Published in final edited form as:

Sci Transl Med. 2015 April 22; 7(284): 284ra60. doi:10.1126/scitranslmed.aaa0282.

Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma

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Abstract

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SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/7/284/284ra60/DC1

Materials and Methods

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Reference (50)

Author contributions: P.L.Y., A.L.S., V.S., R.D.B., M.A.T., A.P.P.L., M.F., B.A., S.C.B., M.S., T.D., S.Y., Z.C., K.R., M.E.B.K., and D.T.W. performed the experiments. W.C. generated the SM22a LoxP CaSR mice. Y.S.P., H.K., P.L.Y., P.J.K., and D.R. designed and analyzed the experiments and performed the statistical analyses. E.J.K., W.R.F., and K.J.B. developed the OVA-sensitized mouse asthma model. C.M.P. developed the MA mouse asthma model. C.J.C., J.P.T.W., P.J.K., Y.S.P., P.L.Y., and D.R. wrote the manuscript.

Competing interests: D.R., P.J.K., C.J.C., and J.P.T.W. are co-inventors on a patent (WO2014049351) claiming the use of CaSR antagonists for the treatment of inflammatory lung disorders. The other authors declare that they have no competing interests.

Airway hyperresponsiveness and inflammation are fundamental hallmarks of allergic asthma that are accompanied by increases in certain polycations, such as eosinophil cationic protein. Levels of these cations in body fluids correlate with asthma severity. We show that polycations and elevated extracellular calcium activate the human recombinant and native calcium-sensing receptor (CaSR), leading to intracellular calcium mobilization, cyclic adenosine monophosphate breakdown, and p38 mitogen-activated protein kinase phosphorylation in airway smooth muscle (ASM) cells. These effects can be prevented by CaSR antagonists, termed calcilytics. Moreover, asthmatic patients and allergen-sensitized mice expressed more CaSR in ASMs than did their healthy counterparts. Indeed, polycations induced hyper-reactivity in mouse bronchi, and this effect was prevented by calcilytics and absent in mice with CaSR ablation from ASM. Calcilytics also reduced airway hyperresponsiveness and inflammation in allergen-sensitized mice in vivo. These data show that a functional CaSR is up-regulated in asthmatic ASM and targeted by locally produced polycations to induce hyperresponsiveness and inflammation. Thus, calcilytics may represent effective asthma therapeutics.

INTRODUCTION

Despite substantial advances in our understanding of its pathophysiology and improved therapeutic regimens, asthma remains a tremendous worldwide health care burden with around 300 million individual sufferers. Although the symptoms of asthma are potentially controllable in most asthma sufferers using conventional therapy such as topical bronchodilators and corticosteroids, these are troublesome to administer efficiently and present unwanted side effects. There remains a significant minority of patients whose symptoms fail to be controlled with these approaches and who face chronically impaired quality of life with increased risk of hospital admission and even death, although in a minority such patients account for the major share of asthma health care costs. Accordingly, there is an urgent unmet need for identification of novel asthma therapies that target the root cause of the disease rather than its clinical sequelae.

Asthma is characterized by inflammation-driven exaggeration of airway narrowing in response to specific and nonspecific environmental stimuli [nonspecific airway hyperresponsiveness (AHR)], as well as chronic remodeling of the conducting airways (1). A number of mechanisms, many driven by inflammation, have been hypothesized to contribute to AHR and/or remodeling. Among these, there is increasing recognition that airway inflammation results in augmented local concentrations of polycations (2–7). The polycations eosinophil cationic protein (ECP) and major basic protein are well-established markers for asthma severity and stability, with some evidence that they may contribute directly to the pathogenesis of asthma (6, 8–10). Furthermore, in asthma, increased arginase activity diverts L-arginine toward increased production of the polycations spermine, spermidine, and putrescine (4, 5, 11). Although in human peripheral blood monocytes spermine exhibits anti-inflammatory properties (12), associations between increases in polycations in the asthmatic airway mucosa and AHR/airway remodeling and inflammation (4, 5, 13) have long been apparent and ascribed to their positive charge (9). However, the cause-effect relationship remains hitherto unexplained. Here, we provide evidence that activation of the cell surface, G protein (heterotrimeric guanine nucleotide-binding protein)–

coupled calcium-sensing receptor (CaSR) by polycations drives AHR and inflammation in allergic asthma.

The CaSR is the master controller of extracellular free ionized calcium ion (Ca^{2+}_o) concentration via the regulation of parathyroid hormone (PTH) secretion (14). Accordingly, CaSR-based therapeutics is used for the treatment of systemic disorders of mineral ion metabolism. Pharmacological activators of the CaSR (calcimimetics) are used to treat hyperparathyroidism, and negative allosteric modulators of the CaSR (calcilytics) are in clinical development for treating autosomal dominant hypocalcemia (15).

In addition to its pivotal role in divalent cation homeostasis, the CaSR is expressed in tissues not involved in mineral ion metabolism such as the blood vessels, breast, and placenta, where the CaSR regulates many fundamental processes including gene expression, ion channel activity, and cell fate (16). Furthermore, altered CaSR expression has also been associated with several pathological conditions including inflammation, vascular calcification, and certain cancers (16–19). In these noncalcitropic tissues, the CaSR responds to a range of stimuli including not only Ca^{2+}_o but also polyvalent cations, amino acids, ionic strength, and pH, making this receptor uniquely capable of integrating multiple environmental signals. Owing to its ability to act as a multimodal chemosensor, the potential relevance of CaSR to asthma pathophysiology is manifold, yet there is currently no evidence regarding CaSR expression or function in asthma. In this regard, a fundamental aspect of asthma pathophysiology is elevated intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) in airway smooth muscle (ASM) cells that is not only critical to the enhanced bronchoconstriction of nonspecific AHR but also implicated in longer-term, likely genomic effects that result in airway remodeling such as increased ASM cell proliferation (leading to airway wall thickening) and deposition of extracellular matrix components (20, 21). There is currently no information as to whether the CaSR can regulate $[\text{Ca}^{2+}]_i$ in the asthmatic airways, even though a polycation sensor such as the CaSR, whose activation leads to an increase in $[\text{Ca}^{2+}]_i$, seems a likely candidate. Therefore, we hypothesized that if a CaSR was to be found in the airways, it would sense and respond not only to inflammation-enhanced Ca^{2+}_o but also to polycations such as the ECPs and L-arginine-derived polyamines putrescine, spermidine, and spermine, whose production is markedly increased during asthma (3–7,11) or by many RNA respiratory viruses that exacerbate asthma, such as influenza A and Newcastle disease virus, which either contain polyamines in the viral envelope or produce them as part of their requirement for replication (22, 23). To test our hypothesis, we examined human ASM samples from nonasthmatic and asthmatic subjects, and used two models of allergen-induced airway inflammation, together with a mouse model of targeted CaSR gene ablation from ASM.

RESULTS

CaSR expression in human and mouse airways is increased during asthma

In human bronchial biopsies and in mouse interlobular bronchi, CaSR was immunolocalized within the SM22 α -positive smooth muscle layer, with additional expression in bronchial epithelium (Fig. 1A). Isolated human and mouse ASM cells retained CaSR expression (Fig. 1B).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western analysis of human ASM cells demonstrated that both CaSR mRNA (Fig. 1C) and protein (Fig. 1D) expression were increased about threefold in moderate asthmatics compared to nonasthmatics (“healthy”). Furthermore, in human ASM cells from healthy individuals, 48 hours of exposure to the asthma-associated proinflammatory cytokines, tumor necrosis factor – α (TNF- α) and interleukin-13 (IL-13), significantly increased CaSR protein expression (Fig. 1E and fig. S1C). qRT-PCR of laser capture microdissected ASM layers of intralobular bronchi in lung sections showed an about threefold increase in CaSR mRNA expression in mixed allergen (MA)–sensitized mice compared to ASM from unsensitized mice (Fig. 1F). Together, these results indicate that the CaSR is present in human and mouse ASM and its expression is increased in asthma. Furthermore, in vitro effects of cytokines on human ASM, and the effects of MA sensitization in a mouse model of allergic asthma, provide an evidence for the role of inflammation in up-regulation of CaSR expression.

Polycations implicated in asthma pathogenesis activate the human CaSR

In many cell types, CaSR activation results in an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) arising from mobilization of Ca^{2+}_i (14). To test the hypothesis that polycations that are up-regulated during asthma activate the CaSR, we measured changes in $[\text{Ca}^{2+}]_i$ in human embryonic kidney (HEK) 293 cells stably expressing the human CaSR (HEK-CaSR), or HEK293 cells stably expressing an empty vector (HEK-0). A representative Western blot of HEK-CaSR and HEK-0 is shown in fig. S2A. Consistent with this hypothesis, HEK-CaSR, but not HEK-0, cells exhibited significant increases in $[\text{Ca}^{2+}]_i$ after exposure to (i) ECP [10 $\mu\text{g}/\text{ml}$, a concentration well below the cytotoxic levels (24) and comparable to those measured in the sputum of some asthmatic subjects (6, 7)], (ii) the polycationic peptide poly-L-arginine [PLA; 300 nM; a mimetic of major basic protein (8)], and (iii) the polycation spermine (1 mM) (Fig. 2A and fig. S2, B to D, for single traces). For each of these agonists, the increase in $[\text{Ca}^{2+}]_i$ was inhibited by the calcilytic NPS89636 (100 nM) (Fig. 2A and fig. S2, B to D, for single traces). Additional calcilytics, NPS2143 (1 μM) or Calhex 231 (1 μM), also prevented spermine-induced CaSR activation (Fig. 2A and fig. S2D).

Calcilytics prevent increases in Ca^{2+}_i in ASM from asthmatic patients

In human ASM, several endogenously produced agents such as acetylcholine (ACh) and histamine evoke increases in $[\text{Ca}^{2+}]_i$, which drive AHR, remodeling, and production of a range of inflammatory cytokines and other mediators in asthma (20, 25). Accordingly, we hypothesized that activation of CaSR in ASM also leads to an increase in $[\text{Ca}^{2+}]_i$, and predicted that this effect would be enhanced in asthmatics.

We found that the sensitivity of human asthmatic ASM in the absence of agents that increase $[\text{Ca}^{2+}]_i$ was significantly higher than that of ASM from nonasthmatics in the presence of 2 mM $[\text{Ca}^{2+}]_o$ (Fig. 2B, left panel, and fig. S3, A and B, for single traces). Inhibition of the CaSR with the calcilytic NPS2143 (1 mM) blunted the $[\text{Ca}^{2+}]_o$ hyperresponsiveness of asthmatic ASM cells, highlighting the functional role of CaSR in this setting (Fig. 2B, right panel, and fig. S3, A and B, for single traces).

Having determined the sensitivity of human ASM to $[Ca^{2+}]_o$, we then tested the ability of the CaSR to alter $[Ca^{2+}]_i$ responses to ACh in human asthmatic and healthy ASM. In the presence of physiological $[Ca^{2+}]_o$ [that is, 1 μ M, at which the CaSR is half-maximally active (14)], exposure to 1 μ M ACh produced the expected increase in $[Ca^{2+}]_i$, which was significantly greater in ASM from asthmatic patients (Fig. 2C). Inhibiting CaSR with NPS2143 reduced the $[Ca^{2+}]_i$ response to ACh to levels that were not significantly different from those measured in healthy ASM (Fig. 2C and fig. S3C). These effects were even more pronounced in the presence of 2 mM Ca^{2+}_o , a concentration at which the CaSR is fully active, whereas they could not be observed in the presence of 0.5 mM $[Ca^{2+}]_o$, which is below the threshold for CaSR activation (fig. S3C).

Histamine (1 mM) also evoked an increase in $[Ca^{2+}]_i$ in both healthy and asthmatic ASM, which was significantly greater in asthmatic ASM (Fig. 2D). Preexposure of asthmatic ASM to calcilytic also reduced $[Ca^{2+}]_i$ responses to histamine so that they did not differ statistically from those in healthy ASM (Fig. 2D). Furthermore, an alternative, membrane impermeant CaSR agonist, Gd^{3+} (0.1 mM), evoked a further increase in $[Ca^{2+}]_i$ in human ASM in the absence (fig. S3D) or presence (fig. S3E) of histamine, effects that were greater in asthmatic than in nonasthmatic ASM cells. Together, these results demonstrate that, in ASM cells, the CaSR is functional and contributes to the regulation of baseline ASM $[Ca^{2+}]_i$. Accordingly, in asthmatic ASM cells, the CaSR may contribute to a higher baseline $[Ca^{2+}]_i$, a leading cause of AHR, whereas calcilytics restore baseline $[Ca^{2+}]_i$.

Calcilytics abrogate signaling pathways characteristic of airway contractility and asthma in human ASM

To determine potential mechanisms by which CaSR modulates contractility relevant to asthma, we explored two mechanisms in non-asthmatic and asthmatic ASM: cyclic adenosine monophosphate (cAMP), which should induce bronchodilation, and phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP_3), an important contributor to bronchoconstriction, with the idea that CaSR activation should suppress cAMP but elevate IP_3 (16). Indeed, in the presence of 2 mM Ca^{2+}_o , cAMP levels were low, and calcilytics increased cAMP, particularly in asthmatic ASM (fig. S3F). Measurements of cellular IP_3 content showed that, particularly in asthmatic ASM, CaSR antagonist suppressed the elevated levels of IP_3 in the presence of 2 mM Ca^{2+}_o (fig. S3G).

In addition to targeting phosphodiesterases to inhibit cAMP breakdown, many of the pipeline or existing drugs for asthma target activation of signaling pathways dependent on extracellular signal–regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase/Akt phosphorylation (26, 27). Therefore, we examined the effect of activation of the ASM CaSR on these pathways in human ASM cells. In healthy ASM, CaSR activation with 5 mM Ca^{2+}_o induced a significant increase in p38 MAPK phosphorylation, an effect that was prevented by co-incubation with a calcilytic. Calcilytic treatment reduced ERK1/2 and Akt phosphorylation at 5 mM Ca^{2+}_o (Fig. 2, E and F, and fig. S4 for technical replicates). Overall, these data highlight the ability of CaSR to modulate signaling pathways activated during asthma, which may contribute to altered ASM function beyond $[Ca^{2+}]_i$.

M22a CaSR *flox/flox* mice are protected from polycation-induced bronchoconstriction

To determine whether activation of the CaSR in ASM leads to AHR in vivo, we generated mice with targeted CaSR ablation from visceral SM cells by breeding LoxP-CaSR mice with SM22 α -Cre mice (28, 29). The resulting SM22 α CaSR *flox/flox* mice [knockout (KO); fig. S5] were comparable to Cre-negative [wild-type (WT)] littermates in appearance, reproductive abilities, body weight, and life expectancy (fig. S5, B and C). Fluorescence-activated cell sorting analysis shows that molecular CaSR ablation from ASM resulted in a significant reduction in CaSR immunoreactivity in KO cells, which was 27% of that seen in WT cells. In contrast, CaSR ablation from ASM cells did not significantly alter the expression of the smooth muscle marker SM22 α (fig. S5D). Successful ASM CaSR ablation was demonstrated functionally by the evidence that Ca²⁺_o (1 to 5 mM) and an alternative, membrane-impermeant CaSR agonist, Gd³⁺ (100 μ M to 1 mM), evoked an increase in [Ca²⁺]_i in WT ASM cells, which was significantly greater than that measured in cells from KO mice (fig. S5E, upper panels for single traces and lower panels for biological replicates). Nevertheless, lungs from KO mice appeared histomorphologically comparable to those of WT mice and did not exhibit fibrosis, inflammation, or impaired alveolarization (fig. S5F). Intralobular bronchi from WT and KO mice also had comparable luminal diameters (fig. S5G).

The intrinsic baseline contractility of intralobular bronchi was not affected by CaSR ablation from ASM cells, as shown by exposure either to high K⁺ (40 mM, fig. S5H) or to increasing concentrations of ACh (1 nM to 30 mM), both of which evoked bronchoconstriction of comparable magnitude in WT and KO mouse bronchi (Fig. 3A). In intralobular bronchi from WT mice, treatment with either spermine (300 μ M, Fig. 3B) or 2.5 mM [Ca²⁺]_o (Fig. 3E) enhanced the broncho-constrictor response to ACh. CaSR ablation from ASM blunted both spermine-induced (Fig. 3C) and [Ca²⁺]_o-induced (Fig. 3F) sensitization of the ACh response. Furthermore, spermine (10 μ M to 3 mM) induced sensitization of the ACh response in precontracted WT, but not in KO mouse bronchi (Fig. 3D). Consistent with these findings, spermine also enhanced the response to ACh (0.5 μ M) in precision-cut lung slices from WT animals (Fig. 3G, and summary in Fig. 3H). This effect was prevented by calcilytic treatment (NPS89636, 300 nM, Fig. 3H). However, the effects of either spermine or calcilytic were not observed in lung slices from KO mice (Fig. 3G, and summary in Fig. 3I). Together, these observations suggest that activation of the ASM CaSR leads to AHR, an effect that can be prevented by calcilytic treatment.

Calcilytics reduce airway resistance in MA-sensitized mice in vivo

To test the effects of pharmacological CaSR activators and inhibitors on pulmonary resistance, we directly measured airflow resistance (R_L) in anaesthetized, paralyzed, mechanically ventilated mice. In naïve mice, acute preexposure (10 min) to the nebulized calcimimetic R568 (1 μ M) resulted in an increase in R_L across the lungs after inhalational challenge with the synthetic muscarinic receptor agonist metha-choline (MCh; 0 to 50 mg/ml), whereas the calcilytic NPS2143 (1 μ M) was able to reverse this effect (Fig. 4A). Next, we developed an MA murine asthma model that leads to robust inflammation and remodeling in the lungs of MA-sensitized mice (fig. S6). In these mice, there was a marked increase in R_L after MCh challenge, and preexposure to R568 resulted in an even greater

increase in R_L . Moreover, the calcilytic was able to significantly reduce AHR in these mice (Fig. 4B).

Calcilytics reduce AHR and inflammation in ovalbumin-sensitized, ovalbumin-challenged mice in vivo

Increased arginase activity drives AHR via the production of polyamines (4, 5, 11), but whether the CaSR is involved in this process is unknown. To test the ability of calcilytics to prevent polycation-induced AHR in vivo, we assessed the effects of nebulized PLA (3 μ M) in the presence or absence of the calcilytic NPS89636 (3 μ M). As an alternative, noninvasive method for measurement of AHR (30, 31), we performed whole-body plethysmography in conscious, unrestrained naïve mice by measuring enhanced pause (Penh). Although Penh does not directly measure airway resistance, particularly in obligate nasal breathers such as mice, it has been widely used as an indicator of airway obstruction in response to inhaled MCh (0.1 to 100 mg/ml) (31). PLA significantly increased Penh at MCh concentrations greater than 10 mg/ml, an effect that was abolished by cotreatment with the nebulized calcilytic. Calcilytic treatment per se significantly reduced Penh in naïve animals treated with MCh (30 mg/ml) (Fig. 5A).

Having demonstrated the ability of calcilytics to reduce AHR evoked by polyamines, we tested their anti-inflammatory properties in an established model of allergic asthma (31), the ovalbumin (OVA)–sensitized, OVA-challenged mouse. Calcilytic inhalation significantly reduced AHR induced by OVA sensitization (Fig. 5B). Bronchoalveolar lavage fluid (BALF) collected from the calcilytic-treated mice also showed a significant reduction in inflammatory cell infiltration (total numbers, macrophages, eosinophils, and lymphocytes; Fig. 5C) and concentrations of ECP, IL-5, IL-13, and TNF- α (Fig. 5D) when compared to their vehicle-treated counterparts. Biochemical analysis of terminal blood samples showed that inhaled calcilytic did not significantly affect serum ionized calcium at 1 hour after inhalation (vehicle control 0.9 ± 0.1 mM versus calcilytic 0.8 ± 0.1 mM; $P > 0.05$; $n = 3$ per experimental group) and up to 24 hours (1.0 ± 0.1 mM and 1.0 ± 0.1 mM, 4 and 24 hours after calcilytic treatment, respectively; $P > 0.05$; $n = 3$ per experimental group). These results suggest that the observed effects of the calcilytics are not ascribed to systemic changes in Ca^{2+}_o homeostasis.

DISCUSSION

Our study highlights the expression of the CaSR in ASM and identifies a fundamental pathophysiological role for this receptor in the context of asthma. The fact that inflammatory cationic proteins known to correlate with asthma severity can activate the CaSR expressed by ASM cells at physiologically relevant concentrations to elevate $[\text{Ca}^{2+}]_i$ and increase the contractility of the ASM nonspecifically provides both a rational explanation for the genesis of nonspecific AHR in asthma and a basis for the direct mechanistic link between this phenomenon and airway inflammation. These findings raise the possibility that the CaSR directly influences mechanisms involved in inflammatory cell recruitment and activation. In turn, production of asthma-relevant cytokines can further increase CaSR expression, thereby generating a positive feedback loop. Thus, locally delivered calcilytics would have the

advantage of breaking this cycle by reducing inflammation and by blunting ASM hyperresponsiveness. Indeed, in two different *in vivo* models of allergic asthma, interfering with CaSR signaling positively affects multiple aspects of airway disease, benefits not achieved by single-drug therapies. In this sense, the CaSR represents a truly novel potential therapeutic target in asthma.

$[Ca^{2+}]_o$ is known to be increased at inflammation sites (18, 32), therefore activating the CaSR, leading to an increase in $[Ca^{2+}]_i$ and p38 MAPK activation and a decrease in the intracellular cAMP pool. In addition, the CaSR is activated by a plethora of molecules, particularly polyamines, which act orthosterically (independently of $[Ca^{2+}]_o$) to help stabilize the unique conformations of the receptor. This leads to preferential coupling to different G proteins, a process defined as ligand-directed targeting of receptor stimulus (33). The relevance of the CaSR to local and systemic symptoms in asthma and other airway diseases is potentially immense, extending beyond its innate expression in ASM and any local regulation of $[Ca^{2+}]_o$. In asthma, airway inflammation leads to increased release of polycations, which are accepted markers of asthma severity, locally and into the systemic circulation (2–7). Sputum ECP concentrations in asthmatics have been reported to attain about 10 $\mu\text{g/ml}$ (6, 7), which here we show to be well within the concentration range sufficient to activate the CaSR. Added to this is arginase-driven production of spermine, spermidine, and putrescine, which are increasingly implicated in asthma pathophysiology (4, 5, 11). From an environmental perspective, CaSR agonists may also be presented to the airways in the form of smoke (Ni^{2+}) or car fumes (Pb^{2+} and Cd^{2+}) (34) and bacterial/viral infections (polyamines) (22, 23).

In addition to elevating $[Ca^{2+}]_i$ and, therefore, priming ASM cells to respond with a lower threshold to pathophysiological stimuli, CaSR may also enhance sensitization of airways to Ca^{2+} , for example, via coupling to protein kinase C and Rho kinase, as demonstrated in other cell systems, namely, HEK-CaSR (35), a topic that is currently unexplored in the lung but is highly relevant to the increasing interest in targeting sensitization mechanisms (36). Beyond contributions to AHR and airway remodeling (25), calcilytics prevent activation of intracellular pathways, which are currently being targeted by pipeline asthma drugs, specifically p38 MAPK and phosphodiesterase inhibitors (26). Indeed, both classes of inhibitors target various inflammatory cells, which release key mediators responsible for the remodeling and inflammation characteristic of these diseases. For this reason, local delivery of calcilytics has the potential to target not only one of the key possible causes for asthma but also the production of proinflammatory cytokines that contribute to its exacerbations. Consistent with this hypothesis is the ability of the calcilytics to reduce inflammatory cell infiltration in the BALF of OVA-sensitized mice.

Our *ex vivo* experiments show that activation of the airway CaSR increases responses to bronchoconstrictors by about 20 to 25%. Albeit apparently small, this effect is substantial if we consider that resistance to the air flow increases to the reciprocal of the fourth power of the bronchial radius. Indeed, direct measurements of airway resistance in naïve animals show that CaSR activators increase R_L by roughly threefold. Thus, antagonizing the CaSR might provide a highly beneficial maneuver for the treatment of AHR *in vivo*.

Individually, each of the approaches used in the current study has its inherent limitation. Concerning the ex vivo studies, wire myography records small airway tension in isolation, and although lung slices allow airway lumen size measurements in a system where the local paracrine environment is intact, there is no active innervation. For in vivo studies, flexiVent data are obtained in anaesthetized mice, which are mechanically ventilated, and even though whole-animal plethysmography records lung function in freely moving, spontaneously breathing animals, it can only report indirectly on airway resistance. However, this broad set of experimental approaches has generated complementary data sets that, as the complexity of the measuring systems steadily builds, have provided comprehensive, overlapping evidence to show calcilytic-dependent diminution of airway responsiveness in normal and pathological paradigms. This idea is central to the thesis that locally delivered calcilytics may represent a brand new therapeutic approach to the treatment of asthma.

Asthma represents a multifactorial disease, involving many cell types in the airway beyond immune cells, including the epithelium and ASM. Accordingly, the expression and potential role of CaSR in cells of the airways become important. Although our study focused on the ASM CaSR, it is worth noting that our observations demonstrate CaSR expression in airway epithelial cells. Epithelial cell damage is pathognomonic of asthma, whereas the presence of environmental polyamines and other CaSR activators might directly activate a functional epithelial CaSR, which might in turn contribute to airway remodeling and altered epithelial permeability in asthma, as demonstrated by CaSR activation in other epithelia (16). On the other hand, CaSR is functionally expressed in human and mouse macrophages, where it plays a crucial role in activation of NLRP3 inflammasome and release of IL-1 β (17, 18), known to be involved in asthma pathogenesis. In addition, we found CaSR expression in human eosinophils (fig. S7), and previous studies have shown that eosinophil degranulation (37) and migration across the lung epithelium (38) is also Ca²⁺_o-dependent. Although our data clearly show a role for the ASM CaSR, particularly in the context of airway inflammation and asthma, given the expression of CaSR on both immune and epithelial cells, exploring their role will be important in future studies in the context of identifying CaSR modulators to alleviate AHR and allergic asthma.

Owing to their ability to evoke rapid fluctuations in plasma PTH, a known anabolic stimulus to bone growth, systemic calcilytics were initially developed as anti-osteoporotic drugs and reached phase 2 clinical trials for this purpose in humans (39). Our in vivo data indicate that locally delivered calcilytics do not significantly affect plasma [Ca²⁺]_o levels (hence, presumably PTH levels) up to 24 hours after treatment, suggesting that calcilytic administration directly to the lung in humans should not negatively affect mineral ion homeostasis.

A major implication of CaSR in the airway is its potential for targeting in the context of disease. Accordingly, calcilytic-based therapeutics could do both, prevent as well as relieve AHR. What is unclear at present is whether CaSR overexpression and/or its responsiveness to polycations and calcilytics is uniform across the entire spectrum of asthma, particularly in view of the understanding that severe asthma may differ in pathophysiology and responsiveness to conventional pharmacotherapy (40). This reservation notwithstanding, it would certainly seem likely that one appealing line of future research will be the possibility

that the CaSR can contribute to the development of asthma in some patients by creating a permissive environment for polycation action, with the corollary that such patients can be identified and treated prophylactically. Furthermore, given the involvement of polycations in other environmental airway insults, such as from pollution and respiratory infection, one might speculate that the potential exists for CaSR-targeted approaches to alleviate other inflammatory airway diseases.

MATERIALS AND METHODS

Study design

The objectives of the study were to test the hypothesis that the CaSR is a potential anti-AHR and anti-inflammatory target for asthma therapy.

For experiments in primary human ASM cells, all protocols were approved by the Mayo Clinic Institutional Review Board. Surgical lung specimens of patients undergoing lobectomy for focal, noninfectious disease were obtained, and normal areas of third- to sixth-generation bronchi were identified and dissected for further use. Patient clinical data (combination of physician diagnosis, pulmonary function tests including bronchodilator responses, and imaging results) were used to identify those with moderate asthma versus not. However, once these data were recorded, all patient identifiers were deleted, and samples were stored and processed with unique number identifiers, preventing retrospective identification of patients. Accordingly, the protocol was considered “minimal risk” and did not require explicit patient consent. For both asthmatics (all moderate, $n = 5$) and nonasthmatics (healthy; patients with no documented history of asthma, $n = 5$), patient ages ranged from 40 to 80 years. Both groups included only those patients undergoing thoracic surgery for focal, noninfectious pathology (for example, localized tumor with negative lymph nodes; bronchoalveolar carcinoma was excluded). Samples in either group were used for a range of experimental protocols, although not all five patient samples were used for every protocol.

All animal procedures were approved by local ethical review and conformed with the regulations of the UK Home Office and the Animal Care and Use Committees of all the participating institutions. Procedures were in strict accordance to the guidelines of the American Physiological Society.

Mice with CaSR-targeted gene ablation from ASM cells were generated by breeding SM22 α -Cre recombinase mice (28) with LoxP CaSR (flanking exon 7 of CaSR) (29). The floxed CaSR mouse strain was generated from C57BL/6 $\times$ SVJ129 mice backcrossed with C57BL/6 for at least eight generations. SM22 α -Cre⁺ were bred with floxed-CaSR^{+/+} to generate *SM CaSR^{flox/flox}* mice (lacking full-length CaSR in ASM), which were used as KO mice, and SM22 α -Cre^{-/-} $\times$ floxed-CaSR^{+/+} (expressing full-length CaSR in ASM) acted as WT, control mice. CaSR-LoxP $\times$ SM22 α -Cre mice were inbred for at least three generations before being used for experiments. Both WT and KO mice are fertile and viable with a normal life span (fig. S5, B and C). For the MA model, 6- to 8-week-old C57/Bl6 mice were purchased from The Jackson Laboratory, and for noninvasive Penh measurements, 6- to 8-week-old BalbC male mice were used (Harlan). For laser capture

microscopy experiments, lungs from four mice (10 airways per mouse) were used. For Ca^{2+}_i imaging in human ASM, wire myography, and lung slice experiments, on the basis of our previous experience, a minimum of three patients per condition (at least 15 cells per experiment per patient) or a minimum of three mice per genotype are required to achieve statistical significance. For experiments in human ASM, wire myography, lung slices, and in vivo plethysmography, lack of responses to ACh (in vitro and ex vivo experiments) or MCh (in vivo experiments) was a preestablished exclusion criterion, as was obvious epithelial damage or denudation in lung slice experiments when samples were observed under light microscopy. Data are presented as average $\pm$ SEM, which was calculated invariably from n (the number of patients or animals, biological replicates), with the exception of Western analysis of Akt, p38 MAPK, and ERK1/2 phosphorylation, where n represents the number of individual experiments (technical replicates). Animals were assigned to the experimental groups at random, but the investigators were not blinded. Where appropriate, data were tested for normality (Shapiro-Wilk test).

In vitro studies

Human ASM cells—Human ASM cells were isolated and cultured as previously described (41) in Dulbecco's modified Eagle's medium/F12 (Life Technologies) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Culturing was limited to less than four passages, and retention of the ASM phenotype was verified by expression of smooth muscle cell markers SM22 α or calponin.

HEK-293 studies—Cells stably transfected with human CaSR (HEK-CaSR) or empty vector (HEK-0, negative control) were generated and cultured as described previously (42). All cells tested negative for mycoplasma.

Mouse ASM cells—Cells were obtained using previously described techniques (43). Passage 1 to 4 cells were serum-starved for 24 hours before experimentation.

Ca^{2+}_i imaging—Techniques using the ratiometric Ca^{2+} indicator fura-2 AM have been previously described (25, 41). An inverted microscope (Olympus IX71) and fluorescence source (Xenon arc or LED) along with rapid perfusion system was used to alter $[\text{Ca}^{2+}]_o$ (1 to 5 mM), add agonists (ACh, histamine) and Gd^{3+} (100 μM to 1 mM), or add the polycations ECP (10 mg/ml), PLA (300 μM), or spermine (1 mM). During experimentation requiring different $[\text{Ca}^{2+}]_o$, these changes were made $\sim$ 30 min before experiment (but after dye loading to ensure no confounding effects of Ca^{2+}_o on CaSR or on the loading per se). Where stated, cells were incubated with calcilytics (NPS89636, NPS2143, or Calhex 231) for 20 min.

Phospho-Akt, p38^{MAPK}, and phospho-ERK cell signaling—Human ASM cells isolated from two healthy subjects ($n = 17$ to 19 technical repeats) were passaged up to 10 times and plated for phosphorylation experiments. Cells were exposed to 0.5 mM Ca^{2+}_o (control), 5 mM Ca^{2+}_o , or 5 mM Ca^{2+}_o in the presence of NPS2143 (1 μM), and experiments were carried out and as described previously (44).

Protein analysis—Standard SDS–polyacrylamide gel electrophoresis with 4 to 15% gels and polyvinylidene difluoride membranes were used with protein detection using far-red (LI-COR Odyssey XL) or horseradish peroxidase–conjugated secondary antibodies. CaSR protein expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Ex vivo studies

Force measurements in intralobular bronchi—Second- to third-order intralobular bronchial rings (2 mm in length) were isolated from the left lobe, cleaned, and mounted in a wire myograph (610M, DMT) for measurement of isometric force as described previously (45) at a passive tension of 2 mN. For the nonpaired experiments (WT versus KO), the data were normalized to the mean maximum for WT, whereas for the paired experiments (control versus treated with spermine or high $[Ca^{2+}]_o$), each data point was normalized to the maximum of its own control. To obtain the spermine concentration-response curve, bronchi were first precontracted with ACh to achieve about 50% of maximal tone, and then rising concentrations of spermine were added to the bath. The averaged data points of each set were fitted with sigmoidal dose-response curve, variable slope (ACh), or second-order polynomial curve (spermine).

Precision-cut lung slices—Samples were prepared as previously described (46). Intralobular bronchi were identified under a light microscope (Nikon Diaphot) and imaged during bronchoconstrictor stimulation. Bronchial lumen areas were measured with ImageJ. Tone was established using 1 μ M ACh, and the effects of polyamine spermine (300 μ M) were determined in the absence and presence of calcilytic NPS89636 (300 nM).

Laser capture microdissection and qRT-PCR—Air-inflated lungs from control and MA-challenged cohort of mice were rapidly frozen under ribonuclease-free conditions (47). Samples were cryosectioned, and total RNA was isolated as described previously (47). CaSR mRNA was standardized against ribosomal protein S16 mRNA (C_t). Individual C_t values were standardized against the mean C_t of the control group (nonasthmatic humans and control mice, C_t) on which statistical comparisons were performed. For graphical representation, the mean fold difference $\pm$ SD between the groups was calculated as $2^{-C_t \pm SD}$.

Immunofluorescence—Standard techniques were applied to cryosections of paraformaldehyde-fixed biopsies from human lung and vibratome-cut, paraformaldehyde postfixed murine lung slices. A TCS-SP2 AOBS confocal laser-scanning microscope (Leica) was used for image acquisition.

In vivo studies

Measurements of airway resistance (R_L)— R_L was measured by flexiVent (SCIREQ) under pentobarbital anesthesia and pancuronium paralysis using established techniques (47, 48). In select cases, animals were prenebulized with the CaSR-positive (R568; 1 μ M) and/or CaSR-negative (NPS2143; 1 μ M) allosteric modulators (Tocris) 10 min before MCh challenge.

MA model—C57/Bl6 mice received daily intranasal mixture of 10 mg of OVA and extracts from *Alternaria*, *Aspergillus*, and *Dermatophagoides* (house dust mite) for 4 weeks (Greer Labs), each dose in 50 ml of phosphate-buffered saline (PBS). Control mice received intranasal PBS. Animals were analyzed 24 hours after the last sensitization.

Whole-body plethysmography—Noninvasive barometric plethysmography (Buxco Research Systems) was carried out in unrestrained, conscious mice as described previously (31). After establishment of baseline enhanced pause (Penh) (49), standard nebulized MCh challenge was performed (0.1 to 100 mg/ml in saline; 3-min recording per dose; PulmoStar nebulizer, Sunrise Medical), and Penh values were calculated and expressed as percentage change (Penh, %). Although the physiological data provided by a Penh-based approach differ from those using the forced oscillation technique of the flexiVent system, the noninvasive approach allowed for longitudinal measurements of baseline and chronic drug effects in the same animals (as below).

Polycation-induced AHR—Twenty-four hours after baseline measurements of Penh with MCh challenge, mice were exposed to aerosolized PLA (3 mM), NPS89636 (3 μ M), PLA + NPS89636, or vehicle [0.3% (v/v) dimethyl sulfoxide (DMSO)] for 1 hour, and MCh challenge was repeated. For experiments with PLA + NPS89636 or vehicle, mice were pretreated for 30 min with NPS89636 (or vehicle) and then cotreated with PLA. Mice were allowed to recover for 1 week between each set of experiments. Separately, naïve animals were exposed to nebulized NPS89636, and 0, 4, and 24 hours later, the blood was collected and analyzed for serum Ca^{2+} .

OVA-induced AHR—Male BalbC mice were sensitized on days 0 and 5 by intraperitoneal injection of 100 μ g of OVA per mouse and 50 mg of aluminum hydroxide per mouse in PBS. Thirteen days after the final injection, Penh was recorded during MCh challenge. The next day, mice were challenged twice with 0.5% nebulized OVA (in PBS, w/v) and nebulized NPS89636 (3 μ M, or 0.03% DMSO vehicle) by inhalation, 4 hours apart. Twenty-nine hours after the first OVA inhalation, Penh was again recorded during MCh challenge.

BALF analysis—Bronchoalveolar lavage was performed after the terminal experiment, cells were isolated by CytoSpin centrifugation (Thermo Scientific), and total and differential cell counts were performed after Leishman's staining. Enzyme-linked immunosorbent assay (R&D) and ECP (Aviscera Bioscience Inc.) measurements were performed according to the manufacturer's instructions.

Materials and antibodies

NPS89636 was a gift from NPS Pharmaceuticals Inc. NPS2143 and Calhex 231 were purchased from Tocris. All other chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Primary antibodies used were as follows: anti-SM22 α (Abcam); anti-CaSR (AnaSpec or Abcam); and anti-phospho-ERK, anti-phospho-Akt, and anti-p38 MAPK (Cell Signaling). Secondary antibodies used were as follows: Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 (Life Technologies). Nuclei were counterstained using Hoechst. Omission of the primary antibodies acted as negative control.

Statistics

Statistical significance was determined using GraphPad Prism 6 software. Student's two-sided, unpaired, or paired *t* test was used to compare a group of two data sets; one- or two-way ANOVA with Bonferroni post hoc test, or nonparametric (Friedman) with Dunn post hoc test, as stated in the figure legends, was used to compare three or more data sets. Where applicable, statistical comparisons were made between nonnormalized data groups, but normalized data are presented in the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge D. H. Edwards for access to wire myography equipment, D. Wright for his help with human ASM cells, D. Richards for the human eosinophil cytospins, and E. F. Nemeth for the gift of NPS89636 and helpful discussions.

Funding: This work was supported by grants from Asthma UK (11/056, to C.J.C., J.P.T.W., D.R., and P.J.K.), the Cardiff Partnership Fund (to D.R., P.J.K., E.J.K., and W.R.F.), a Marie Curie Initial Training Network "Multifaceted CaSR" (to D.R. and P.J.K.), the Biotechnology and Biological Sciences Research Council (BB/D01591X to D.R. and P.J.K.), and the U.S. NIH [HL056470, HL088029 (to Y.S.P.), and HL090595 (to C.M.P.)]. We also acknowledge support from the Department of Health via the National Institute for Health Research Comprehensive Biomedical Research Centre award to Guy's & King's College London and King's College Hospital NHS Foundation Trust.

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Fig. 1. CaSR immunolocalizes to human and mouse airways and is overexpressed in asthma
(A) Human airway biopsy (upper panels) or mouse intralobular bronchi (lower panels) stained with CaSR antibody (red) and SM22 α (green) show immunoreactivity in both smooth muscle and epithelium. Scale bars, 10 mm. **(B)** Human and mouse ASM cells stained with anti-SM22 α antibody and showing CaSR immunoreactivity. Scale bars, 100 mm. **(C)** qRT-PCR shows higher CaSR expression in moderate asthmatics than in healthy subjects [$n = 4$ patients per group, fold change versus healthy, mean (line) $\pm$ SD (box)]. **(D)** Western analysis of CaSR protein shows substantially elevated CaSR expression in

moderate asthmatics ($n = 5$ patients per group). (E) Exposure of healthy human ASM cells to TNF- α (20 ng/ml) or IL-13 (50 ng/ml) for 48 hours significantly increased CaSR protein expression ($n = 5$ patients per group) compared to vehicle control for either cytokine. (F) CaSR mRNA expression was significantly greater in mice after induction of airway inflammation with MAs in comparison to unsensitized mice [$n = 4$ mice per group; 10 airways per mouse, fold change versus unsensitized, mean (line) $\pm$ SD (box)]. Statistical comparisons were performed [on C_t values for (C) and (F)] by two-tailed, unpaired Student's t tests (C, D, and F) and one-way analysis of variance (ANOVA) with Bonferroni post hoc test (E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus healthy, control, or unsensitized, as shown. Source data, details of the statistical analysis, and P values are given in the Supplementary Materials.

Fig. 2. Polycations activate the human CaSR in recombinant systems and human ASM cells, particularly those from asthmatics

(A) ECPs ($n = 7$), PLA ($n = 6$), or spermine ($n = 17$) each increased $[Ca^{2+}]_i$ in HEK-CaSR, but not in HEK-0 cells (ECP, $n = 3$; PLA, $n = 6$; spermine, $n = 6$). In HEK-CaSR cells, the calcilytic NPS89636 prevented these increases (ECP, $n = 3$; PLA, $n = 3$; and spermine, $n = 4$). Two alternative calcilytics, NPS2143 ($n = 4$) and Calhex 231 ($n = 5$), also prevented spermine-induced CaSR activation. (B) In human ASM cells, exposure to 2 mM $[Ca^{2+}]_o$ increased $[Ca^{2+}]_i$ in asthmatic but not in healthy ASM cells (left), an effect prevented by the

calcilytic NPS2143 (right) ($n = 3$ each). (**C** and **D**) In the presence of 1 mM Ca^{2+}_o , exposure to ACh (**C**) ($n = 4$ healthy, $n = 4$ asthmatic) or histamine (**D**) ($n = 5$ healthy, $n = 4$ asthmatic) resulted in increases in $[\text{Ca}^{2+}]_i$, which was greater in asthmatic ASM cells. This effect was prevented by NPS2143. ns, not significant. (**E** and **F**) Western analysis [exemplar gel (**E**) and summary data (**F**)] of healthy ASM cell lysates shows the effects of 5 mM Ca^{2+}_o in the absence or presence of NPS2143 on Akt, p38 MAPK, and ERK phosphorylation ($n = 17$ to 19 independent experiments from cells isolated from $n = 2$ nonasthmatic patients). Statistical significance was determined by one-way ANOVA with Bonferroni post hoc test (**A**), two-way ANOVA with Bonferroni post hoc test (**B** to **D**), or one-way ANOVA with Dunn post hoc test (**F**). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, significantly different from control HEK-CaSR (**A**), from control healthy or asthmatic ASM (**B** to **D**), or from 5 mM Ca^{2+}_o (**B**). Source data, details of the statistical analysis, and P values are given in the Supplementary Excel spreadsheet.

Fig. 3. CaSR ablation from ASM cells blunts polycation-induced bronchoconstriction

(A to F) Tension measurements in intralobular bronchi from mice with targeted CaSR ablation from ASM cells (*SM22a* CaSR *flax/flax*, KO) and from *SM22a*-Cre mice (WT). (A) Under control conditions, bronchial contractility to ACh was not affected by CaSR ablation (WT, *n* = 14; KO, *n* = 12). Exposure to spermine enhanced contractility to ACh in WT (B, *n* = 6) but not in KO mice (C, *n* = 7). (D) Spermine alone induced constriction in WT but not in KO mouse intralobular bronchi, which was significant at 1 mM (WT, *n* = 4; KO, *n* = 7) and above (WT, *n* = 3; KO, *n* = 7). The sensitivity to ACh was increased when $[Ca^{2+}]_o$ was

raised from the physiological 1 to 2.5 mM in WT mice (E, $n = 6$) but not in KO mice (F, $n = 7$). (G to I) In precision-cut lung slices from WT mice, ACh-induced contraction of intralobular bronchi was potentiated by spermine and prevented by the calcilytic NPS89636 (G, upper panels) [representative of seven experiments; summary in (H); $n = 7$], whereas there was no spermine potentiation or calcilytic effect in KO mice (G, lower panels) [representative of four experiments; summary in (I); $n = 4$]. Scale bar, 100 μ m. Statistical comparisons were made by two-way ANOVA (A to F) (between curves and for WT versus KO for identical agonist concentrations) or two-tailed, paired Student's t test (H and I) (performed on the nonnormalized data). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, statistically different from respective WT controls. Source data, details of the statistical analysis, and P values are given in the Supplementary Materials.

Fig. 4. Activation of the airway CaSR exacerbates AHR in vivo

(A) In mechanically ventilated, unsensitized mice, acute exposure to the calcimimetic R568 increased bronchoconstriction to MCh challenge, measured as increased airway resistance (R_L). The calcilytic prevented the AHR induced by R568, but evinced little effect on its own. (B) MA-sensitized mice showed enhanced response to MCh. Preexposure to the calcimimetic resulted in a further increase in R_L , whereas the calcilytic NPS2143 reduced AHR. Statistical comparisons between the curves were made by two-way ANOVA, Bonferroni post hoc test. $n = 5$ per condition. ** $P < 0.01$, *** $P < 0.001$ statistically different

from control, $^{###}P < 0.001$ statistically different from calcimimetic. Source data, details of the statistical analysis, and P values are given in the Supplementary Materials.

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Fig. 5. Nebulized calcilytics prevent AHR and inflammation in mice in vivo

(A) Nebulized calcilytic (NPS89636) prevented PLA-induced AHR in unsensitized, conscious mice. Data are presented as percentage changes in enhanced pause, Penh (Penh, %), in MCh-challenged mice ($n = 6$ mice per condition). (B to D) Calcilytic abrogated hyperresponsiveness (B) ($n = 5$ for control, $n = 6$ each for vehicle and calcilytic), reduced inflammatory cell infiltration (C) ($n = 11$), and the concentrations of ECP ($n = 11$), IL-5, IL-13, and TNF- α (D) ($n = 10$ for vehicle, and $n = 11$ for calcilytic) into the BALF from OVA-sensitized, OVA-challenged mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, statistically different from vehicle control; ### $P < 0.05$, ### $P < 0.001$, statistically different from treatment with PLA. For (A) and (B), statistical comparisons were made by two-way ANOVA with Bonferroni post hoc test. For (C) and (D), statistical significance was determined by two-tailed, unpaired Student's t test. Source data, details of the statistical analysis, and P values are given in the Supplementary Materials