airway inflammation model.[153] Taken together, the potential benefits of CaSR NAMs in directly reducing aberrant airway smooth muscle $Ca^{2+}{}_i$ signaling and contraction as well as attenuating airway inflammation, remodelling, and inhibiting AHR in chronic disease models suggest the CaSR may be a novel therapeutic target in asthma.

CONCLUSIONS

The CaSR is a multimodal chemosensor that responds to diverse exogenous and endogenous stimuli via multiple allosteric binding sites. While great efforts have been made to therapeutically target the CaSR, to date, only CaSR PAMs have reached the clinic. Despite CaSR NAMs demonstrating promise as treatments for ADH, the reasons for the potential interpatient variability in responsiveness to NAMs need to be established to progress development of such compounds. Thus, much can still be learned about how CaSR NAMs bind to the receptor and how naturally occurring mutations alter the binding and function of the NAMs. A better identification of the CaSR's many allosteric binding sites may enable drug discovery efforts that target novel CaSR binding sites with potential to identify distinct chemotypes of allosteric modulators with unique pharmacological properties.

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Notes

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