Topical therapy with negative allosteric modulators of the calcium-sensing receptor (calcilytics) for the management of asthma: the beginning of a new era?

Authors:

Daniela Riccardi[1], Jeremy PT Ward[2], Polina L Yarova[3], Luke J Janssen[4], Tak Hong Lee[2,5], Sun Ying[6], Chris J Corrigan[2]

Affiliations:

1. Cardiff School of Biosciences, University of Cardiff, UK

2. Faculty of Life Sciences and Medicine and Asthma UK Centre in Allergic Mechanisms of Asthma, King's College London, UK

3. Translational and Clinical Research Institute, School of Medical Sciences, Newcastle University, UK

4. Department of Medicine, McMaster University, Hamilton, Ontario, Canada

5. Hong Kong Sanatorium and Hospital, Hong Kong, China

6. Department of Immunology, School of Basic Medical Sciences, Capital Medical University, Beijing, China

Corresponding Author:

Professor C J Corrigan, King's College London Faculty of Life Sciences and Medicine, 5th Floor, Tower Wing, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK.

E-mail: chris.corrigan@kcl.ac.uk

Tel: +44 207 188 0610

Take home message: Negative allosteric modulators of the calcium-sensing receptor (calcilytics) delivered topically to the airways have the potential to revolutionise asthma therapy

Abstract

In this review article we present the evidence to date supporting the role of the calcium-sensing receptor (CaSR) as a key, pluripotential molecular trigger for asthma and speculate on the likely benefits of topical therapy of asthma with negative allosteric modulators of the CaSR: calcilytics.

What is the calcium-sensing receptor (CaSR) and what are calcilytics (Figure 1)?

The extracellular calcium-sensing receptor (CaSR) is a G protein-coupled receptor (GPCR) originally identified as the body's master controller for extracellular, free ionised calcium (Ca^{2+}). It is widely expressed as a regulator of global calcium metabolism: for example, it is responsible for homoeostasis of extracellular [Ca^{2+}] by regulating parathyroid hormone secretion, Ca^{2+} resorption in the renal loop of Henlé, calcitonin release in the thyroid and osteoclast-mediated bone resorption. The CaSR provides the means by which these cell types sense the extracellular ionised calcium concentration [Ca^{2+}], and maintain it within a narrow physiological range [1]. It is now recognised, however, that the CaSR is widely expressed in many other cell types and has functions unrelated to regulation of extracellular Ca^{2+} homoeostasis. Other important physiological roles include sensing of dietary nutrients in the gut, glucose-mediated insulin secretion, taste satiety and vascular smooth muscle function [1 - 3]. In this review we propose a novel, causal role for the CaSR in the pathophysiology of airways smooth muscle hyperresponsiveness, airways inflammation and the key mechanism by which this inflammation exacerbates bronchial smooth muscle spasm in human asthma.

The CaSR is expressed in the cell membrane as a constitutive homodimer with large extracellular domains which enclose a ligand-binding cleft (Figure 1). In addition to Ca^{2+} it responds to other di-, tri- and polyvalent cations such as Mg^{2+}, Gd^{3+} and other orthosteric agonists including polyamines and polycationic proteins [2, 3]: as will be explained below, this ability to respond to cationic proteins forms a keystone of its potential importance in asthma pathogenesis. The CaSR couples through several different G-protein-mediated signalling pathways, including $G_{q,11}$ (releases Ca^{2+} from intracellular stores; activates protein kinase C), $G_{i/o}$ (inhibits the generation of cyclic adenosine monophosphate; cAMP), and $G_{12,13}$ (activates Rho-kinase and other effector kinases). In addition, the CaSR can also signal via mitogen-activated protein kinase (MAPK) cascades, including extracellular signal-

regulated kinase (ERK), p38 MAPK, c-Jun N-terminal kinase MAP-kinase, and the phosphoinositide 3-kinase (PI3 kinase) and mechanistic target of rapamycin (mTOR) pathways [1, 2]. Because of its ability to couple to multiple G proteins, the CaSR is complex and exhibits “biased agonism”, whereby different ligands activate (or inhibit) specific subsets of signalling pathways preferentially [1 - 3].

The CaSR also binds a range of positive and negative allosteric modulators. Natural positive allosteric modulators (PAMs) of the CaSR include aliphatic and aromatic amino acids that enhance the sensitivity of the CaSR to its primary ligand, Ca^{2+} [4]. Synthetic PAMs of the CaSR, also known as calcimimetics, include the clinically available drugs cinacalcet, evocalcet and etelcalcetide. These compounds are currently in general clinical therapeutic use to treat hypercalcaemia arising from hyperparathyroidism of various aetiologies, including parathyroid carcinoma, hyperparathyroidism secondary to renal failure, familial hypocalciuric hypercalcaemia and primary hyperparathyroidism in neonates or those patients with parathyroid carcinoma who are not suitable for surgical excision. Calcimimetics exhibit ligand biased signalling, with preferential activation of CaSR-mediated phosphorylation of ERK1/2 over Ca^{2+} mobilisation (reviewed in [5]).

Synthetic, negative allosteric modulators (NAMs) of the CaSR, also known as calcilytics, were first discovered as a result of high throughput screening of compounds based on an arylalkylamine scaffold (reviewed in [6]) and now include the amino alcohol compounds such as ronacaleret, JTT-305 (also known as MK-5442), NPSP795 and the quinazolin-2-one derivatives ATF936 and AXT914 [7]. Some of these calcilytics have been evaluated for therapy of osteoporosis but found to be clinically ineffective, likely because they exert opposing effects on key processes such as calcium mobilisation and osteoblast activity (reviewed in [1]). Recently the calcilytic NPSP795 has been repurposed to treat autosomal dominant hypocalcaemia with hypercalciuria, which is caused by activating mutations in the *CASR* gene [8], while others are under investigation for therapy of other hypocalcaemic disorders such as idiopathic hypercalciuria.

What is the clinical evidence that the CaSR is involved in the pathogenesis of airways smooth muscle hyperresponsiveness in human asthma?

From the account above it will be self-evident that the function of the CaSR is influenced not only by its binding of Ca^{2+} and other inorganic cations, but also by sensing organic, polycationic species, the local concentrations of which may be greatly increased in the

airways as a result of inflammation and environmental exposure. It has long been known from studies on murine surrogates [9] and patients with occupational asthma caused by exposure to aliphatic polyamines [10] that exposure to these compounds increases the risk of manifestation of airways smooth muscle hyperresponsiveness. Similarly, it is well recognised that elevated airways concentrations of the eosinophilic cationic proteins, while not specific for asthma, correlate with disease severity in the context of asthma [11]. In asthma, polyamines and polycationic protein products of airways inflammatory cells in particular have the potential to bind to and activate the CaSR directly, functioning as orthosteric agonists which markedly heighten the signal output of the CaSR. Thus, it is entirely plausible to assume that over-expression of the CaSR and/or activation of the CaSR by local, environmental stimuli accounts for the phenomenon of airways smooth muscle hyperresponsiveness which characterises human asthma, and for the regulation of the degree of this hyperresponsiveness by the concentrations of cationic products of local, asthma-relevant inflammatory cells. In this article we present data, from our group and others, in direct support of this hypothesis and discuss in more detail the role of the CaSR in regulating ASM contraction and airways inflammation. We also summarise and discuss the evidence that the concentrations of polyamines and polycationic proteins are elevated in the airways in asthma and, in many previous studies, have been shown to correlate with disease severity.

Airways smooth muscle (ASM) hyperresponsiveness is responsible for the short-term, spontaneous variability in airways obstruction which causes asthmatics (but not non-asthmatics) to develop sudden wheezing and breathlessness when exposed to a range of specific and non-specific stimuli such as smoke, cold air, allergens in sensitised subjects, exercise and respiratory tract infections. In a recent key study [12] it was demonstrated that human asthma is accompanied~~characterised~~ by over-expression of the CaSR on airway smooth muscle (ASM) cells compared with non-asthmatic controls, and furthermore that exposure of this receptor to negative allosteric modulators abrogated asthmatic ASM hyperreactivity to contractile stimuli *ex vivo* and *in vitro*. It was also demonstrated that expression of the CaSR is up regulated on human ASM cells exposed to asthma-associated cytokines: it was hypothesised that this is driven by the signal transducer and activator of transcription (STAT) and κB response elements in the CaSR gene promoters [13]. Exposure of murine lung slices to the CaSR agonist spermine *ex vivo* potentiated ASM contraction induced by acetylcholine; this effect was abolished in lung slices from animals with selective CaSR ablation in their ASM cells and abrogated by calcilytics in lung slices from wild type mice but not those with the selective CaSR ablation [12]. Moreover, wild type

mice exhibited AHR following exposure to inhaled poly-L-arginine (another CaSR agonist*) in vivo*, an effect which was abolished by inhaled calcilytics [12].~~Exposure of murine lung slices to spermine *in vitro* or poly-L-arginine *in vivo*, both CaSR agonists, potentiated ASM contraction induced by acetylcholine, an effect that was abolished in lung slices from animals with selective CaSR ablation in their ASM cells [12]. Furthermore, t~~In addition, the calcilytic drug NPS2143 attenuated basal, elevated intracellular Ca^{2+} concentrations as well as Ca^{2+} release in response to acetylcholine or histamine in ASM cells from asthmatic, but not non-asthmatic control patients [12]. This latter observation is particularly notable because it demonstrates that calcilytics, while normalising the Ca^{2+} concentrations in ASM cells from asthmatic patients, do not appear to alter the function of ASM cells in non-asthmatic individuals.

In the same study [12] it was discovered that, in addition to the arginase products spermine, spermidine and putrescine, the CaSR was also activated by products of eosinophils, including eosinophil cationic proteins and major basic protein, providing a clear functional basis for the regulation of asthma severity by the products of these cells.

It is also noteworthy that exposure of foetal lung ASM cells to hyperoxia has been reported to up-regulate CaSR expression, inducing hyperresponsiveness to histamine and increased proliferation. Again these effects were attenuated by calcilytics, providing a therapeutic avenue to the management of neonatal airways diseases including hyperoxia-induced, neonatal asthma [14].

~~Finally, i~~It may also be relevant that a recent bioinformatics study of genetic variants of the CaSR uncovered clinically relevant associations with several diseases unrelated to regulation of circulating Ca^{2+}, including asthma (15): it remains to be seen if and how these genetic mutations which dysregulate total body calcium homoeostasis might also affect polycation-sensing of CaSR in inflamed ASM.

Pathophysiological mechanism of airways smooth muscle hyperresponsiveness in asthma (Figure 2)

There is good evidence that bronchial hyperresponsiveness in asthma is associated with increased ASM contractile function, the mechanisms of which have yet to be fully defined but include alterations to the ASM cell intracellular Ca^{2+} concentration $[Ca^{2+}_i]$ handling and sensitivity, contractile machinery and cytoskeletal dynamics and structure [16 – 20]. In this

section we briefly outline the mechanisms underlying ASM contraction and relaxation, how they may be altered is asthma, and the potential key role of the CaSR.

Elevation of $[Ca^{2+}]_I$ is central to ASM contraction, and activation of other cell types including epithelial and inflammatory cells. Multiple pathways contribute to $Ca^{2+}{}_i$ homoeostasis, including Ca^{2+} release and sequestration by the sarcoplasmic reticulum (SR) and mitochondria, and Ca^{2+} flux into and out of the cell [19, 21]. These are activated (or inhibited) by a variety of GPCRs [22] (Figure 2). Most bronchoconstrictors activate GPCR coupled via $G_{q,11}$ to phospholipase Cβ, generating inositol trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3 elicits Ca^{2+} release from the SR, whereas DAG activates non-selective (Ca^{2+} and Na^+ permeable) receptor-operated channels (ROC), and protein kinase C (PKC). Ca^{2+} released by IP_3 activates adjacent ryanodine receptors (RyR; Ca^{2+}-induced Ca^{2+} release), amplifying the response [21]. RyR are also activated by cyclic ADP ribose, generated by CD38 [23]. Depletion of SR Ca^{2+} content activates store operated channels (SOC) via STIM and thus further increases Ca^{2+} entry. Voltage gated Ca^{2+} channels (L-type) appear to be of limited significance in ASM or indeed the pathogenesis of asthma, which is why calcium channel blockers have proven ineffective for asthma prophylaxis and therapy [24, 25].

Cytosolic Ca^{2+} is sequestered back into the SR by the sarcoendoplasmic reticulum ATPase (SERCA), and expelled from the cell by a Na^+-Ca^{2+} exchanger (NCX) and plasma membrane Ca^{2+} ATPase (PMCA) [19]. As NCX exchanges three Na^+ for each Ca^{2+} it is membrane potential- and Na^+ gradient-dependent; the resulting depolarisation and local increases in $[Na^+]_i$ following Na^+ entry via ROC causes NCX to operate in reverse mode, facilitating Ca^{2+} entry [26].

The ASM cell is highly organised, with cell membrane, peripheral SR and mitochondria creating signalling micro-domains that allow rapid regulation of the temporal and spatial aspects of changes in $[Ca^{2+}]_i$. This facilitates generation of oscillations in $[Ca^{2+}]_i$ on stimulation by bronchoconstrictors [27, 28], while bronchodilators reduce the Ca^{2+}-oscillation frequency [29]. Importantly, the magnitude of ASM shortening correlates with Ca^{2+}-oscillation frequency and not amplitude [27]. ASM also exhibits slower oscillations in membrane potential on stimulation, which may switch NCX between forward and reverse modes, producing corresponding $[Ca^{2+}]_i$ oscillations [30]. Many enzymes decode Ca^{2+}-oscillation frequency in a wide variety of cell types, and may contribute to both contraction and proliferation of ASM cells [19, 30].

Elevation of ASM $[Ca^{2+}]_i$ leads to Ca^{2+}-calmodulin-mediated activation of myosin light chain kinase (MLCK), phosphorylation of myosin light chain (MLC) and consequently activation of myosin ATPase and cell shortening. Relaxation requires dephosphorylation of MLC by its phosphatase (MLCP), so force generation depends on the balance between MLCK and MLCP activities. MLCP is constitutively active, but can be inhibited by RhoA-kinase (ROCK). ROCK is activated by the monomeric G-protein RhoA, which is itself activated by $G_{12,13}$ coupled GPCRs [31]. Inhibition of MLCP means more force is generated for the same elevation of $[Ca^{2+}]_i$ (Ca^{2+} sensitisation); PKC similarly induces Ca^{2+} sensitisation via phosphorylation of the MLCP inhibitor CPI-17 [32]. Notably, many bronchoconstrictors act through both $G_{q,11}$ and $G_{12,13}$ coupled pathways. For example, acetylcholine activates M3 muscarinic receptors which couple to both $G_{q,11}$ and $G_{12,13}$, thus stimulating IP_3-induced SR $Ca^{2+}{}_i$ release, activation of ROC and PKC, simultaneously with RhoA/ROCK-mediated Ca^{2+} sensitisation [33]. Notably, some bronchoconstrictors also activate G_i-coupled GPCR, thus inhibiting adenylyl cyclase (see below).

β_2-adrenoreceptor agonists and most endogenous bronchodilators (e.g. catecholamines, vasoactive intestinal peptide, PGE_2) elicit relaxation by activating adenylyl cyclase via G_s-coupled GPCRs to elevate cAMP; cAMP is degraded by phosphodiesterases (PDEs), so PDE inhibitors (e.g. theophylline) also elevate cAMP. Elevation of cAMP supresses multiple bronchoconstrictor pathways, mediated either by protein kinase A (PKA) or Epac (exchange factor directly activated by cAMP); these include Ca^{2+} influx and mobilisation, RhoA activation and MLC phosphorylation (reviewed in [22, 32]). It also stimulates SERCA, thus reducing $[Ca^{2+}]_I$ [35], slows Ca^{2+} oscillations [29] and enhances degradation of bronchoconstrictor GPCRs [34].

Numerous studies have shown asthma-associated perturbations of the pathways discussed above, either using ASM derived from asthmatics or animal surrogates, or treated with asthma-associated mediators; as these have been extensively reviewed [16, 19, 20, 22, 36], discussion here is limited to a few salient points. There is a wide consensus that asthma is associated with ASM hyperresponsiveness, while enhanced $[Ca^{2+}]_i$ mobilisation is well documented, being attributed to increased Ca^{2+} entry and release [28, 37, 38] and reduced activity of SERCA and Ca^{2+} reuptake into the SR [39, 40]. Similarly, Ca^{2+} sensitisation and RhoA/ROCK have also been strongly implicated in ASM hyperresponsiveness [31, 41 - 43].

The role of the CaSR in airways smooth muscle hyperresponsiveness and the potential of calcilytics

Under normal, “healthy” conditions, CaSR expressed on ASM cells would be expected to reside in a continuous state of low level activation in the presence of normal concentrations of interstitial [Ca^{2+}]; this is consistent with the finding that calcilytics reduced [Ca^{2+}]$_i$ in ACh-stimulated human ASM cells from both healthy and asthmatic donors [12]. Asthma is however accompanied both by elevated expression of the CaSR in ASM [12] and elevated concentrations in the airways of potent CaSR activators, including eosinophil cationic protein and major basic protein, and cationic polyamines (putrescine, spermidine, spermine). The latter are elevated in asthma owing to both increased arginase activity and reduced polyamine catabolism [12, 44 - 47], and have been previously associated with the pathophysiology of bronchial hyperresponsiveness [48 - 50]. It has also been proposed that inflammation leads to localised elevations of extracellular [Ca^{2+}] which may also increase CaSR activity [51, 52].

The effects of all of these stimuli acting in concert would inevitably result in a leftward shift in the CaSR [Ca^{2+}]-response relationship and greatly increased signal output, potentiated by the elevated expression of CaSR [1, 3, 12]. Thus ASM Ca^{2+} mobilisation and RhoA/ROCK- and PKC-mediated Ca^{2+} sensitivity would be elevated (via $G_{q,11}$, $G_{12,13}$, G_i and MAPK cascades), whereas adenylyl cyclase and cAMP generation would be inhibited (via G_i). Collectively, this would potentiate ASM contractility and sensitivity to other bronchoconstrictor autacoids that act through these same intracellular signalling pathways (e.g. ACh, histamine, neuropeptides, prostaglandins, leukotrienes; see Figure 2). These effects would be further amplified by the asthma-associated perturbations in ASM signal transduction pathways discussed in the previous section [16, 19, 20, 22, 36] and sufficient to account for ASM hyperresponsiveness in asthma.

This scenario again underlines the concept that ASM hyperresponsiveness in asthma is highly dependent upon the environment of the ASM (where the ASM cells are immersed in cationic proteins bathing the interstitium), and not entirely an intrinsic, functional abnormality of the ASM itself. This might in turn explain why not all studies performed on human ASM obtained from asthmatic patients and studied *ex vivo* report differences in ASM contractility or Ca^{2+} homoeostasis when compared to ASM from healthy donors [16, 17, 53]. When ASM is excised for study *ex vivo*, it is perforce removed from its surrounding inflammatory milieu and exposure to asthma-associated mediators, including polycationic CaSR ligands. It is possible to hypothesise that the continued presence of such stimuli is necessary to effect detectable functional alterations in the ASM under some circumstances *ex vivo*. Similar reasoning underlies the suggestion that ASM may be normal in asthma, but its function

altered by an abnormal environment (discussed in [16]). It is interesting to speculate that one of the effects of this "abnormal environment" might be to up-regulate CaSR expression on ASM, at least in susceptible patients who develop asthma [12]. It is also possible to hypothesise, although this seems less likely, that this phenomenon might also be partly attributable to variation in disease severity and therapy and/or the anatomical source of the ASM, since ASM from the trachea and main bronchi is known to differ functionally from that of the more relevant, intrapulmonary bronchi. In particular, ASM from the latter has been shown to exhibit hyperreactivity in asthma when this was not the case for the larger airways [17, 36].

Taken together, the data above are consistent with the hypothesis that over-expression and activation of the CaSR by relevant, asthma-associated extracellular ligands in the immediate vicinity of the ASM is a critical driver of ASM hyperresponsiveness in asthma, with the corollary that therapy with calcilytics has the potential to abolish it. This is consistent with the recent observation referred to above [12] that inhibition of the CaSR by calcilytics attenuates or ablates bronchial hyperresponsiveness in animal surrogates of asthma.

The potential for calcilytics to inhibit airways inflammation and its effects on ASM hyperresponsiveness in human asthma (Figure 3)

In addition to its putative role in engendering ASM hyperresponsiveness in human asthma, signalling mediated through the CaSR is increasingly recognised to have key roles both in immune surveillance and the regulation of ongoing inflammation in the airways and elsewhere [54 - 56], for example via activation of the NLRP3 inflammasome [51]. Both of these key immunological functions of the CaSR have recently been implicated in the pathogenesis of other chronic inflammatory diseases such as rheumatoid arthritis [52].

The CaSR is known to be expressed on monocyte/macrophages, neutrophils [57] and T cells [58], rendering them sensitive to activation by extracellular, locally released, inflammation-associated ligands of the CaSR as well as elevation of local extracellular [Ca^{2+}] which has been shown to activate the NLRP3 inflammasome in macrophages [51] and NF-κB and other downstream signalling pathways in neutrophils and T cells. The additional, recent demonstration that eosinophils express the CaSR [12] raises the possibility that release of eosinophil cationic proteins in the course of asthmatic mucosal inflammation may further activate other local eosinophils as well as other cells via the CaSR, in addition to prolonging their lifespan by inhibition of apoptosis [47] through an autocrine, feedback loop. Finally it is noteworthy that structural cells of the airways may also contribute to polyamine-

driven inflammation in asthma: for example airways epithelial damage, a pathognomonic feature of asthma, in addition to triggering alarmin release results in the loss of activity of polyamine catabolic enzymes, resulting in further local injury to the epithelium [45].

These recent experimental findings likely underlie, at least in part, the longer established findings that the expression of CaSR agonists such as polyamines and eosinophil cationic proteins in many previous studies of asthma involving both human subjects and animal surrogates from a multidisciplinary literature correlates positively with airways inflammation and remodelling, as well as disease activity [46 - 49, 59]. Conversely, in murine asthma surrogates, chronic treatment with calcilytics has been reported to attenuate airways hyperresponsiveness, inflammation and remodelling [12, 60]. Taken together, all of these data suggest that, in addition to abolishing ASM hyperresponsiveness, topical therapy of asthmatic patients with calcilytics has the potential to exert an anti-inflammatory effect. Furthermore, regardless of any possible anti-inflammatory effects, calcilytic therapy would be expected to abolish direct exacerbation of ASM hyperresponsiveness in patients with asthma by stimulation of the over-expressed CaSR by products of inflammatory cells such as polyamines and eosinophil-derived proteins.

In closing this section, it is worth noting that other recent studies are consistent with the hypothesis that signalling via the CaSR may play a role in other aspects of asthma pathophysiology. For example, signalling via the CaSR has also been reported to be responsible for hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells [61], suggesting a role in the pathogenesis of hypoxic pulmonary hypertension, and consistent with the hypothesis that it similarly contributes to ASM hypertrophy in asthma. Similarly, a very recent study referred to above [14] has presented evidence that signalling via the CaSR is responsible for the ASM hyperreactivity observed in some premature infants ventilated with supplemental oxygen.

The potential to deliver established calcilytics topically to the airways of patients with asthma

Several small molecule calcilytics have been developed and assessed for oral therapy of osteoporosis as mentioned above, including the amino alcohols ronacaleret and JTT-305 in Phase 2 clinical trials and NPSP795 (a zwitterion amino alcohol) and AXT914 (a quinazolin-2-one) in Phase 1 trials. Although, when administered systemically by oral dosing, these drugs were not found to be efficacious for the treatment of post-menopausal osteoporosis, an indication for which they were originally developed [62, 63] they were importantly

observed to be safe and tolerable in human subjects (some were observed to cause mild hypercalcaemia in a small fraction of normal human volunteers). A very recent study [64] addressed the likely suitability of these four calcilytics for topical delivery to the airways of human asthmatic patients, potentially using devices similar or identical to existing inhaler devices. This included an assessment of the feasibility of delivering them to the airways in sufficient quantities to abolish bronchial hyperresponsiveness and airways inflammation in a murine surrogate of chronic asthma. All four calcilytics [8, 65 - 67], when delivered topically inhibited poly-L-arginine-induced airways hyperresponsiveness in naïve mice and suppressed both airways hyperresponsiveness and inflammation in an asthma surrogate, confirming class specificity. Repeated exposure to inhaled calcilytics did not alter blood pressure, heart rate or serum calcium concentrations, providing considerable precedent for the expectation that topically delivered drugs will not disrupt systemic calcium regulation in human asthmatic patients. Optimal candidates for repurposing for topical therapy of human asthma were identified based on their effects on airways hyperresponsiveness and inflammation, pharmacokinetics and pharmacodynamics, formulation and micronisation properties. In this study, whereas both inhaled calcilytics and inhaled corticosteroids were observed to reduce airways inflammation, only the former obviated features of airways remodelling such as goblet cell hyperplasia.

Outstanding Issues

The CaSR is expressed by many structural and inflammatory cells of the lung, from the airways smooth muscle to the bronchial mucosa. Furthermore, it is capable of being activated by many ligands other than Ca^{2+}, including basic proteins and arginine metabolites at sites of inflammation. It is possible, therefore, that the consequences of CaSR activation or blockade may vary according to the precise situation in the lungs and the nature of the local environment, which may be influenced both by intrinsic inflammation and the effects of external factors such as infectious agents and inhaled, environmental pollutants.

As with all drugs, aside from the potential beneficial effects of calcilytics on hyperoxia-induced bronchial hyperresponsiveness in premature neonates referred to above [14], the potential for topically delivered calcilytics to exert unwanted effects on lung development or immune surveillance can only be determined by clinical experience. The fact that a range of systemically delivered calcilytics has been used successfully in human subjects for many years to manage a range of disorders of calcium metabolism provides considerable reassurance, however, that topically delivered drugs will be both safe and tolerable. Furthermore, as has been emphasised throughout this article, calcilytics are least likely to

exert any functional effects in “normal” tissue, where the CaSR is expressed at baseline density and is not in an “inflammatory” environment of orthosteric agonists.

The future positioning of calcilytic therapy for asthma

In summary, the data presented herein suggest that it should be possible to repurpose calcilytics as topical therapy, with a favourable PKPD, safety and tolerability profile, for human asthma delivered using inhaler devices familiar to patients, and that this therapy has the potential to abolish ASM hyperresponsiveness, and thereby wheezing and breathlessness. As long as they are compliant with therapy, patients need not live in fear of sudden wheezing or breathlessness, which is also a likely cause of sudden death from asthma (according to the most recent National Review of Asthma Deaths in the UK [685], at least half of the patients who die from asthma in the UK are deemed to have “mild/moderate” disease, and presumably therefore die as a result of bronchospasm possibly exacerbated by mucous plugging). The suitability of topical calcilytic therapy to replace conventional bronchodilator therapy for asthma is further underlined by recent evidence that calcilytics elevate cAMP concentrations in human ASM cells, particularly from asthmatic patients [12] and dilate acetylcholine pre-contracted murine airways [64] or lung slices, an effect at which they demonstrate greater potency than conventional bronchodilators such as salbutamol and formoterol [696]. This is most likely attributable to signalling via $G_{i/o}$ and not off-target L-type calcium channel inhibition [64], and provides yet another therapeutic avenue through which calcilytics may relieve bronchospasm. Further, experimental evidence suggests that topical calcilytics can inhibit airways mucosal inflammation in asthmatic patients at least as efficiently as corticosteroids (but with none of their unwanted effects), in addition to suppressing the effects of polyamine and other cationic protein CaSR ligands released from inflammatory and other cells on ASM and other local inflammatory and airways structural cells. Above all, calcilytic therapy has the potential directly to target the receptor triggering a wide range of pathophysiological and pro-inflammatory events in asthma rather than intervening in downstream signalling (see Table 1).

	β2 agonists	Anti-muscarinics	PDE4 inhibitors	Steroids	Biologicals	Calcilytics
Airway hyperresponsiveness						☑
Inflammation			☑	☑	☑	☑
Remodelling/fibrosis						☑
Bronchoconstriction	☑	☑				☑
Restrictions/adverse effects	Black box warning		Diarrhoea, weight loss	Pneumonia, Osteoporosis Bone fracture		

Table 1: The scope of calcilytic therapy compared with "conventional" therapy for asthma (black box warning refers to the potential danger of treating asthma with long-acting β2-agonists in the absence of corticosteroids). PDE4 = phosphodiesterase 4.

If these expectations are fulfilled, calcilytic therapy has the potential completely to replace stepwise therapy with inhaled bronchodilators and corticosteroids advocated in current asthma guidelines, enabling administration of the therapy once or twice daily to adults and children in a limited range of devices, thus facilitating compliance, and teaching and valid checking of inhaler technique.

With this background and the recent identification of calcilytics that appear both safe and suitable for topical delivery to the airways in asthma, these data firmly suggest that first in human studies will be feasible, desirable and achievable in the short term. In the first instance we propose experiments firstly to address the hypothesis that inhaled calcilytics abolish ASM hyperresponsiveness in mild asthmatics, and secondly to address the hypothesis that they abolish both early- and late-phase bronchoconstriction following allergen bronchial challenge of mild, atopic asthmatic patients prior to studies in wider groups of asthmatic patients. It will be of particular interest, in the longer term, to follow up clinically the initial indications that, unlike corticosteroids, calcilytics have the capacity to alter the natural history of airways remodelling in asthma and thereby reduce or obviate irreversible airways obstruction.

Financial support

Some of the research by the authors cited in this article was supported by an Initial Foundation grant “Polycations and the calcium-sensing receptor (CaSR) in asthma” from Asthma UK awarded to Chris J Corrigan, Jeremy PT Ward, Paul J Kemp & Daniela Riccardi), grants "Multifaceted CaSR” and “Biomedicine” awarded to Paul J Kemp and Daniela Riccardi from the Marie Curie Integrated Training Network, a KESS 2 Studentship awarded to Daniela Riccardi and a Wellcome Trust Fellowship awarded to Polina Yarova.

Conflict of Interest Statement

Chris J Corrigan, Jeremy PT Ward and Daniela Riccardi hold a patent for the development of calcium receptor antagonists for the treatment of inflammatory lung disease (https://patents.google.com/patent/WO2014049351A1/en) and Daniela Riccardi and Polina L Yarova have a pending Composition of Matter patent currently undergoing filing for development of new chemical entities: “Novel calcilytics for pulmonary disease” (IP from NCE GB1719023.2). These authors and the remaining authors Luke J Janssen, Tak H Lee and Sun Ying declare no other conflicts of interest relevant to the content of this manuscript, including grants or contracts from any other entity, royalties or licences, consulting fees, payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events, payment for expert testimony, support for attending meetings and/or travel, participation on a Data Safety Monitoring Board or Advisory Board, leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid, stock or stock options, receipt of equipment, materials, drugs, medical writing, gifts or other services or other financial or non-financial interests.

Legends to Figures

Figure 1: ***Overview of ligand binding sites and signalling pathways of the CaSR.*** The CaSR functions as a constitutive homodimer comprising of (i) a large extracellular domain consisting of a cysteine-rich domain and bi-lobar "Venus flytrap" structure which binds to cations, anions and allosteric modulators, (ii) a seven transmembrane domain (7TMD) and (iii) an intracellular domain containing a binding site for hypoxia-induced mitogenic factor (HIMF). Ca^{2+} binding sites are indicated as red circles. The CaSR couples to three heterotrimeric G-proteins: (i) $G_{q,11}$ which activates phospholipase C (PLC) to generate inositol trisphosphate (IP3) and diacylglycerol (DAG) with subsequent Ca^{2+} release from the SR, Ca^{2+} entry via receptor operated channel (ROC), and activation of protein kinase C (PKC), and consequently the mitogen-activated protein kinase (MAPK) cascade and extracellular signal-regulated kinase 1 and 2 (ERK1/2); (ii) G_i which both inhibits adenylate cyclase, thus supressing PKA-mediated inactivation of MAPK, and also activates MAPK via a $G_{\beta/\gamma}$ and Ras pathway; (iii) $G_{12,13}$ which activates the RhoA/ROCK pathway. The CaSR also signals via the phosphoinositide 3-kinase (PI3K) and mTOR (mechanistic target of rapamycin complex) pathways, leading to activation of Akt (protein kinase B) and nuclear factor kappa B (NFκB)-mediated IL-1β release. Akt also induces reactive oxygen species (ROS) production and suppresses caspase activation in mitochondria.

Figure 2: ***Signalling pathways underlying bronchial smooth muscle contraction and hyperresponsiveness.*** The figure displays key pathways regulating intracellular Ca^{2+} homoeostasis and Ca^{2+} sensitivity and thus contractile function in bronchial smooth muscle cells. Those reported to be altered in tissue from asthmatics or asthma surrogates and implicated in hyperresponsiveness are indicated. Some intermediate components are omitted for clarity. cADPR, cyclic ADP ribose; CD38, cyclic ADP ribose hydrolase; DAG, diacylglycerol; GPCR, G-protein coupled receptor; IP3, inositol trisphosphate; IP3R, inositol trisphosphate receptor; M3/M2, muscarinic receptors; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NCX, Na^+/Ca^{2+} exchanger; PKC, protein kinase C; PLCβ ,phospholipase C β; PMCA, plasma membrane Ca^{2+} ATPase; ROC, receptor operated channel; ROCK, Rho kinase; RyR, Ryanodine receptor (Ca^{2+} release channel); SERCA, sarcoendoplasmic reticulum Ca^{2+} ATPase; SOC, store operated channel; STIM, stromal interaction molecule.

Figure 3: ***Signalling pathways in the asthmatic airway: role of the CaSR and likely actions of its negative allosteric modulators***. The figure represents a simplified schematic of key cell types, cytokines, chemokines and growth factors involved in the pathogenesis of

asthma. All cell types illustrated have been shown to express CaSR, and would thus be affected by modulators of CaSR activity. CCL5, CC chemokine 5 (RANTES); CCL11, CC chemokine 11 (eotaxin); LTC4, cysteinyl leukotriene C4; ECP, MBP, eosinophil cationic protein, major basic protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1β, interleukin 1β; ILC2, type-2 innate lymphoid cell; TGFβ, transforming growth factor β; Th2, T helper 2 cell; TNFα, tumour necrosis factor α; TSLP, thymic stromal lymphopoietin.

References

(1) Leach K, Fadil M Hannan FM, Josephs TM, Keller AN, Møller TC, Ward DT, Kallay E, Mason RS, Thakker RV, Riccardi D, Conigrave AD, Bräuner-Osborne H. International Union of Basic and Clinical Pharmacology. CVIII. Calcium-Sensing Receptor Nomenclature, Pharmacology, and Function. Pharmacol Rev 2020; 72(3): 558-604. doi: 10.1124/pr.119.018531.

(2) Lopez-Fernandez I, Schepelmann M, Brennan SC, Yarova PL, Riccardi D. The calcium-sensing receptor: one of a kind. Exp Physiol 2015; 100(12): 1392-1399.

(3) Quinn SJ, Ye CP, Diaz R, Kifor O, Bai M, Vassilev P, Brown E. The Ca2+-sensing receptor: a target for polyamines. Am J Physiol 1997; 273(4): C1315-23.

(4) Shenglong Ling , Pan Shi, Sanling Liu, Xianyu Meng, Yingxin Zhou, Wenjing Sun, Shenghai Chang, Xing Zhang, Longhua Zhang, Chaowei Shi, Demeng Sun, Lei Liu, Changlin Tian. Structural mechanism of cooperative activation of the human calcium-sensing receptor by Ca^{2+} ions and L-tryptophan. Cell Research 2021; 31:383–394.

(5) Diayo J, DeBono A, Josephs TM, Bourke JE, Capuano B, Gregory KJ, Leach K. Therapeutic opportunities of targeting allosteric binding sites on the calcium-sensing receptor. ACS Pharmacol Transl Sci 2021; 4(2): 666-679.

(6) Marquis RW, Lago AM, Callahan JF, Rahman A, Dong X, Stroup GB, Hoffman S, Gowen M, DelMar EG, Van Wagenen BC, Logan S, Shimizu S, Fox J, Nemeth EF, Roethke T, Smith BR, Ward KW, Bhatnagar P. Antagonists of the calcium sensing receptor. 2. Amino alcohol-based parathyroid hormone secretagogues. J Med Chem 2009; 52(21): 6599-605.

(7) Letz S, Haag C, Schulze E, Frank-Raue K, Raue F, Hofner B, Mayr B, Schöfl C. Amino alcohol- (NPS-2143) and quinazolinone-derived calcilytics (ATF936 and AXT914) differentially mitigate excessive signalling of calcium-sensing receptor mutants causing Bartter syndrome type 5 and autosomal dominant hypocalcemia. PLOS ONE 2014; 9(12): e115178.

(8) Roberts MS, Gafni RI, Brillante B, Guthrie LC, Streit J, Gash D, Gelb J, Krusinska E, Brennan SC, Schepelmann M, Riccardi D, Khayat MEB, Ward DT, Nemeth EF, Rosskamp R, Collins MT. Treatment of autosomal dominant hypocalcemia Type 1 with the calcilytic NPSP795 (SHP635). J Bone Miner Res 2019; 34(9): 1609-1618. doi: 10.1002/jbmr.3747.

(9) North ML, Grasemann H, Khanna N, Inman MD, Gauvreau GM, Scott JA. Increased ornithine-derived polyamines cause airway hyperresponsiveness in a mouse model of asthma. Am J Respir Cell Mol Biol 2013; 48(6): 694–702.

(10) Ng TP, Lee HS, Malik MA, Chee CBE, Cheong TH, Wang YT. Asthma in chemical workers exposed to aliphatic polyamines. Occupational Medicine 1995; 45(1): 45–48.

(11) Koh GCH, Shek LPC, Goh DYT, van Bever H, Koh DSQ. Eosinophil cationic protein: is it useful in asthma? A systematic review. Respir Med 2007; 101(4): 696-705.
(12) Yarova PL, Stewart AL, Sathish V, Britt RD Jr, Thompson MA, Lowe AP, Freeman M, Aravamudan B, Kita H, Brennan SC, Schepelmann M, Davies T, Yung S, Cholisoh Z, Kidd EJ, Ford WR, Broadley KJ, Rietdorf K, Chang W, Bin Khayat ME, Ward DT, Corrigan CJ, Ward JP, Kemp PJ, Pabelick CM, Prakash YS, Riccardi D. Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma. Sci Transl Med 2015; 7(284): 284ra260.
(13) Hendy GN, Canaff L. Calcium-sensing receptor gene: regulation of expression. Front Physiol 2016; 7: 394.
(14) Roesler AM, Ravix J, Bartman CM, Patel BS, Marta S, Roos B, Nesbitt L, Pabelick CM, Martin RJ, MacFarlan PM, Prakash YS. Calcium-sensing receptor contributes to hyperoxia effects on human fetal airway smooth muscle. Frontiers in Physiology 2021; 12: 287. DOI: 10.3389/fphys.2021.585895
(15) Dershem R, Gorvin CM, Raghu RPR, Metpally RPR, Krishnamurthy S, Smelser DT, Hannan FM, Carey DJ, Thakker RV, Breitwieser GE, Regeneron Genetics Center. Familial hypocalciuric hypercalcemia Type 1 and autosomal-dominant hypocalcemia Type 1: prevalence in a large healthcare population. Am J Hum Genet 2020; 106(6): 734-747.
(16) Camoretti-Mercado B, Lockey RF. Airway smooth muscle pathophysiology in asthma. J Allergy Clin Immunol 2021 Jun; 147(6): 1983-1995.
(17) Ijpma G, Kachmar L, Matusovsky OS, Bates JH, Benedetti A, Martin JG, Lauzon AM. Human trachealis and main bronchi smooth muscle are normoresponsive in asthma. Am J Respir Crit Care Med 2015 Apr 15; 191(8): 884-93.
(18) Leguillette R, Laviolette M, Bergeron C, Zitouni N, Kogut P, Solway J, Kachmar L, Hamid Q, Lauzon AM. Myosin, transgelin, and myosin light chain kinase: expression and function in asthma. Am J Respir Crit Care Med 2009; 179: 194-204.
(19) Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JP, Lee TH. Ca(2+) homeostasis and structural and functional remodelling of airway smooth muscle in asthma. Thorax 2010; 65(6): 547-52. doi: 10.1136/thx.2009.129296.
(20) Zhang W, Gunst SJ. Molecular mechanisms for the mechanical modulation of airway responsiveness. J Eng Sci Med Diagn Ther 2019; 2: 0108051-0108058.
(21) Berridge MJ. Smooth muscle cell calcium activation mechanisms. J Physiol 2008; 586: 5047-5061.
(22) Fuentes N, McCullough M, Panettieri Jr RA, Druey KM. RGS proteins, GRKs, and beta-arrestins modulate G protein-mediated signaling pathways in asthma. Pharmacol Ther 2021; 223: 107818.

(23) Deshpande DA, White TA, Dogan S, Walseth TF, Panettieri RA, Kannan MS. CD38/cyclic ADP-ribose signaling: role in the regulation of calcium homeostasis in airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2005; 288: L773-L788.
(24) Boie S, Chen J, Sanderson MJ, Sneyd J. The relative contributions of store-operated and voltage-gated Ca(2+) channels to the control of Ca(2+) oscillations in airway smooth muscle. J Physiol 2017; 595: 3129-3141.
(25) Barnes PJ. Clinical studies with calcium antagonists in asthma. Br J Clin Pharmacol 1985; 20 (Suppl 2): 289S-298S.
(26) Hirota S, Pertens E, Janssen LJ. The reverse mode of the Na(+)/Ca(2+) exchanger provides a source of Ca(2+) for store refilling following agonist-induced Ca(2+) mobilization. Am J Physiol Lung Cell Mol Physiol 2007b; 292: L438-L447.
(27) Perez JF, Sanderson MJ. The frequency of calcium oscillations induced by 5-HT, ACH, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. J Gen Physiol 2005; 125: 535-553.
(28) Perez-Zoghbi JF, Karner C, Ito S, Shepherd M, Alrashdan Y, Sanderson MJ. Ion channel regulation of intracellular calcium and airway smooth muscle function. Pulm Pharmacol Ther 2009; 22: 388-397.
(29) Bai Y, Sanderson MJ. Airway smooth muscle relaxation results from a reduction in the frequency of Ca2+ oscillations induced by a cAMP-mediated inhibition of the IP3 receptor. Respir Res 2006; 7: 34.
(30) Hirota S, Helli P, Janssen LJ. Ionic mechanisms and Ca2+ handling in airway smooth muscle. Eur Respir J 2007a; 30: 114-133.
(31) Schaafsma D, Bos IS, Zuidhof AB, Zaagsma J, Meurs H. The inhaled Rho kinase inhibitor Y-27632 protects against allergen-induced acute bronchoconstriction, airway hyperresponsiveness, and inflammation. Am J Physiol Lung Cell Mol Physiol 2008; 295: L214-L219.
(32) Koopmans T, Anaparti V, Castro-Piedras I, Yarova P, Irechukwu N, Nelson C, Perez-Zoghbi J, Tan X, Ward JP, Wright DB. Ca2+ handling and sensitivity in airway smooth muscle: emerging concepts for mechanistic understanding and therapeutic targeting. Pulm Pharmacol Ther 2014; 29: 108-120.
(33) Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. Respir Res 2006; 7: 73.
(34) Billington CK, Ojo OO, Penn RB, Ito S. cAMP regulation of airway smooth muscle function. Pulm Pharmacol Ther 2013; 26: 112–120.
(35) East JM. Sarco(endo)plasmic reticulum calcium pumps: recent advances in our understanding of structure/function and biology (review). Mol Membr Biol 2000; 17: 189-200.
(36) Ijpma G, Kachmar L, Panariti A, Matusovsky OS, Torgerson D, Benedetti A, Lauzon AM.

Intrapulmonary airway smooth muscle is hyperreactive with a distinct proteome in asthma. Eur Respir J 2020; 56(1):1902178.

(37) White TA, Xue A, Chini EN, Thompson M, Sieck GC, Wylam ME. Role of transient receptor potential C3 in TNF-alpha-enhanced calcium influx in human airway myocytes. Am J Respir Cell Mol Biol 2006; 35: 243-251.

(38) Guedes AG, Dileepan M, Jude JA, Deshpande DA, Walseth TF, Kannan MS. Role of CD38/cADPR signaling in obstructive pulmonary diseases. Curr Opin Pharmacol 2020; 51: 29-33.

(39) Mahn K, Hirst SJ, Ying S, Holt MR, Lavender P, Ojo OO, Siew L, Simcock DE, McVicker CG, Kanabar V, Snetkov VA, O'Connor BJ, Karner C, Cousins DJ, Macedo P, Chung KF, Corrigan CJ, Ward JP, Lee TH. Diminished sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) expression contributes to airway remodelling in bronchial asthma. Proc Natl Acad Sci USA 2009; 106: 10775-10780.

(40) Sathish V, Thompson MA, Bailey JP, Pabelick CM, Prakash YS, Sieck GC. Effect of proinflammatory cytokines on regulation of sarcoplasmic reticulum Ca2+ reuptake in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2009; 297: L26-L34.

(41) Zhang Y, Saradna A, Ratan R, Ke X, Tu W, Do DC, Hu C, Gao P. RhoA/Rho-kinases in asthma: from pathogenesis to therapeutic targets. Clin Transl Immunology 2020; 9, e01134.

(42) Ojiaku CA, Cao G, Zhu W, Yoo EJ, Shumyatcher M, Himes BE, An SS, Panettieri RA, Jr. TGF-β1 evokes human airway smooth muscle cell shortening and hyperresponsiveness via Smad3. Am J Respir Cell Mol Biol 2018; 58: 575-584.

(43) Shaifta Y, MacKay CE, Irechukwu N, O'Brien KA, Wright DB, Ward JPT, Knock GA. Transforming growth factor-β enhances Rho-kinase activity and contraction in airway smooth muscle via the nucleotide exchange factor ARHGEF1. J Physiol 2018; 596: 47-66.

(44) Maarsingh H, Zaagsma J, Meurs H. Arginase: a key enzyme in the pathophysiology of allergic asthma opening novel therapeutic perspectives. Br J Pharmacol 2009; 158(3): 652-664.

(45) Jain V, Raina S, Gheware AP, Singh R, Rehman R, Negi V, Murray Stewart T, Mabalirajan U, Mishra AK, Casero RA, Jr., Agrawal A, Ghosh B. Reduction in polyamine catabolism leads to spermine-mediated airway epithelial injury and induces asthma features. Allergy 2018; 73(10): 2033-2045.

(46) Kurosawa M, Shimizu Y, Tsukagoshi H, Ueki M. Elevated levels of peripheral blood, naturally occurring aliphatic polyamines in bronchial asthmatic patients with active symptoms. Allergy 1992; 47: 638-643.

(47) Jain V. Role of polyamines in asthma pathophysiology. Med Sci (Basel) 2018: 6(1).

(48) Coyle AJ, Ackerman SJ, Irvin CG. Cationic proteins induce airway hyperresponsiveness dependent on charge interactions. Am Rev Respir Dis 1993; 147: 896-900.

(49) Homma T, Bates JH, Irvin CG. Airway hyperresponsiveness induced by cationic proteins in vivo: site of action. Am J Physiol - Lung Cellular and Molecular Physiology 2005; 289: L413-L418.
(50) North ML, Grasemann H, Khanna N, Inman MD, Gauvreau GM, Scott JA. Increased ornithine-derived polyamines cause airway hyperresponsiveness in a mouse model of asthma. Am J Resp Cell Mol Biol 2013; 48: 694-702.
(51) Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, Germain RN, Kastner DL, Chae JJ. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca2+ and cAMP. Nature 2012; 492(7427): 123-127.
(52) Jager E, Murthy S, Schmidt C, Hahn M, Strobel S, Peters A et al. Calcium-sensing receptor-mediated NLRP3 inflammasome response to calciprotein particles drives inflammation in rheumatoid arthritis. Nat Commun 2020 Aug 25; 11(1):4243. doi: 10.1038/s41467-020-17749-6.
(53) Sweeney D, Hollins F, Gomez E, Mistry R, Saunders R, Challiss RAJ, Brightling CE. No evidence for altered intracellular calcium handling in airway smooth muscle cells from human subjects with asthma. BMC Pulmonary Medicine 2015; 15: 12.
(54) Canton J, Schlam D, Breuer C, Gütschow M, Glogauer M, Grinstein S. Calcium-sensing receptors signal constitutive macropinocytosis and facilitate the uptake of NOD2 ligands in macrophages. Nat Commun 2016 Apr 6; 7: 11284. doi: 10.1038/ncomms11284.
(55) Hendy GN, Canaff L. Calcium-sensing receptor, proinflammatory cytokines and calcium homeostasis. Semin Cell Dev Biol 2016; 49: 37-43.
(56) Klein GL, Castro SM, Garofalo RP. The calcium-sensing receptor as a mediator of inflammation. Semin Cell Dev Biol 2016; 49: 52-56.
(57) Zhai TY, Cui BH, Zou L, Zeng JY, Gao S, Zhao Q, Wang Y, Xie WL, Sun YH. Expression and role of the calcium-sensing receptor in rat peripheral blood polymorphonuclear neutrophils. Oxid Med Cell Longev 2017; 2017:3869561. doi: 10.1155/2017/3869561.
(58) Li T, Sun M, Yin X, Wu C, Wu Q, Feng S, Li H, Luan Y, Wen J, Yan L, Zhao B, Xu C, Sun Y. Expression of the calcium sensing receptor in human peripheral blood T lymphocyte and its contribution to cytokine secretion through MAPKs or NF-kappaB pathways. Molecular Immunology 2013: 53(4): 414-420.
(59) Koller DY, Halmerbauer G, Frischer T, Roithner B. Assessment of eosinophil granule proteins in various body fluids: is there a relation to clinical variables in childhood asthma? Clin Exp Allergy 1999; 29: 786-793.
(60) Lee JW, Park HA, Kwon OK, Park JW, Lee G, Lee HJ, et al. NPS 2143, a selective calcium-sensing receptor antagonist inhibits lipopolysaccharide-induced pulmonary inflammation. Molec Immunol 2017; 90: 150-157.

(61) Li GW, Xing WJ, Bai SZ, Hao JH, Guo J, Li HZ, Li HX, Zhang WH, Yang BF, Wu LY, Wang R, Yang GD, Xu CQ. The calcium-sensing receptor mediates hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells through MEK1/ERK1,2 and PI3K pathways. Basic Clin Pharmacol Toxicol 2011; 108(3): 185-193.
(62) Nemeth EF, Goodman WG. Calcimimetic and Calcilytic Drugs: Feats, Flops, and Futures. Calcif Tissue Int 2016; 98(4): 341-58.
(63) Widler L. Calcilytics: antagonists of the calcium-sensing receptor for the treatment of osteoporosis. Future Med Chem 2011; 3(5): 535-547.
(64) Yarova PL, Huang P, Schepelmann MW, Bruce R, Ecker R, Nica R, Telezhkin V, Traini D, Gomes Dos Reis L, Kidd EJ, Ford WR, Broadley KJ, Kariuki BM, Corrigan CJ, Ward JPT, Kemp PJ, Riccardi D. Characterization of negative allosteric modulators of the calcium-sensing receptor for repurposing as a treatment of asthma. J Pharmacol Exp Ther 2021 Jan; 376(1): 51-63. doi: 10.1124/jpet.120.000281.
(65) Caltabiano S, Dollery CT, Hossain M, Kurtinecza MT, Desjardins JP, Favus MJ, Kumar R, Fitzpatrick LA. Characterization of the effect of chronic administration of a calcium-sensing receptor antagonist, ronacaleret, on renal calcium excretion and serum calcium in postmenopausal women. Bone 2013; 56: 154-162.
(66) Cosman F, Gilchrist N, McClung M, Foldes J, de Villiers T, Santora A, Leung A, Samanta S, Heyden N, McGinnis JP, Rosenberg E, Denker AE. A phase 2 study of MK-5442, a calcium-sensing receptor antagonist, in postmenopausal women with osteoporosis after long-term use of oral bisphosphonates. Osteoporosis International 2016; 27: 377–386.
(67) John MR, Harfst E, Loeffler J, Bellelia R, Mason J, Bruin GJM, Seuwen K, Klickstein LB, Mindeholm L, Widler L, Kneissel M. AXT914 a novel, orally-active parathyroid hormone-releasing drug in two early studies of healthy volunteers and postmenopausal women. Bone 2014; 64: 204-210.

(685) The Royal Colleges of Physicians of the United Kingdom. Why asthma still kills. The National Review of Asthma Deaths (NRAD) Confidential Enquiry Report May 2014. Available at: https://www.rcplondon.ac.uk/projects/outputs/why-asthma-still-kills
(696) Diao J, Lam M, Gregory K, Leach K, Bourke JE. Biased negative allosteric modulators of the calcium-sensing receptor differentially oppose airway contraction in mouse precision cut lung slices. Am J Respir Crit Care Med 2021; 203: A4516.

Topical therapy with negative allosteric modulators of the calcium-sensing receptor (calcilytics) for the management of asthma: the beginning of a new era?

Authors:

Daniela Riccardi[1], Jeremy PT Ward[2], Polina L Yarova[3], Luke J Janssen[4], Tak Hong Lee[2,5], Sun Ying[6], Chris J Corrigan[2]

Affiliations:

1. Cardiff School of Biosciences, University of Cardiff, UK

2. Faculty of Life Sciences and Medicine and Asthma UK Centre in Allergic Mechanisms of Asthma, King's College London, UK

3. Translational and Clinical Research Institute, School of Medical Sciences, Newcastle University, UK

4. Department of Medicine, McMaster University, Hamilton, Ontario, Canada

5. Hong Kong Sanatorium and Hospital, Hong Kong, China

6. Department of Immunology, School of Basic Medical Sciences, Capital Medical University, Beijing, China

Corresponding Author:

Professor C J Corrigan, King's College London Faculty of Life Sciences and Medicine, 5th Floor, Tower Wing, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK.

E-mail: chris.corrigan@kcl.ac.uk

Tel: +44 207 188 0610

Take home message: Negative allosteric modulators of the calcium-sensing receptor (calcilytics) delivered topically to the airways have the potential to revolutionise asthma therapy

Abstract

In this review article we present the evidence to date supporting the role of the calcium-sensing receptor (CaSR) as a key, pluripotential molecular trigger for asthma and speculate on the likely benefits of topical therapy of asthma with negative allosteric modulators of the CaSR: calcilytics.

What is the calcium-sensing receptor (CaSR) and what are calcilytics (Figure 1)?

The extracellular calcium-sensing receptor (CaSR) is a G protein-coupled receptor (GPCR) originally identified as the body's master controller for extracellular, free ionised calcium (Ca^{2+}). It is widely expressed as a regulator of global calcium metabolism: for example, it is responsible for homoeostasis of extracellular [Ca^{2+}] by regulating parathyroid hormone secretion, Ca^{2+} resorption in the renal loop of Henlé, calcitonin release in the thyroid and osteoclast-mediated bone resorption. The CaSR provides the means by which these cell types sense the extracellular ionised calcium concentration [Ca^{2+}], and maintain it within a narrow physiological range [1]. It is now recognised, however, that the CaSR is widely expressed in many other cell types and has functions unrelated to regulation of extracellular Ca^{2+} homoeostasis. Other important physiological roles include sensing of dietary nutrients in the gut, glucose-mediated insulin secretion, taste satiety and vascular smooth muscle function [1 - 3]. In this review we propose a novel, causal role for the CaSR in the pathophysiology of airways smooth muscle hyperresponsiveness, airways inflammation and the key mechanism by which this inflammation exacerbates bronchial smooth muscle spasm in human asthma.

The CaSR is expressed in the cell membrane as a constitutive homodimer with large extracellular domains which enclose a ligand-binding cleft (Figure 1). In addition to Ca^{2+} it responds to other di-, tri- and polyvalent cations such as Mg^{2+}, Gd^{3+} and other orthosteric agonists including polyamines and polycationic proteins [2, 3]: as will be explained below, this ability to respond to cationic proteins forms a keystone of its potential importance in asthma pathogenesis. The CaSR couples through several different G-protein-mediated signalling pathways, including $G_{q,11}$ (releases Ca^{2+} from intracellular stores; activates protein kinase C), $G_{i/o}$ (inhibits the generation of cyclic adenosine monophosphate; cAMP), and $G_{12,13}$ (activates Rho-kinase and other effector kinases). In addition, the CaSR can also signal via mitogen-activated protein kinase (MAPK) cascades, including extracellular signal-

regulated kinase (ERK), p38 MAPK, c-Jun N-terminal kinase MAP-kinase, and the phosphoinositide 3-kinase (PI3 kinase) and mechanistic target of rapamycin (mTOR) pathways [1, 2]. Because of its ability to couple to multiple G proteins, the CaSR is complex and exhibits “biased agonism”, whereby different ligands activate (or inhibit) specific subsets of signalling pathways preferentially [1 - 3].

The CaSR also binds a range of positive and negative allosteric modulators. Natural positive allosteric modulators (PAMs) of the CaSR include aliphatic and aromatic amino acids that enhance the sensitivity of the CaSR to its primary ligand, Ca^{2+} [4]. Synthetic PAMs of the CaSR, also known as calcimimetics, include the clinically available drugs cinacalcet, evocalcet and etelcalcetide. These compounds are currently in general clinical therapeutic use to treat hypercalcaemia arising from hyperparathyroidism of various aetiologies, including parathyroid carcinoma, hyperparathyroidism secondary to renal failure, familial hypocalciuric hypercalcaemia and primary hyperparathyroidism in neonates or those patients with parathyroid carcinoma who are not suitable for surgical excision. Calcimimetics exhibit ligand biased signalling, with preferential activation of CaSR-mediated phosphorylation of ERK1/2 over Ca^{2+} mobilisation (reviewed in [5]).

Synthetic, negative allosteric modulators (NAMs) of the CaSR, also known as calcilytics, were first discovered as a result of high throughput screening of compounds based on an arylalkylamine scaffold (reviewed in [6]) and now include the amino alcohol compounds such as ronacaleret, JTT-305 (also known as MK-5442), NPSP795 and the quinazolin-2-one derivatives ATF936 and AXT914 [7]. Some of these calcilytics have been evaluated for therapy of osteoporosis but found to be clinically ineffective, likely because they exert opposing effects on key processes such as calcium mobilisation and osteoblast activity (reviewed in [1]). Recently the calcilytic NPSP795 has been repurposed to treat autosomal dominant hypocalcaemia with hypercalciuria, which is caused by activating mutations in the *CASR* gene [8], while others are under investigation for therapy of other hypocalcaemic disorders such as idiopathic hypercalciuria.

What is the clinical evidence that the CaSR is involved in the pathogenesis of airways smooth muscle hyperresponsiveness in human asthma?

From the account above it will be self-evident that the function of the CaSR is influenced not only by its binding of Ca^{2+} and other inorganic cations, but also by sensing organic, polycationic species, the local concentrations of which may be greatly increased in the

airways as a result of inflammation and environmental exposure. It has long been known from studies on murine surrogates [9] and patients with occupational asthma caused by exposure to aliphatic polyamines [10] that exposure to these compounds increases the risk of manifestation of airways smooth muscle hyperresponsiveness. Similarly, it is well recognised that elevated airways concentrations of the eosinophilic cationic proteins, while not specific for asthma, correlate with disease severity in the context of asthma [11]. In asthma, polyamines and polycationic protein products of airways inflammatory cells in particular have the potential to bind to and activate the CaSR directly, functioning as orthosteric agonists which markedly heighten the signal output of the CaSR. Thus, it is entirely plausible to assume that over-expression of the CaSR and/or activation of the CaSR by local, environmental stimuli accounts for the phenomenon of airways smooth muscle hyperresponsiveness which characterises human asthma, and for the regulation of the degree of this hyperresponsiveness by the concentrations of cationic products of local, asthma-relevant inflammatory cells. In this article we present data, from our group and others, in direct support of this hypothesis and discuss in more detail the role of the CaSR in regulating ASM contraction and airways inflammation. We also summarise and discuss the evidence that the concentrations of polyamines and polycationic proteins are elevated in the airways in asthma and, in many previous studies, have been shown to correlate with disease severity.

Airways smooth muscle (ASM) hyperresponsiveness is responsible for the short-term, spontaneous variability in airways obstruction which causes asthmatics (but not non-asthmatics) to develop sudden wheezing and breathlessness when exposed to a range of specific and non-specific stimuli such as smoke, cold air, allergens in sensitised subjects, exercise and respiratory tract infections. In a recent key study [12] it was demonstrated that human asthma is accompanied by over-expression of the CaSR on airway smooth muscle (ASM) cells compared with non-asthmatic controls, and furthermore that exposure of this receptor to negative allosteric modulators abrogated asthmatic ASM hyperreactivity to contractile stimuli *ex vivo* and *in vitro*. It was also demonstrated that expression of the CaSR is up regulated on human ASM cells exposed to asthma-associated cytokines: it was hypothesised that this is driven by the signal transducer and activator of transcription (STAT) and κB response elements in the CaSR gene promoters [13]. Exposure of murine lung slices to the CaSR agonist spermine *ex vivo* potentiated ASM contraction induced by acetylcholine; this effect was abolished in lung slices from animals with selective CaSR ablation in their ASM cells and abrogated by calcilytics in lung slices from wild type mice but not those with the selective CaSR ablation [12]. Moreover, wild type mice exhibited AHR following

exposure to inhaled poly-L-arginine (another CaSR agonist*) in vivo*, an effect which was abolished by inhaled calcilytics [12]. In addition, the calcilytic drug NPS2143 attenuated basal, elevated intracellular Ca^{2+} concentrations as well as Ca^{2+} release in response to acetylcholine or histamine in ASM cells from asthmatic, but not non-asthmatic control patients [12]. This latter observation is particularly notable because it demonstrates that calcilytics, while normalising the Ca^{2+} concentrations in ASM cells from asthmatic patients, do not appear to alter the function of ASM cells in non-asthmatic individuals.

In the same study [12] it was discovered that, in addition to the arginase products spermine, spermidine and putrescine, the CaSR was also activated by products of eosinophils, including eosinophil cationic proteins and major basic protein, providing a clear functional basis for the regulation of asthma severity by the products of these cells.

It is also noteworthy that exposure of foetal lung ASM cells to hyperoxia has been reported to up-regulate CaSR expression, inducing hyperresponsiveness to histamine and increased proliferation. Again these effects were attenuated by calcilytics, providing a therapeutic avenue to the management of neonatal airways diseases including hyperoxia-induced, neonatal asthma [14].

It may also be relevant that a recent bioinformatics study of genetic variants of the CaSR uncovered clinically relevant associations with several diseases unrelated to regulation of circulating Ca^{2+}, including asthma (15): it remains to be seen if and how these genetic mutations which dysregulate total body calcium homoeostasis might also affect polycation-sensing of CaSR in inflamed ASM.

Pathophysiological mechanism of airways smooth muscle hyperresponsiveness in asthma (Figure 2)

There is good evidence that bronchial hyperresponsiveness in asthma is associated with increased ASM contractile function, the mechanisms of which have yet to be fully defined but include alterations to the ASM cell intracellular Ca^{2+} concentration [Ca^{2+}_i] handling and sensitivity, contractile machinery and cytoskeletal dynamics and structure [16 – 20]. In this section we briefly outline the mechanisms underlying ASM contraction and relaxation, how they may be altered is asthma, and the potential key role of the CaSR.

Elevation of $[Ca^{2+}]_I$ is central to ASM contraction, and activation of other cell types including epithelial and inflammatory cells. Multiple pathways contribute to $Ca^{2+}{}_i$ homoeostasis, including Ca^{2+} release and sequestration by the sarcoplasmic reticulum (SR) and mitochondria, and Ca^{2+} flux into and out of the cell [19, 21]. These are activated (or inhibited) by a variety of GPCRs [22] (Figure 2). Most bronchoconstrictors activate GPCR coupled via $G_{q,11}$ to phospholipase Cβ, generating inositol trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3 elicits Ca^{2+} release from the SR, whereas DAG activates non-selective (Ca^{2+} and Na^+ permeable) receptor-operated channels (ROC), and protein kinase C (PKC). Ca^{2+} released by IP_3 activates adjacent ryanodine receptors (RyR; Ca^{2+}-induced Ca^{2+} release), amplifying the response [21]. RyR are also activated by cyclic ADP ribose, generated by CD38 [23]. Depletion of SR Ca^{2+} content activates store operated channels (SOC) via STIM and thus further increases Ca^{2+} entry. Voltage gated Ca^{2+} channels (L-type) appear to be of limited significance in ASM or indeed the pathogenesis of asthma, which is why calcium channel blockers have proven ineffective for asthma prophylaxis and therapy [24, 25].

Cytosolic Ca^{2+} is sequestered back into the SR by the sarcoendoplasmic reticulum ATPase (SERCA), and expelled from the cell by a Na^+-Ca^{2+} exchanger (NCX) and plasma membrane Ca^{2+} ATPase (PMCA) [19]. As NCX exchanges three Na^+ for each Ca^{2+} it is membrane potential- and Na^+ gradient-dependent; the resulting depolarisation and local increases in $[Na^+]_i$ following Na^+ entry via ROC causes NCX to operate in reverse mode, facilitating Ca^{2+} entry [26].

The ASM cell is highly organised, with cell membrane, peripheral SR and mitochondria creating signalling micro-domains that allow rapid regulation of the temporal and spatial aspects of changes in $[Ca^{2+}]_i$. This facilitates generation of oscillations in $[Ca^{2+}]_i$ on stimulation by bronchoconstrictors [27, 28], while bronchodilators reduce the Ca^{2+}-oscillation frequency [29]. Importantly, the magnitude of ASM shortening correlates with Ca^{2+}-oscillation frequency and not amplitude [27]. ASM also exhibits slower oscillations in membrane potential on stimulation, which may switch NCX between forward and reverse modes, producing corresponding $[Ca^{2+}]_i$ oscillations [30]. Many enzymes decode Ca^{2+}-oscillation frequency in a wide variety of cell types, and may contribute to both contraction and proliferation of ASM cells [19, 30].

Elevation of ASM $[Ca^{2+}]_i$ leads to Ca^{2+}-calmodulin-mediated activation of myosin light chain kinase (MLCK), phosphorylation of myosin light chain (MLC) and consequently activation of myosin ATPase and cell shortening. Relaxation requires dephosphorylation of MLC by its phosphatase (MLCP), so force generation depends on the balance between MLCK and

MLCP activities. MLCP is constitutively active, but can be inhibited by RhoA-kinase (ROCK). ROCK is activated by the monomeric G-protein RhoA, which is itself activated by $G_{12,13}$ coupled GPCRs [31]. Inhibition of MLCP means more force is generated for the same elevation of $[Ca^{2+}]_i$ (Ca^{2+} sensitisation); PKC similarly induces Ca^{2+} sensitisation via phosphorylation of the MLCP inhibitor CPI-17 [32]. Notably, many bronchoconstrictors act through both $G_{q,11}$ and $G_{12,13}$ coupled pathways. For example, acetylcholine activates M3 muscarinic receptors which couple to both $G_{q,11}$ and $G_{12,13}$, thus stimulating IP_3-induced SR $Ca^{2+}{}_i$ release, activation of ROC and PKC, simultaneously with RhoA/ROCK-mediated Ca^{2+} sensitisation [33]. Notably, some bronchoconstrictors also activate G_i-coupled GPCR, thus inhibiting adenylyl cyclase (see below).

β_2-adrenoreceptor agonists and most endogenous bronchodilators (e.g. catecholamines, vasoactive intestinal peptide, PGE_2) elicit relaxation by activating adenylyl cyclase via G_s-coupled GPCRs to elevate cAMP; cAMP is degraded by phosphodiesterases (PDEs), so PDE inhibitors (e.g. theophylline) also elevate cAMP. Elevation of cAMP supresses multiple bronchoconstrictor pathways, mediated either by protein kinase A (PKA) or Epac (exchange factor directly activated by cAMP); these include Ca^{2+} influx and mobilisation, RhoA activation and MLC phosphorylation (reviewed in [22, 32]). It also stimulates SERCA, thus reducing $[Ca^{2+}]_I$ [35], slows Ca^{2+} oscillations [29] and enhances degradation of bronchoconstrictor GPCRs [34].

Numerous studies have shown asthma-associated perturbations of the pathways discussed above, either using ASM derived from asthmatics or animal surrogates, or treated with asthma-associated mediators; as these have been extensively reviewed [16, 19, 20, 22, 36], discussion here is limited to a few salient points. There is a wide consensus that asthma is associated with ASM hyperresponsiveness, while enhanced $[Ca^{2+}]_i$ mobilisation is well documented, being attributed to increased Ca^{2+} entry and release [28, 37, 38] and reduced activity of SERCA and Ca^{2+} reuptake into the SR [39, 40]. Similarly, Ca^{2+} sensitisation and RhoA/ROCK have also been strongly implicated in ASM hyperresponsiveness [31, 41 - 43].

The role of the CaSR in airways smooth muscle hyperresponsiveness and the potential of calcilytics

Under normal, “healthy” conditions, CaSR expressed on ASM cells would be expected to reside in a continuous state of low level activation in the presence of normal concentrations of interstitial $[Ca^{2+}]$; this is consistent with the finding that calcilytics reduced $[Ca^{2+}]_i$ in ACh-

stimulated human ASM cells from both healthy and asthmatic donors [12]. Asthma is however accompanied both by elevated expression of the CaSR in ASM [12] and elevated concentrations in the airways of potent CaSR activators, including eosinophil cationic protein and major basic protein, and cationic polyamines (putrescine, spermidine, spermine). The latter are elevated in asthma owing to both increased arginase activity and reduced polyamine catabolism [12, 44 - 47], and have been previously associated with the pathophysiology of bronchial hyperresponsiveness [48 - 50]. It has also been proposed that inflammation leads to localised elevations of extracellular [Ca^{2+}] which may also increase CaSR activity [51, 52].

The effects of all of these stimuli acting in concert would inevitably result in a leftward shift in the CaSR [Ca^{2+}]-response relationship and greatly increased signal output, potentiated by the elevated expression of CaSR [1, 3, 12]. Thus ASM Ca^{2+} mobilisation and RhoA/ROCK- and PKC-mediated Ca^{2+} sensitivity would be elevated (via $G_{q,11}$, $G_{12,13}$, G_i and MAPK cascades), whereas adenylyl cyclase and cAMP generation would be inhibited (via G_i). Collectively, this would potentiate ASM contractility and sensitivity to other bronchoconstrictor autacoids that act through these same intracellular signalling pathways (e.g. ACh, histamine, neuropeptides, prostaglandins, leukotrienes; see Figure 2). These effects would be further amplified by the asthma-associated perturbations in ASM signal transduction pathways discussed in the previous section [16, 19, 20, 22, 36] and sufficient to account for ASM hyperresponsiveness in asthma.

This scenario again underlines the concept that ASM hyperresponsiveness in asthma is highly dependent upon the environment of the ASM (where the ASM cells are immersed in cationic proteins bathing the interstitium), and not entirely an intrinsic, functional abnormality of the ASM itself. This might in turn explain why not all studies performed on human ASM obtained from asthmatic patients and studied *ex vivo* report differences in ASM contractility or Ca^{2+} homoeostasis when compared to ASM from healthy donors [16, 17, 53]. When ASM is excised for study *ex vivo*, it is perforce removed from its surrounding inflammatory milieu and exposure to asthma-associated mediators, including polycationic CaSR ligands. It is possible to hypothesise that the continued presence of such stimuli is necessary to effect detectable functional alterations in the ASM under some circumstances *ex vivo*. Similar reasoning underlies the suggestion that ASM may be normal in asthma, but its function altered by an abnormal environment (discussed in [16]). It is interesting to speculate that one of the effects of this “abnormal environment” might be to up-regulate CaSR expression on ASM, at least in susceptible patients who develop asthma [12]. It is also possible to hypothesise, although this seems less likely, that this phenomenon might also be partly

attributable to variation in disease severity and therapy and/or the anatomical source of the ASM, since ASM from the trachea and main bronchi is known to differ functionally from that of the more relevant, intrapulmonary bronchi. In particular, ASM from the latter has been shown to exhibit hyperreactivity in asthma when this was not the case for the larger airways [17, 36].

Taken together, the data above are consistent with the hypothesis that over-expression and activation of the CaSR by relevant, asthma-associated extracellular ligands in the immediate vicinity of the ASM is a critical driver of ASM hyperresponsiveness in asthma, with the corollary that therapy with calcilytics has the potential to abolish it. This is consistent with the recent observation referred to above [12] that inhibition of the CaSR by calcilytics attenuates or ablates bronchial hyperresponsiveness in animal surrogates of asthma.

The potential for calcilytics to inhibit airways inflammation and its effects on ASM hyperresponsiveness in human asthma (Figure 3)

In addition to its putative role in engendering ASM hyperresponsiveness in human asthma, signalling mediated through the CaSR is increasingly recognised to have key roles both in immune surveillance and the regulation of ongoing inflammation in the airways and elsewhere [54 - 56], for example via activation of the NLRP3 inflammasome [51]. Both of these key immunological functions of the CaSR have recently been implicated in the pathogenesis of other chronic inflammatory diseases such as rheumatoid arthritis [52].

The CaSR is known to be expressed on monocyte/macrophages, neutrophils [57] and T cells [58], rendering them sensitive to activation by extracellular, locally released, inflammation-associated ligands of the CaSR as well as elevation of local extracellular [Ca^{2+}] which has been shown to activate the NLRP3 inflammasome in macrophages [51] and NF-κB and other downstream signalling pathways in neutrophils and T cells. The additional, recent demonstration that eosinophils express the CaSR [12] raises the possibility that release of eosinophil cationic proteins in the course of asthmatic mucosal inflammation may further activate other local eosinophils as well as other cells via the CaSR, in addition to prolonging their lifespan by inhibition of apoptosis [47] through an autocrine, feedback loop. Finally it is noteworthy that structural cells of the airways may also contribute to polyamine-driven inflammation in asthma: for example airways epithelial damage, a pathognomonic feature of asthma, in addition to triggering alarmin release results in the loss of activity of polyamine catabolic enzymes, resulting in further local injury to the epithelium [45].

These recent experimental findings likely underlie, at least in part, the longer established findings that the expression of CaSR agonists such as polyamines and eosinophil cationic proteins in many previous studies of asthma involving both human subjects and animal surrogates from a multidisciplinary literature correlates positively with airways inflammation and remodelling, as well as disease activity [46 - 49, 59]. Conversely, in murine asthma surrogates, chronic treatment with calcilytics has been reported to attenuate airways hyperresponsiveness, inflammation and remodelling [12, 60]. Taken together, all of these data suggest that, in addition to abolishing ASM hyperresponsiveness, topical therapy of asthmatic patients with calcilytics has the potential to exert an anti-inflammatory effect. Furthermore, regardless of any possible anti-inflammatory effects, calcilytic therapy would be expected to abolish direct exacerbation of ASM hyperresponsiveness in patients with asthma by stimulation of the over-expressed CaSR by products of inflammatory cells such as polyamines and eosinophil-derived proteins.

In closing this section, it is worth noting that other recent studies are consistent with the hypothesis that signalling via the CaSR may play a role in other aspects of asthma pathophysiology. For example, signalling via the CaSR has also been reported to be responsible for hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells [61], suggesting a role in the pathogenesis of hypoxic pulmonary hypertension, and consistent with the hypothesis that it similarly contributes to ASM hypertrophy in asthma. Similarly, a very recent study referred to above [14] has presented evidence that signalling via the CaSR is responsible for the ASM hyperreactivity observed in some premature infants ventilated with supplemental oxygen.

The potential to deliver established calcilytics topically to the airways of patients with asthma

Several small molecule calcilytics have been developed and assessed for oral therapy of osteoporosis as mentioned above, including the amino alcohols ronacaleret and JTT-305 in Phase 2 clinical trials and NPSP795 (a zwitterion amino alcohol) and AXT914 (a quinazolin-2-one) in Phase 1 trials. Although, when administered systemically by oral dosing, these drugs were not found to be efficacious for the treatment of post-menopausal osteoporosis, an indication for which they were originally developed [62, 63] they were importantly observed to be safe and tolerable in human subjects (some were observed to cause mild hypercalcaemia in a small fraction of normal human volunteers). A very recent study [64] addressed the likely suitability of these four calcilytics for topical delivery to the airways of human asthmatic patients, potentially using devices similar or identical to existing inhaler

devices. This included an assessment of the feasibility of delivering them to the airways in sufficient quantities to abolish bronchial hyperresponsiveness and airways inflammation in a murine surrogate of chronic asthma. All four calcilytics [8, 65 - 67], when delivered topically inhibited poly-L-arginine-induced airways hyperresponsiveness in naïve mice and suppressed both airways hyperresponsiveness and inflammation in an asthma surrogate, confirming class specificity. Repeated exposure to inhaled calcilytics did not alter blood pressure, heart rate or serum calcium concentrations, providing considerable precedent for the expectation that topically delivered drugs will not disrupt systemic calcium regulation in human asthmatic patients. Optimal candidates for repurposing for topical therapy of human asthma were identified based on their effects on airways hyperresponsiveness and inflammation, pharmacokinetics and pharmacodynamics, formulation and micronisation properties. In this study, whereas both inhaled calcilytics and inhaled corticosteroids were observed to reduce airways inflammation, only the former obviated features of airways remodelling such as goblet cell hyperplasia.

Outstanding Issues

The CaSR is expressed by many structural and inflammatory cells of the lung, from the airways smooth muscle to the bronchial mucosa. Furthermore, it is capable of being activated by many ligands other than Ca^{2+}, including basic proteins and arginine metabolites at sites of inflammation. It is possible, therefore, that the consequences of CaSR activation or blockade may vary according to the precise situation in the lungs and the nature of the local environment, which may be influenced both by intrinsic inflammation and the effects of external factors such as infectious agents and inhaled, environmental pollutants.

As with all drugs, aside from the potential beneficial effects of calcilytics on hyperoxia-induced bronchial hyperresponsiveness in premature neonates referred to above [14], the potential for topically delivered calcilytics to exert unwanted effects on lung development or immune surveillance can only be determined by clinical experience. The fact that a range of systemically delivered calcilytics has been used successfully in human subjects for many years to manage a range of disorders of calcium metabolism provides considerable reassurance, however, that topically delivered drugs will be both safe and tolerable. Furthermore, as has been emphasised throughout this article, calcilytics are least likely to exert any functional effects in “normal” tissue, where the CaSR is expressed at baseline density and is not in an “inflammatory” environment of orthosteric agonists.

The future positioning of calcilytic therapy for asthma

In summary, the data presented herein suggest that it should be possible to repurpose calcilytics as topical therapy, with a favourable PKPD, safety and tolerability profile, for human asthma delivered using inhaler devices familiar to patients, and that this therapy has the potential to abolish ASM hyperresponsiveness, and thereby wheezing and breathlessness. As long as they are compliant with therapy, patients need not live in fear of sudden wheezing or breathlessness, which is also a likely cause of sudden death from asthma (according to the most recent National Review of Asthma Deaths in the UK [68], at least half of the patients who die from asthma in the UK are deemed to have “mild/moderate” disease, and presumably therefore die as a result of bronchospasm possibly exacerbated by mucous plugging). The suitability of topical calcilytic therapy to replace conventional bronchodilator therapy for asthma is further underlined by recent evidence that calcilytics elevate cAMP concentrations in human ASM cells, particularly from asthmatic patients [12] and dilate acetylcholine pre-contracted murine airways [64] or lung slices, an effect at which they demonstrate greater potency than conventional bronchodilators such as salbutamol and formoterol [69]. This is most likely attributable to signalling via $G_{i/o}$ and not off-target L-type calcium channel inhibition [64], and provides yet another therapeutic avenue through which calcilytics may relieve bronchospasm. Further, experimental evidence suggests that topical calcilytics can inhibit airways mucosal inflammation in asthmatic patients at least as efficiently as corticosteroids (but with none of their unwanted effects), in addition to suppressing the effects of polyamine and other cationic protein CaSR ligands released from inflammatory and other cells on ASM and other local inflammatory and airways structural cells. Above all, calcilytic therapy has the potential directly to target the receptor triggering a wide range of pathophysiological and pro-inflammatory events in asthma rather than intervening in downstream signalling (see Table 1).

	β2 agonists	Anti-muscarinics	PDE4 inhibitors	Steroids	Biologicals	Calcilytics
Airway hyperresponsiveness						☑
Inflammation			☑	☑	☑	☑
Remodelling/fibrosis						☑
Bronchoconstriction	☑	☑				☑
Restrictions/adverse effects	Black box warning		Diarrhoea, weight loss	Pneumonia, Osteoporosis Bone fracture		

Table 1: The scope of calcilytic therapy compared with "conventional" therapy for asthma (black box warning refers to the potential danger of treating asthma with long-acting β2-agonists in the absence of corticosteroids). PDE4 = phosphodiesterase 4.

If these expectations are fulfilled, calcilytic therapy has the potential completely to replace stepwise therapy with inhaled bronchodilators and corticosteroids advocated in current asthma guidelines, enabling administration of the therapy once or twice daily to adults and children in a limited range of devices, thus facilitating compliance, and teaching and valid checking of inhaler technique.

With this background and the recent identification of calcilytics that appear both safe and suitable for topical delivery to the airways in asthma, these data firmly suggest that first in human studies will be feasible, desirable and achievable in the short term. In the first instance we propose experiments firstly to address the hypothesis that inhaled calcilytics abolish ASM hyperresponsiveness in mild asthmatics, and secondly to address the hypothesis that they abolish both early- and late-phase bronchoconstriction following allergen bronchial challenge of mild, atopic asthmatic patients prior to studies in wider groups of asthmatic patients. It will be of particular interest, in the longer term, to follow up clinically the initial indications that, unlike corticosteroids, calcilytics have the capacity to alter the natural history of airways remodelling in asthma and thereby reduce or obviate irreversible airways obstruction.

Financial support

Some of the research by the authors cited in this article was supported by an Initial Foundation grant “Polycations and the calcium-sensing receptor (CaSR) in asthma” from Asthma UK awarded to Chris J Corrigan, Jeremy PT Ward, Paul J Kemp & Daniela Riccardi), grants "Multifaceted CaSR” and “Biomedicine” awarded to Paul J Kemp and Daniela Riccardi from the Marie Curie Integrated Training Network, a KESS 2 Studentship awarded to Daniela Riccardi and a Wellcome Trust Fellowship awarded to Polina Yarova.

Conflict of Interest Statement

Chris J Corrigan, Jeremy PT Ward and Daniela Riccardi hold a patent for the development of calcium receptor antagonists for the treatment of inflammatory lung disease (https://patents.google.com/patent/WO2014049351A1/en) and Daniela Riccardi and Polina L Yarova have a pending Composition of Matter patent currently undergoing filing for development of new chemical entities: “Novel calcilytics for pulmonary disease” (IP from NCE GB1719023.2). These authors and the remaining authors Luke J Janssen, Tak H Lee and Sun Ying declare no other conflicts of interest relevant to the content of this manuscript, including grants or contracts from any other entity, royalties or licences, consulting fees, payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events, payment for expert testimony, support for attending meetings and/or travel, participation on a Data Safety Monitoring Board or Advisory Board, leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid, stock or stock options, receipt of equipment, materials, drugs, medical writing, gifts or other services or other financial or non-financial interests.

Legends to Figures

Figure 1: ***Overview of ligand binding sites and signalling pathways of the CaSR.*** The CaSR functions as a constitutive homodimer comprising of (i) a large extracellular domain consisting of a cysteine-rich domain and bi-lobar "Venus flytrap" structure which binds to cations, anions and allosteric modulators, (ii) a seven transmembrane domain (7TMD) and (iii) an intracellular domain containing a binding site for hypoxia-induced mitogenic factor (HIMF). Ca^{2+} binding sites are indicated as red circles. The CaSR couples to three heterotrimeric G-proteins: (i) $G_{q,11}$ which activates phospholipase C (PLC) to generate inositol trisphosphate (IP3) and diacylglycerol (DAG) with subsequent Ca^{2+} release from the SR, Ca^{2+} entry via receptor operated channel (ROC), and activation of protein kinase C (PKC), and consequently the mitogen-activated protein kinase (MAPK) cascade and extracellular signal-regulated kinase 1 and 2 (ERK1/2); (ii) G_i which both inhibits adenylate cyclase, thus supressing PKA-mediated inactivation of MAPK, and also activates MAPK via a $G_{\beta/\gamma}$ and Ras pathway; (iii) $G_{12,13}$ which activates the RhoA/ROCK pathway. The CaSR also signals via the phosphoinositide 3-kinase (PI3K) and mTOR (mechanistic target of rapamycin complex) pathways, leading to activation of Akt (protein kinase B) and nuclear factor kappa B (NFκB)-mediated IL-1β release. Akt also induces reactive oxygen species (ROS) production and suppresses caspase activation in mitochondria.

Figure 2: ***Signalling pathways underlying bronchial smooth muscle contraction and hyperresponsiveness.*** The figure displays key pathways regulating intracellular Ca^{2+} homoeostasis and Ca^{2+} sensitivity and thus contractile function in bronchial smooth muscle cells. Those reported to be altered in tissue from asthmatics or asthma surrogates and implicated in hyperresponsiveness are indicated. Some intermediate components are omitted for clarity. cADPR, cyclic ADP ribose; CD38, cyclic ADP ribose hydrolase; DAG, diacylglycerol; GPCR, G-protein coupled receptor; IP3, inositol trisphosphate; IP3R, inositol trisphosphate receptor; M3/M2, muscarinic receptors; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NCX, Na^+/Ca^{2+} exchanger; PKC, protein kinase C; PLCβ ,phospholipase C β; PMCA, plasma membrane Ca^{2+} ATPase; ROC, receptor operated channel; ROCK, Rho kinase; RyR, Ryanodine receptor (Ca^{2+} release channel); SERCA, sarcoendoplasmic reticulum Ca^{2+} ATPase; SOC, store operated channel; STIM, stromal interaction molecule.

Figure 3: ***Signalling pathways in the asthmatic airway: role of the CaSR and likely actions of its negative allosteric modulators***. The figure represents a simplified schematic of key cell types, cytokines, chemokines and growth factors involved in the pathogenesis of

asthma. All cell types illustrated have been shown to express CaSR, and would thus be affected by modulators of CaSR activity. CCL5, CC chemokine 5 (RANTES); CCL11, CC chemokine 11 (eotaxin); LTC4, cysteinyl leukotriene C4; ECP, MBP, eosinophil cationic protein, major basic protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1β, interleukin 1β; ILC2, type-2 innate lymphoid cell; TGFβ, transforming growth factor β; Th2, T helper 2 cell; TNFα, tumour necrosis factor α; TSLP, thymic stromal lymphopoietin.

References

(1) Leach K, Fadil M Hannan FM, Josephs TM, Keller AN, Møller TC, Ward DT, Kallay E, Mason RS, Thakker RV, Riccardi D, Conigrave AD, Bräuner-Osborne H. International Union of Basic and Clinical Pharmacology. CVIII. Calcium-Sensing Receptor Nomenclature, Pharmacology, and Function. Pharmacol Rev 2020; 72(3): 558-604. doi: 10.1124/pr.119.018531.

(2) Lopez-Fernandez I, Schepelmann M, Brennan SC, Yarova PL, Riccardi D. The calcium-sensing receptor: one of a kind. Exp Physiol 2015; 100(12): 1392-1399.

(3) Quinn SJ, Ye CP, Diaz R, Kifor O, Bai M, Vassilev P, Brown E. The Ca2+-sensing receptor: a target for polyamines. Am J Physiol 1997; 273(4): C1315-23.

(4) Shenglong Ling , Pan Shi, Sanling Liu, Xianyu Meng, Yingxin Zhou, Wenjing Sun, Shenghai Chang, Xing Zhang, Longhua Zhang, Chaowei Shi, Demeng Sun, Lei Liu, Changlin Tian. Structural mechanism of cooperative activation of the human calcium-sensing receptor by Ca^{2+} ions and L-tryptophan. Cell Research 2021; 31:383–394.

(5) Diayo J, DeBono A, Josephs TM, Bourke JE, Capuano B, Gregory KJ, Leach K. Therapeutic opportunities of targeting allosteric binding sites on the calcium-sensing receptor. ACS Pharmacol Transl Sci 2021; 4(2): 666-679.

(6) Marquis RW, Lago AM, Callahan JF, Rahman A, Dong X, Stroup GB, Hoffman S, Gowen M, DelMar EG, Van Wagenen BC, Logan S, Shimizu S, Fox J, Nemeth EF, Roethke T, Smith BR, Ward KW, Bhatnagar P. Antagonists of the calcium sensing receptor. 2. Amino alcohol-based parathyroid hormone secretagogues. J Med Chem 2009; 52(21): 6599-605.

(7) Letz S, Haag C, Schulze E, Frank-Raue K, Raue F, Hofner B, Mayr B, Schöfl C. Amino alcohol- (NPS-2143) and quinazolinone-derived calcilytics (ATF936 and AXT914) differentially mitigate excessive signalling of calcium-sensing receptor mutants causing Bartter syndrome type 5 and autosomal dominant hypocalcemia. PLOS ONE 2014; 9(12): e115178.

(8) Roberts MS, Gafni RI, Brillante B, Guthrie LC, Streit J, Gash D, Gelb J, Krusinska E, Brennan SC, Schepelmann M, Riccardi D, Khayat MEB, Ward DT, Nemeth EF, Rosskamp R, Collins MT. Treatment of autosomal dominant hypocalcemia Type 1 with the calcilytic NPSP795 (SHP635). J Bone Miner Res 2019; 34(9): 1609-1618. doi: 10.1002/jbmr.3747.

(9) North ML, Grasemann H, Khanna N, Inman MD, Gauvreau GM, Scott JA. Increased ornithine-derived polyamines cause airway hyperresponsiveness in a mouse model of asthma. Am J Respir Cell Mol Biol 2013; 48(6): 694–702.

(10) Ng TP, Lee HS, Malik MA, Chee CBE, Cheong TH, Wang YT. Asthma in chemical workers exposed to aliphatic polyamines. Occupational Medicine 1995; 45(1): 45–48.

(11) Koh GCH, Shek LPC, Goh DYT, van Bever H, Koh DSQ. Eosinophil cationic protein: is it useful in asthma? A systematic review. Respir Med 2007; 101(4): 696-705.
(12) Yarova PL, Stewart AL, Sathish V, Britt RD Jr, Thompson MA, Lowe AP, Freeman M, Aravamudan B, Kita H, Brennan SC, Schepelmann M, Davies T, Yung S, Cholisoh Z, Kidd EJ, Ford WR, Broadley KJ, Rietdorf K, Chang W, Bin Khayat ME, Ward DT, Corrigan CJ, Ward JP, Kemp PJ, Pabelick CM, Prakash YS, Riccardi D. Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma. Sci Transl Med 2015; 7(284): 284ra260.
(13) Hendy GN, Canaff L. Calcium-sensing receptor gene: regulation of expression. Front Physiol 2016; 7: 394.
(14) Roesler AM, Ravix J, Bartman CM, Patel BS, Marta S, Roos B, Nesbitt L, Pabelick CM, Martin RJ, MacFarlan PM, Prakash YS. Calcium-sensing receptor contributes to hyperoxia effects on human fetal airway smooth muscle. Frontiers in Physiology 2021; 12: 287. DOI: 10.3389/fphys.2021.585895
(15) Dershem R, Gorvin CM, Raghu RPR, Metpally RPR, Krishnamurthy S, Smelser DT, Hannan FM, Carey DJ, Thakker RV, Breitwieser GE, Regeneron Genetics Center. Familial hypocalciuric hypercalcemia Type 1 and autosomal-dominant hypocalcemia Type 1: prevalence in a large healthcare population. Am J Hum Genet 2020; 106(6): 734-747.
(16) Camoretti-Mercado B, Lockey RF. Airway smooth muscle pathophysiology in asthma. J Allergy Clin Immunol 2021 Jun; 147(6): 1983-1995.
(17) Ijpma G, Kachmar L, Matusovsky OS, Bates JH, Benedetti A, Martin JG, Lauzon AM. Human trachealis and main bronchi smooth muscle are normoresponsive in asthma. Am J Respir Crit Care Med 2015 Apr 15; 191(8): 884-93.
(18) Leguillette R, Laviolette M, Bergeron C, Zitouni N, Kogut P, Solway J, Kachmar L, Hamid Q, Lauzon AM. Myosin, transgelin, and myosin light chain kinase: expression and function in asthma. Am J Respir Crit Care Med 2009; 179: 194-204.
(19) Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JP, Lee TH. Ca(2+) homeostasis and structural and functional remodelling of airway smooth muscle in asthma. Thorax 2010; 65(6): 547-52. doi: 10.1136/thx.2009.129296.
(20) Zhang W, Gunst SJ. Molecular mechanisms for the mechanical modulation of airway responsiveness. J Eng Sci Med Diagn Ther 2019; 2: 0108051-0108058.
(21) Berridge MJ. Smooth muscle cell calcium activation mechanisms. J Physiol 2008; 586: 5047-5061.
(22) Fuentes N, McCullough M, Panettieri Jr RA, Druey KM. RGS proteins, GRKs, and beta-arrestins modulate G protein-mediated signaling pathways in asthma. Pharmacol Ther 2021; 223: 107818.

(23) Deshpande DA, White TA, Dogan S, Walseth TF, Panettieri RA, Kannan MS. CD38/cyclic ADP-ribose signaling: role in the regulation of calcium homeostasis in airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2005; 288: L773-L788.
(24) Boie S, Chen J, Sanderson MJ, Sneyd J. The relative contributions of store-operated and voltage-gated Ca(2+) channels to the control of Ca(2+) oscillations in airway smooth muscle. J Physiol 2017; 595: 3129-3141.
(25) Barnes PJ. Clinical studies with calcium antagonists in asthma. Br J Clin Pharmacol 1985; 20 (Suppl 2): 289S-298S.
(26) Hirota S, Pertens E, Janssen LJ. The reverse mode of the Na(+)/Ca(2+) exchanger provides a source of Ca(2+) for store refilling following agonist-induced Ca(2+) mobilization. Am J Physiol Lung Cell Mol Physiol 2007b; 292: L438-L447.
(27) Perez JF, Sanderson MJ. The frequency of calcium oscillations induced by 5-HT, ACH, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. J Gen Physiol 2005; 125: 535-553.
(28) Perez-Zoghbi JF, Karner C, Ito S, Shepherd M, Alrashdan Y, Sanderson MJ. Ion channel regulation of intracellular calcium and airway smooth muscle function. Pulm Pharmacol Ther 2009; 22: 388-397.
(29) Bai Y, Sanderson MJ. Airway smooth muscle relaxation results from a reduction in the frequency of Ca2+ oscillations induced by a cAMP-mediated inhibition of the IP3 receptor. Respir Res 2006; 7: 34.
(30) Hirota S, Helli P, Janssen LJ. Ionic mechanisms and Ca2+ handling in airway smooth muscle. Eur Respir J 2007a; 30: 114-133.
(31) Schaafsma D, Bos IS, Zuidhof AB, Zaagsma J, Meurs H. The inhaled Rho kinase inhibitor Y-27632 protects against allergen-induced acute bronchoconstriction, airway hyperresponsiveness, and inflammation. Am J Physiol Lung Cell Mol Physiol 2008; 295: L214-L219.
(32) Koopmans T, Anaparti V, Castro-Piedras I, Yarova P, Irechukwu N, Nelson C, Perez-Zoghbi J, Tan X, Ward JP, Wright DB. Ca2+ handling and sensitivity in airway smooth muscle: emerging concepts for mechanistic understanding and therapeutic targeting. Pulm Pharmacol Ther 2014; 29: 108-120.
(33) Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. Respir Res 2006; 7: 73.
(34) Billington CK, Ojo OO, Penn RB, Ito S. cAMP regulation of airway smooth muscle function. Pulm Pharmacol Ther 2013; 26: 112–120.
(35) East JM. Sarco(endo)plasmic reticulum calcium pumps: recent advances in our understanding of structure/function and biology (review). Mol Membr Biol 2000; 17: 189-200.
(36) Ijpma G, Kachmar L, Panariti A, Matusovsky OS, Torgerson D, Benedetti A, Lauzon AM.

Intrapulmonary airway smooth muscle is hyperreactive with a distinct proteome in asthma. Eur Respir J 2020; 56(1):1902178.

(37) White TA, Xue A, Chini EN, Thompson M, Sieck GC, Wylam ME. Role of transient receptor potential C3 in TNF-alpha-enhanced calcium influx in human airway myocytes. Am J Respir Cell Mol Biol 2006; 35: 243-251.

(38) Guedes AG, Dileepan M, Jude JA, Deshpande DA, Walseth TF, Kannan MS. Role of CD38/cADPR signaling in obstructive pulmonary diseases. Curr Opin Pharmacol 2020; 51: 29-33.

(39) Mahn K, Hirst SJ, Ying S, Holt MR, Lavender P, Ojo OO, Siew L, Simcock DE, McVicker CG, Kanabar V, Snetkov VA, O'Connor BJ, Karner C, Cousins DJ, Macedo P, Chung KF, Corrigan CJ, Ward JP, Lee TH. Diminished sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) expression contributes to airway remodelling in bronchial asthma. Proc Natl Acad Sci USA 2009; 106: 10775-10780.

(40) Sathish V, Thompson MA, Bailey JP, Pabelick CM, Prakash YS, Sieck GC. Effect of proinflammatory cytokines on regulation of sarcoplasmic reticulum Ca2+ reuptake in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2009; 297: L26-L34.

(41) Zhang Y, Saradna A, Ratan R, Ke X, Tu W, Do DC, Hu C, Gao P. RhoA/Rho-kinases in asthma: from pathogenesis to therapeutic targets. Clin Transl Immunology 2020; 9, e01134.

(42) Ojiaku CA, Cao G, Zhu W, Yoo EJ, Shumyatcher M, Himes BE, An SS, Panettieri RA, Jr. TGF-β1 evokes human airway smooth muscle cell shortening and hyperresponsiveness via Smad3. Am J Respir Cell Mol Biol 2018; 58: 575-584.

(43) Shaifta Y, MacKay CE, Irechukwu N, O'Brien KA, Wright DB, Ward JPT, Knock GA. Transforming growth factor-β enhances Rho-kinase activity and contraction in airway smooth muscle via the nucleotide exchange factor ARHGEF1. J Physiol 2018; 596: 47-66.

(44) Maarsingh H, Zaagsma J, Meurs H. Arginase: a key enzyme in the pathophysiology of allergic asthma opening novel therapeutic perspectives. Br J Pharmacol 2009; 158(3): 652-664.

(45) Jain V, Raina S, Gheware AP, Singh R, Rehman R, Negi V, Murray Stewart T, Mabalirajan U, Mishra AK, Casero RA, Jr., Agrawal A, Ghosh B. Reduction in polyamine catabolism leads to spermine-mediated airway epithelial injury and induces asthma features. Allergy 2018; 73(10): 2033-2045.

(46) Kurosawa M, Shimizu Y, Tsukagoshi H, Ueki M. Elevated levels of peripheral blood, naturally occurring aliphatic polyamines in bronchial asthmatic patients with active symptoms. Allergy 1992; 47: 638-643.

(47) Jain V. Role of polyamines in asthma pathophysiology. Med Sci (Basel) 2018: 6(1).

(48) Coyle AJ, Ackerman SJ, Irvin CG. Cationic proteins induce airway hyperresponsiveness dependent on charge interactions. Am Rev Respir Dis 1993; 147: 896-900.

(49) Homma T, Bates JH, Irvin CG. Airway hyperresponsiveness induced by cationic proteins in vivo: site of action. Am J Physiol - Lung Cellular and Molecular Physiology 2005; 289: L413-L418.
(50) North ML, Grasemann H, Khanna N, Inman MD, Gauvreau GM, Scott JA. Increased ornithine-derived polyamines cause airway hyperresponsiveness in a mouse model of asthma. Am J Resp Cell Mol Biol 2013; 48: 694-702.
(51) Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, Germain RN, Kastner DL, Chae JJ. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca2+ and cAMP. Nature 2012; 492(7427): 123-127.
(52) Jager E, Murthy S, Schmidt C, Hahn M, Strobel S, Peters A et al. Calcium-sensing receptor-mediated NLRP3 inflammasome response to calciprotein particles drives inflammation in rheumatoid arthritis. Nat Commun 2020 Aug 25; 11(1):4243. doi: 10.1038/s41467-020-17749-6.
(53) Sweeney D, Hollins F, Gomez E, Mistry R, Saunders R, Challiss RAJ, Brightling CE. No evidence for altered intracellular calcium handling in airway smooth muscle cells from human subjects with asthma. BMC Pulmonary Medicine 2015; 15: 12.
(54) Canton J, Schlam D, Breuer C, Gütschow M, Glogauer M, Grinstein S. Calcium-sensing receptors signal constitutive macropinocytosis and facilitate the uptake of NOD2 ligands in macrophages. Nat Commun 2016 Apr 6; 7: 11284. doi: 10.1038/ncomms11284.
(55) Hendy GN, Canaff L. Calcium-sensing receptor, proinflammatory cytokines and calcium homeostasis. Semin Cell Dev Biol 2016; 49: 37-43.
(56) Klein GL, Castro SM, Garofalo RP. The calcium-sensing receptor as a mediator of inflammation. Semin Cell Dev Biol 2016; 49: 52-56.
(57) Zhai TY, Cui BH, Zou L, Zeng JY, Gao S, Zhao Q, Wang Y, Xie WL, Sun YH. Expression and role of the calcium-sensing receptor in rat peripheral blood polymorphonuclear neutrophils. Oxid Med Cell Longev 2017; 2017:3869561. doi: 10.1155/2017/3869561.
(58) Li T, Sun M, Yin X, Wu C, Wu Q, Feng S, Li H, Luan Y, Wen J, Yan L, Zhao B, Xu C, Sun Y. Expression of the calcium sensing receptor in human peripheral blood T lymphocyte and its contribution to cytokine secretion through MAPKs or NF-kappaB pathways. Molecular Immunology 2013: 53(4): 414-420.
(59) Koller DY, Halmerbauer G, Frischer T, Roithner B. Assessment of eosinophil granule proteins in various body fluids: is there a relation to clinical variables in childhood asthma? Clin Exp Allergy 1999; 29: 786-793.
(60) Lee JW, Park HA, Kwon OK, Park JW, Lee G, Lee HJ, et al. NPS 2143, a selective calcium-sensing receptor antagonist inhibits lipopolysaccharide-induced pulmonary inflammation. Molec Immunol 2017; 90: 150-157.

(61) Li GW, Xing WJ, Bai SZ, Hao JH, Guo J, Li HZ, Li HX, Zhang WH, Yang BF, Wu LY, Wang R, Yang GD, Xu CQ. The calcium-sensing receptor mediates hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells through MEK1/ERK1,2 and PI3K pathways. Basic Clin Pharmacol Toxicol 2011; 108(3): 185-193.

(62) Nemeth EF, Goodman WG. Calcimimetic and Calcilytic Drugs: Feats, Flops, and Futures. Calcif Tissue Int 2016; 98(4): 341-58.

(63) Widler L. Calcilytics: antagonists of the calcium-sensing receptor for the treatment of osteoporosis. Future Med Chem 2011; 3(5): 535-547.

(64) Yarova PL, Huang P, Schepelmann MW, Bruce R, Ecker R, Nica R, Telezhkin V, Traini D, Gomes Dos Reis L, Kidd EJ, Ford WR, Broadley KJ, Kariuki BM, Corrigan CJ, Ward JPT, Kemp PJ, Riccardi D. Characterization of negative allosteric modulators of the calcium-sensing receptor for repurposing as a treatment of asthma. J Pharmacol Exp Ther 2021 Jan; 376(1): 51-63. doi: 10.1124/jpet.120.000281.

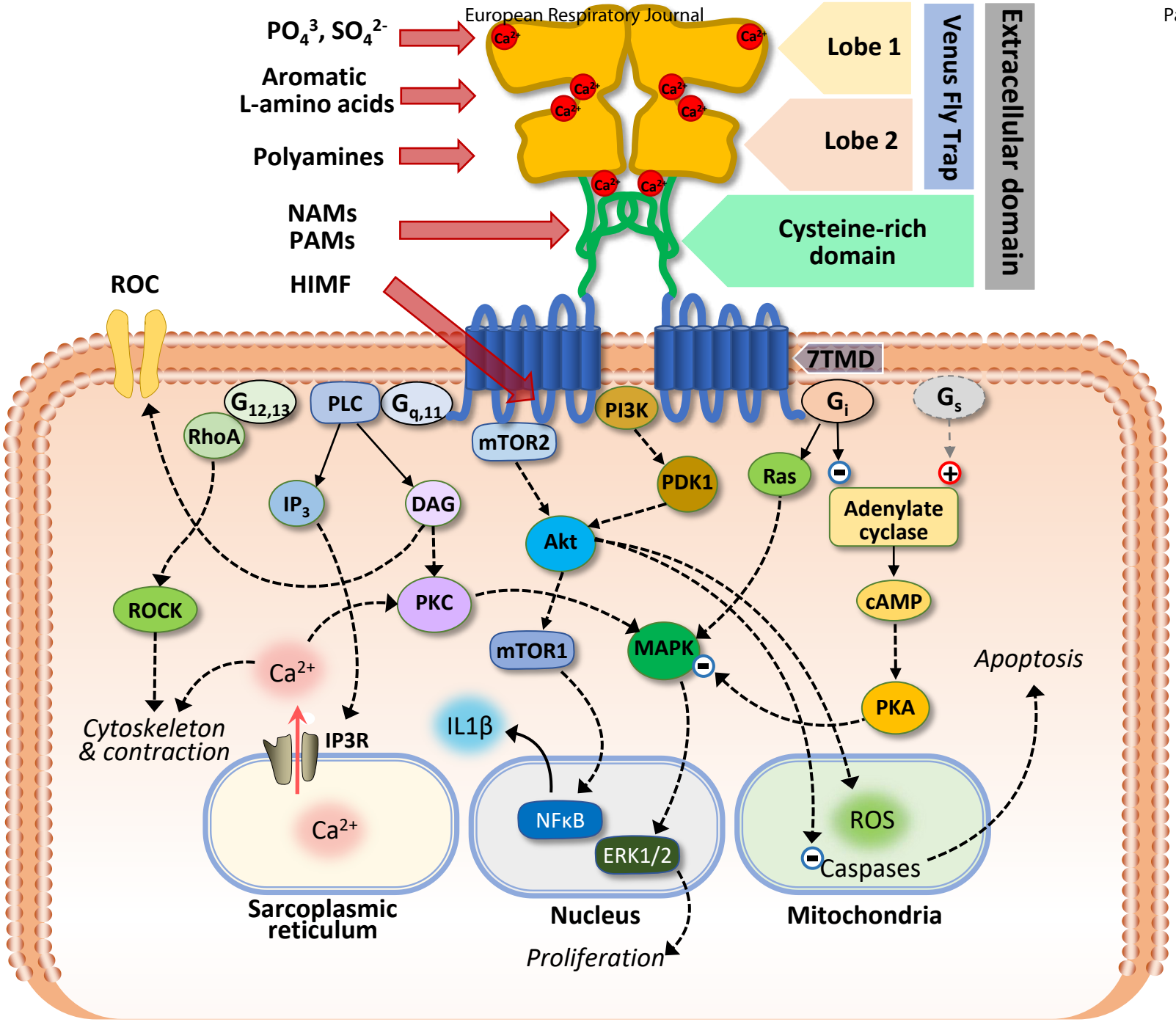
(65) Caltabiano S, Dollery CT, Hossain M, Kurtinecza MT, Desjardins JP, Favus MJ, Kumar R, Fitzpatrick LA. Characterization of the effect of chronic administration of a calcium-sensing receptor antagonist, ronacaleret, on renal calcium excretion and serum calcium in postmenopausal women. Bone 2013; 56: 154-162.

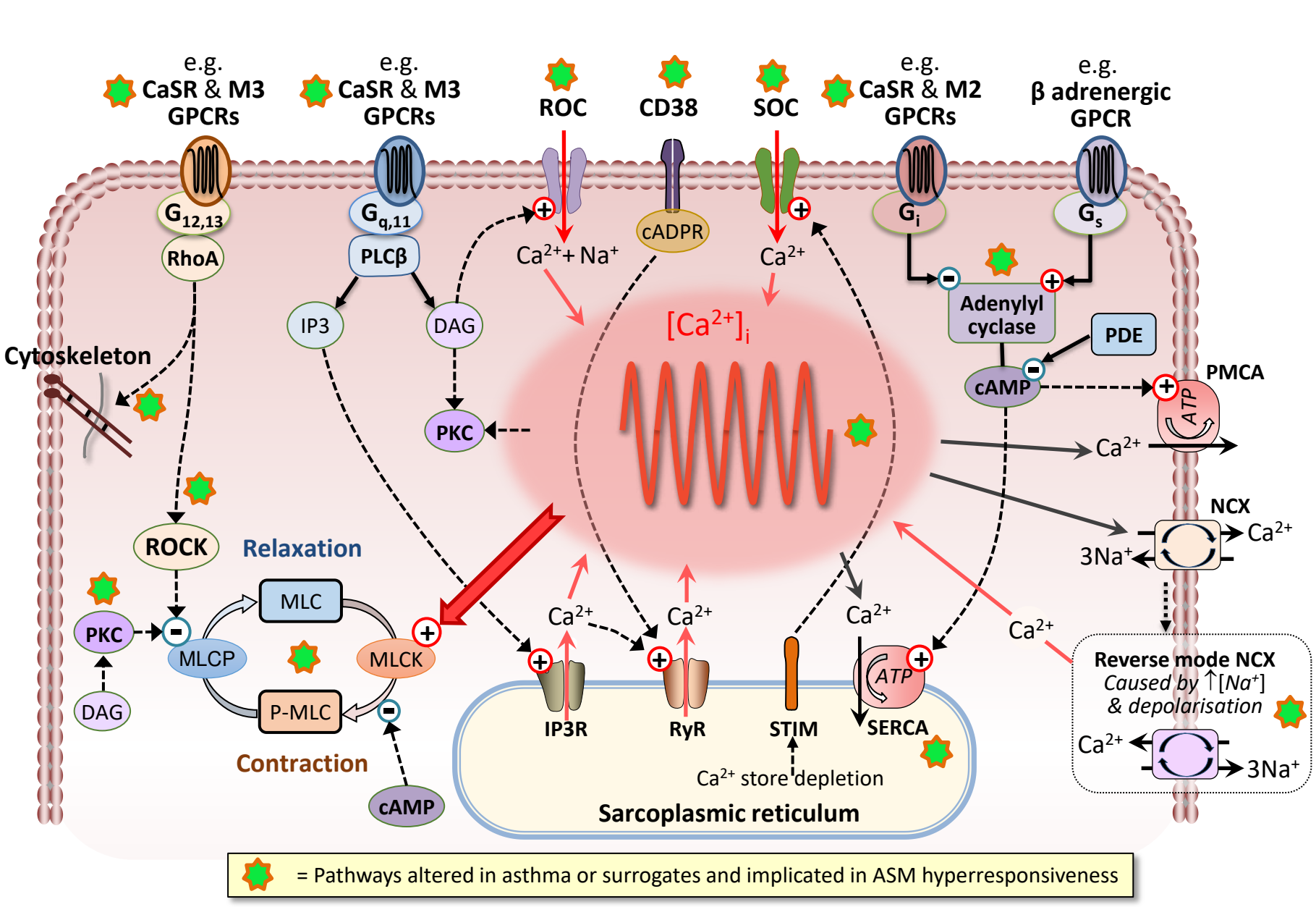
(66) Cosman F, Gilchrist N, McClung M, Foldes J, de Villiers T, Santora A, Leung A, Samanta S, Heyden N, McGinnis JP, Rosenberg E, Denker AE. A phase 2 study of MK-5442, a calcium-sensing receptor antagonist, in postmenopausal women with osteoporosis after long-term use of oral bisphosphonates. Osteoporosis International 2016; 27: 377–386.

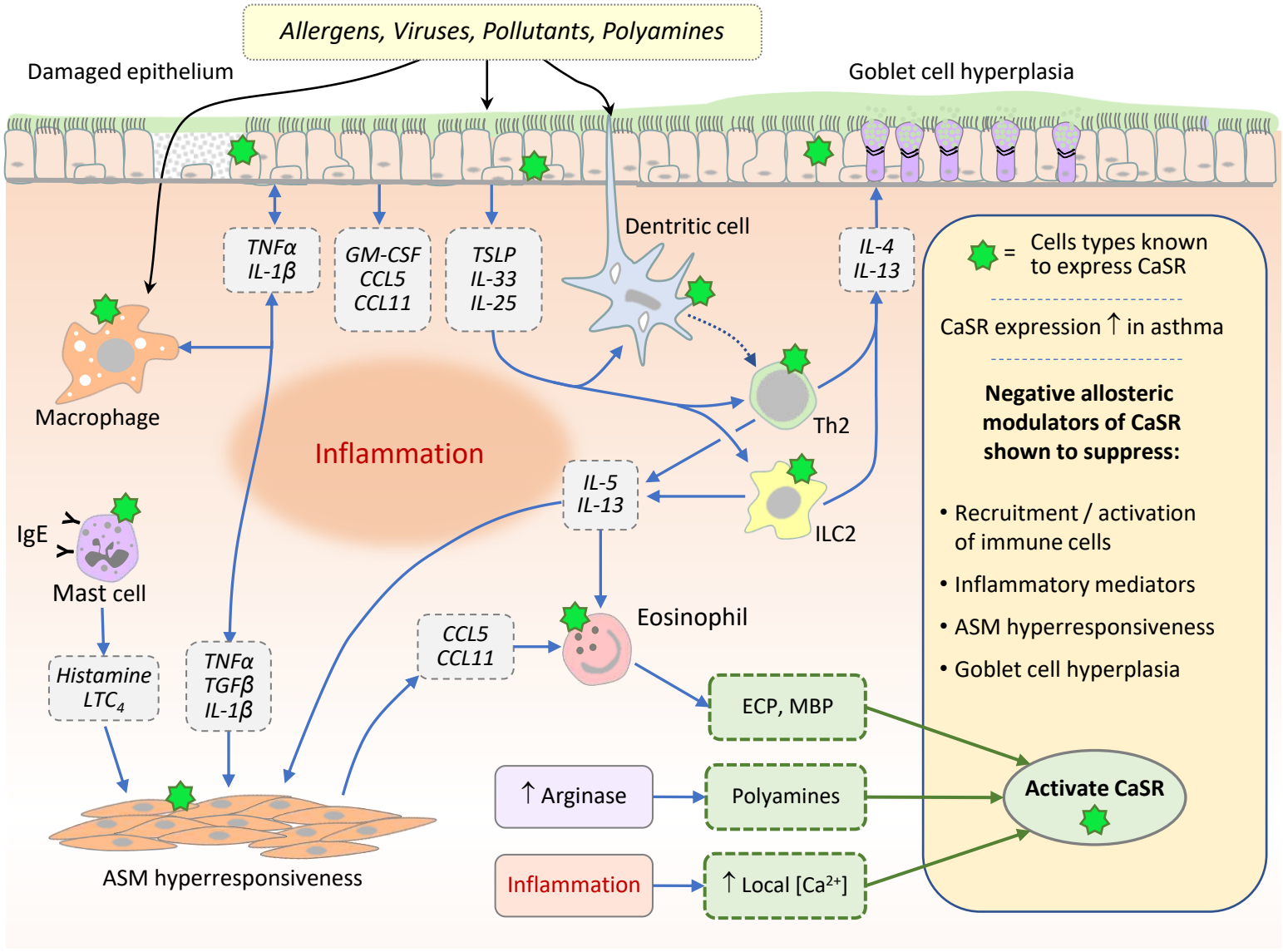
(67) John MR, Harfst E, Loeffler J, Bellelia R, Mason J, Bruin GJM, Seuwen K, Klickstein LB, Mindeholm L, Widler L, Kneissel M. AXT914 a novel, orally-active parathyroid hormone-releasing drug in two early studies of healthy volunteers and postmenopausal women. Bone 2014; 64: 204-210.

(68) The Royal Colleges of Physicians of the United Kingdom. Why asthma still kills. The National Review of Asthma Deaths (NRAD) Confidential Enquiry Report May 2014. Available at: https://www.rcplondon.ac.uk/projects/outputs/why-asthma-still-kills

(69) Diao J, Lam M, Gregory K, Leach K, Bourke JE. Biased negative allosteric modulators of the calcium-sensing receptor differentially oppose airway contraction in mouse precision cut lung slices. Am J Respir Crit Care Med 2021; 203: A4516.


PO_4^{3-}, SO_4^{2-}
Aromatic L-amino acids
Polyamines
NAMs
PAMs
HIMF
ROC
Lobe 1
Lobe 2
Venus Fly Trap
Cysteine-rich domain
Extracellular domain
7TMD
$G_{12,13}$
RhoA
PLC
$G_{q,11}$
PI3K
G_i
G_s
mTOR2
PDK1
Ras
Adenylate cyclase
IP_3
DAG
Akt
ROCK
PKC
cAMP
mTOR1
MAPK
PKA
Apoptosis
Ca^{2+}
Cytoskeleton & contraction
IP3R
IL1β
NFκB
ERK1/2
ROS
Caspases
Sarcoplasmic reticulum
Nucleus
Mitochondria
Proliferation


e.g.
CaSR & M3 GPCRs
e.g.
CaSR & M3 GPCRs
ROC
CD38
SOC
e.g.
CaSR & M2 GPCRs
e.g.
β adrenergic GPCR
$G_{12,13}$
RhoA
$G_{q,11}$
PLCβ
IP3
DAG
Ca^{2+}+ Na^+
cADPR
Ca^{2+}
G_i
G_s
Adenylyl cyclase
PDE
cAMP
PMCA
ATP
Ca^{2+}
$[Ca^{2+}]_i$
Cytoskeleton
PKC
ROCK
Relaxation
MLC
MLCP
MLCK
P-MLC
Contraction
DAG
cAMP
NCX
$3Na^+$
Ca^{2+}
IP3R
RyR
STIM
SERCA
ATP
Ca^{2+} store depletion
Sarcoplasmic reticulum
Reverse mode NCX
Caused by ↑[Na^+]
& depolarisation
= Pathways altered in asthma or surrogates and implicated in ASM hyperresponsiveness


Allergens, Viruses, Pollutants, Polyamines
Damaged epithelium
Goblet cell hyperplasia
TNFα
IL-1β
GM-CSF
CCL5
CCL11
TSLP
IL-33
IL-25
Dentritic cell
IL-4
IL-13
Macrophage
Th2
Inflammation
IL-5
IL-13
ILC2
IgE
Mast cell
Histamine
LTC4
TNFα
TGFβ
IL-1β
CCL5
CCL11
Eosinophil
ECP, MBP
↑ Arginase
Polyamines
Inflammation
↑ Local [Ca2+]
Activate CaSR
ASM hyperresponsiveness
= Cells types known to express CaSR
CaSR expression ↑ in asthma
Negative allosteric modulators of CaSR shown to suppress:
• Recruitment / activation of immune cells
• Inflammatory mediators
• ASM hyperresponsiveness
• Goblet cell hyperplasia