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How to cite:
Yarova, Polina L.; Stewart, Alecia L.; Sathish, Venkatachalem; Britt, Rodney D.; Thompson, Michael A.; Lowe, Alexander P. P.; Freeman, Michelle; Aravamudan, Bharathi; Kita, Hirohito; Brennan, Sarah C.; Schepelmann, Martin; Davies, Thomas; Yung, Sun; Cholisoh, Zakky; Kidd, Emma J.; Ford, William R.; Broadley, Kenneth J.; Rietdorf, Katja; Chang, Wenhan; Bin Khayat, Mohd E.; Ward, Donald T.; Corrigan, Christopher J.; T. Ward, Jeremy P.; Kemp, Paul J.; Pabelick, Christina M.; Prakash, Y. S. and Riccardi, Daniela (2015). Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma. Science Translational Medicine, 7(284) 284ra60.

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Version: Accepted Manuscript

Link(s) to article on publisher’s website:
http://dx.doi.org/doi:10.1126/scitranslmed.aaa0282

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


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Abstract

Airways hyperresponsiveness and inflammation are fundamental hallmarks of allergic asthma and are accompanied by increases in certain polycations, such as eosinophil cationic protein, whose levels in body fluids correlate with asthma severity. Here we show that polycations, or elevated extracellular calcium activate the human recombinant and native calcium-sensing receptor (CaSR), leading to intracellular calcium mobilization, cAMP breakdown and p38 MAPK phosphorylation in airway smooth muscle (ASM) cells, effects prevented by CaSR antagonists, termed calcilytics. Asthmatic patients and allergen-sensitized mice expressed more CaSR in ASMs than their healthy counterparts. Polycations induced hyperreactivity in mouse bronchi, effect 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 upregulated in asthmatic ASM and targeted by locally produced polycations to induce hyperresponsiveness and inflammation. Calcilytics may represent a novel, effective asthma therapeutics.
Introduction

Despite substantial advances in our understanding of its pathophysiology and improved therapeutic regimens, asthma remains a tremendous worldwide healthcare burden with around 300 million individual sufferers. While the symptoms of asthma are potentially controllable in the majority of asthma sufferers using conventional therapy such as topical bronchodilators and corticosteroids, these are troublesome to administer efficiently and present unwanted side effects. Most importantly, 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 healthcare 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 non-specific environmental stimuli (non-specific airway hyperresponsiveness, or 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 towards increased production of the polycations spermine, spermidine and putrescine (4, 5, 11). While in human peripheral blood monocytes spermine exhibits anti-inflammatory properties (12). Although such 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-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\(^{2+}\)) concentration via the regulation of parathyroid hormone secretion (14). Accordingly, CaSR-based therapeutics is employed 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 non-calciotropic tissues, the CaSR responds to a range of stimuli including not only Ca\(^{2+}\), 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 ([Ca\(^{2+}\)]\(_{i}\)) in airway smooth muscle (ASM) cells that is critical not only to the enhanced bronchoconstriction of non-specific AHR but is 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 [Ca\(^{2+}\)]\(_{i}\) in the asthmatic airways, even though a polycation sensor such as the CaSR, whose activation leads to an increase in [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\(^{2+}\), but also to polycations such as the ECPs and L-arginine-derived polyamines putrescine, spermidine and spermine, whose production is dramatically 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 non-asthmatic and asthmatic subjects, and employed two models of allergen-
induced airways 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 SM22α-positive smooth muscle layer, with additional expression in bronchial epithelium (Fig. 1A). Isolated human and mouse ASM cells retained CaSR expression (Fig. 1B).

Quantitative RT-PCR and western analysis of human ASM cells demonstrated that both CaSR mRNA (Fig. 1C) and protein (Fig. 1D) expression were increased approximately three-fold in moderate asthmatics, compared to non-asthmatics (“healthy”). Furthermore, in human ASM cells from healthy individuals, 48h exposure to the asthma-associated pro-inflammatory cytokines, TNFα and 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 approximately three-fold increase in CaSR mRNA expression in mixed allergen-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 in the effects of mixed allergen sensitization in a mouse model of allergic asthma provide an evidence for the role of inflammation in upregulation 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 ([Ca\(^{2+}\)]) arising from mobilization of Ca\(^{2+}\)\(_i\) (14). To test the hypothesis that polycations that are upregulated during asthma activate the CaSR, we measured changes in [Ca\(^{2+}\)], in HEK293 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 [Ca\(^{2+}\)], following exposure to: i) eosinophil cationic protein (ECP, 10 μg/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-D for single traces). For each of these agonists, the increase in [Ca$^{2+}$]$_i$ was inhibited by the calcilytic NPS89636 (100 nM)(Fig. 2A and Fig. S2, B-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$^{2+}$ in ASM from asthmatic patients**

In human ASM, several endogenously produced agents such as acetylcholine (ACh) and histamine evoke increases in [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 [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 [Ca$^{2+}$]$_i$ was significantly higher than those from non-asthmatics in the presence of 2 mM [Ca$^{2+}$]$_o$ (Fig. 2B, left panel and Fig. S3A,B for single traces). Inhibition of the CaSR with the calcilytic NPS2143 (1 µM) blunted the [Ca$^{2+}$]$_o$ hyper-responsiveness of asthmatic ASM cells, highlighting the functional role of CaSR in this setting (Fig. 2B, right panel and Fig. S3A,B for single traces).

Having determined the sensitivity of human asthmatic 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$^{2+}$]$_o$, *i.e.*, 1 mM, at which the CaSR is half-maximally active (14)) exposure to 1 µM 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$, a concentration at which the CaSR is fully active, while they could not be observed in the presence of 0.5 mM [Ca$^{2+}$]$_o$, which is below the threshold for CaSR activation (Fig. S3C).

Histamine (1 µM) also evoked an increase in [Ca$^{2+}$]$_i$, in both healthy and asthmatic ASM, which was significantly greater in asthmatic ASM (Fig. 2D). Importantly, pre-exposure 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 either in the absence (Fig. S3D) or presence (Fig. S3E) of histamine, effects which were greater in asthmatic than in non-asthmatic ASM cells. Together, these results demonstrate that, in ASM cells, the CaSR is functional and contributes to the regulation of baseline ASM $[\text{Ca}^{2+}]_{i}$. Accordingly, in asthmatic ASM cells, the CaSR may contribute to a higher baseline $[\text{Ca}^{2+}]_{i}$, a leading cause of AHR, whereas calcilytics restore baseline $[\text{Ca}^{2+}]_{i}$.

**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: cAMP, which should induce bronchodilation, and phospholipase C (PLC)/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$^{2+}$, 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$^{2+}$ (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 upon ERK1/2, p38 MAPK, and PI3K/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$^{2+}$ induced a significant increase in p38 MAPK phosphorylation, an effect which was prevented by co-incubation with a calcilytic. Calcilytic treatment reduced ERK1/2 and Akt phosphorylation at 5 mM Ca$^{2+}$ (Fig. 2E,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 $[\text{Ca}^{2+}]_{i}$.

**SM22α-CaSR$^{\text{flo}}$/$\text{flo}$ 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 SM22α-Cre mice (28, 29). The resulting SM22α-CaSR$^{\text{flo}}$/$\text{flo}$ mice (knock out, KO, Fig. S5) were comparable to Cre-negative (wild-type, WT) littermates in appearance, reproductive abilities, body weight and life expectancy (Fig. S5 B,C). FACS 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, SM22α (Fig. S5D). Successful ASM CaSR ablation was demonstrated functionally by the evidence that Ca\textsuperscript{2+}\textsubscript{o} (1-5 mM) and an alternative, membrane-impermeant CaSR agonist, Gd\textsuperscript{3+} (100 µM-1mM), evoked an increase in [Ca\textsuperscript{2+}]\textsubscript{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).

Intrinsic baseline contractility of the intralobular bronchi was not affected by CaSR ablation from ASM cells, as shown by exposure to either high K\textsuperscript{+} (40 mM, Fig. S5H) or to increasing concentrations of ACh (1 nM - 30 µM), 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 µM, Fig. 3B) or 2.5 mM [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 3E) enhanced the bronchoconstrictor response to ACh. CaSR ablation from ASM blunted both spermine- (Fig. 3C) and [Ca\textsuperscript{2+}]\textsubscript{o}- (Fig. 3F) induced sensitization of the ACh response. Furthermore, spermine (10 µM – 3 mM) induced sensitization of the ACh response in pre-contracted WT, but not in KO mouse bronchi (Fig. 3D). Consistent with these findings, spermine also enhanced the response to ACh (0.5 µM) 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). Taken together, these observations suggest that activation of the ASM CaSR leads to AHR, an effect which can be prevented by calcilytic treatment.

**Calcilytics reduce airway resistance in mixed allergen-sensitized mice in vivo**

To test the effects of pharmacological CaSR activators and inhibitors on pulmonary resistance, we directly measured airflow resistance (R\textsubscript{L}) in anaesthetized, paralyzed, mechanically ventilated mice. In naïve mice, acute pre-exposure (10 min) to the nebulized calcimimetic, R568 (1 µM), resulted in an increase in R\textsubscript{L} across the lungs following inhalational challenge with the synthetic muscarinic receptor agonist, methacholine (MCh, 0
– 50 mg/ml), whereas the calcilytic NPS2143 (1 µM) was able to reverse this effect (Fig. 4A). Next, we developed a novel, mixed allergen (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 $R_L$ following MCh challenge, and pre-exposure 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-sensitised, 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, the effects of nebulized PLA (3 µM) were assessed in the presence or absence of the calcilytic NPS89636 (3 µM). As an alternative, non-invasive method for measurement of AHR (30, 31), we performed whole-body plethysmography in conscious, unrestrained naïve mice by measuring enhanced pause (Penh). While 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 - 100 mg/ml) (31). PLA significantly increased Penh at MCh concentrations greater than 10 mg/ml, an effect that was abolished by co-treatment with the nebulized calcilytic. Calcilytic treatment per se significantly reduced Penh in naïve animals treated with 30 mg/ml MCh (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). Importantly, 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 vs. 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, four 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 is the first to highlight the expression of the CaSR in ASM, and to identify 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 $[Ca^{2+}]_{i}$, and increase the contractility of the ASM non-specifically provides both a rational explanation for the genesis of non-specific AHR in asthma and a basis for the direct mechanistic link between this phenomenon and airways 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 both 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 impacts on 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 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 airways 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 approximately 10 μg/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\(^{2+}\)], and, therefore, priming ASM cells to respond with a lower threshold to pathophysiological stimuli, CaSR may also enhance sensitization of airways to Ca\(^{2+}\), e.g. via coupling to PKC 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 pro-inflammatory 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 airways CaSR increases responses to bronchoconstrictors by approximately 20-25%. 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 three folds. 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 anaesthetised 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 airways resistance. However, this broad set
of experimental approaches has generated complementary data sets which, as the complexity of the measuring systems steadily builds, have provided comprehensive, overlapping evidence to show calcilytic-dependent diminution of airways 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 multi-factorial 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 becomes important. While our study focused on the ASM CaSR, it is worth noting that our observations demonstrate CaSR expression in airways epithelial cells. Epithelial cell damage is pathognomonic of asthma, while the presence of environmental polyamines and other CaSR activators might directly activate a functional epithelial CaSR, which might in turn contribute to airways 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-1β (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⁺ dependent. While 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 II clinical trials for this purpose in humans (39). Our *in vivo* data indicate that locally delivered calcilytics do not significantly affect plasma [Ca²⁺]₀ levels (hence presumably PTH levels) up to 24 hours post-treatment, suggesting that calcilytic administration directly to the lung in humans should not negatively impact on 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, non-infectious disease were obtained and normal areas of 3rd to 6th generation bronchi identified, and dissected for further use. Patient clinical data (combination of physician diagnosis, pulmonary function tests including bronchodilator responses, imaging results) were used to identify those with moderate asthma vs. not. However, once these data were recorded, all patient identifiers were deleted, and samples 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 non-asthmatics (“healthy”, patients with no documented history of asthma, N = 5), patient ages ranged from 40 - 80 years. Both groups included only those patients undergoing thoracic surgery for focal, non-infectious pathology (e.g. 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 5 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 sm22α-Cre recombinase mice (28), with LoxP CaSR (flanking exon 7 of CaSR) (29). The floxed CaSR mouse strain was generated from C57BL/6 x SVJ129 mice backcrossed with C57BL/6 for at least 8 generations. SM22α-Cre+ were bred with floxed-CaSR+/+ to generate SM22α-Cre+/CaSR−/− mice (lacking full-length CaSR in ASM), which were used as KO mice and SM22α-Cre+/CaSR+/+ (expressing full-length CaSR in ASM) acted as WT, control mice. CaSR-LoxP x SM22α-Cre mice were inbred for at least 3 generations before being used for experiments. Both WT and KO mice are fertile, and viable with a normal lifespan (Fig. S5 B,C). For the mixed allergen model, 6-8 week C57/Bl6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine), and for non-invasive Penh measurements, 6-8
week BalbC male mice were used (Harlan). For laser-capture microscopy experiments, lungs from four mice (10 airways per mouse) were used. For Ca\(^{2+}\), imaging in human ASM, wire myography and lung slice experiments, based on our previous experience a minimum of 3 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 \textit{in vivo} plethysmography, lack of responses to ACh (\textit{in vitro} and \textit{ex vivo} experiments) or MCh (\textit{in vivo} experiments) were a pre-established 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 ± standard error of the mean (S.E.M.), 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).

\textit{In vitro studies}

\textit{Human ASM cells:} Human ASM cells were isolated and cultured as previously described (41) in DMEM/F12 (Life Technologies) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Culturing was limited to <4 passages and retention of the ASM phenotype was verified by expression of smooth muscle cell markers SM22α or calponin.

\textit{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.

\textit{Mouse ASM cells:} Cells were obtained using previously described techniques (43). Passage 1-4 cells were serum-starved for 24 hours prior to experimentation.

\textit{Ca}\(^{2+}\) imaging: Techniques using the ratiometric Ca\(^{2+}\) indicator fura-2 AM have been previously described (25, 41). An inverted microscope (Olympus IX71, Southend-on-Sea, United Kingdom) and fluorescence source (Xenon arc or LED) along with rapid perfusion system was used to alter \([\text{Ca}^{2+}]_o\) (1-5 mM), add agonists (ACh, histamine), Gd\(^{3+}\) (100 μM-1mM) 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 ~30 min prior to experiment (but after dye loading to ensure no confounding effects of \([\text{Ca}^{2+}]_o\) on CaSR or on
the loading per se). Where stated, cells were incubated with calcilytics (NPS89636, NPS2143 or Calhex) for 20 minutes.

**pAkt, p38MAPK and pERK cell signaling:** Human ASM isolated from two healthy subjects (n = 17-19 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) experiments were carried out and as described previously (44).

**Protein analysis:** Standard SDS-PAGE with 4-15% gels and PVDF membranes were used with protein detection using far-red (LiCor Odyssey XL) or HRP-conjugated secondary antibodies. CaSR protein expression was normalized to GAPDH.

**Ex vivo studies**

**Force measurements in intralobular bronchi:** 2\(^{\text{nd}}\)-3\(^{\text{rd}}\) order intralobular bronchial rings (2 mm length) were isolated from the left lobe, cleaned and mounted in a wire myograph (610 M, DMT) for measurement of isometric force as described previously (45) at a passive tension of 2 mN. For the non-paired experiments (WT vs. KO), the data were normalized to the mean maximum for WT, whilst for the paired experiments (control vs. 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 pre-contracted with ACh to achieve approximately 50% of maximal tone, 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 light microscopy (Nikon Diaphot) and imaged during bronchoconstrictor stimulation. Bronchial lumen areas were measured with ImageJ. Tone was established using 1 μM ACh and the effects of polyamine spermine (300 μM) were determined in the absence and presence of calcilytic NPS89636 (300 nM).

**Laser capture microdissection (LCM) and quantitative RT-PCR:** Air-inflated lungs from control and mixed allergen challenged cohort of mice were rapidly frozen under RNase-free conditions (47). Samples were cryosectioned and total RNA was isolated as described previously (47). CaSR mRNA was standardized against ribosomal protein S16 mRNA (ΔCt). Individual ΔCt values were standardized against mean ΔCt of the control group (non-asthmatic humans and control mice, ΔΔCt) on which statistical comparisons were performed.
For graphical representation, the mean fold difference ± SD between the groups was calculated as $2^{-\Delta\Delta C_t \pm SD}$.

**Immunofluorescence:** Standard techniques were applied to cryosections of paraformaldehyde-fixed biopsies from human lung and vibratome-cut, paraformaldehyde post-fixed 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 (Montreal, Canada) under pentobarbital anesthesia and pancuronium paralysis using established techniques (47, 48). In select cases, animals were pre-nebulized with the CaSR positive (R568; 1 µM) and/or negative (NPS2143; 1 µM) allosteric modulators (Tocris) 10 min prior to MCh challenge.

**Mixed allergen model:** Each C57BL6 mice received daily intranasal mixture of 10 µg of OVA, and extracts from *Alternaria, Aspergillus* and *Dermatophagoides* (house dust mite) for 4 weeks (Greer Labs), each dose in 50 µl in PBS. Control mice received intranasal PBS. Animals were analyzed 24h after the last sensitization.

**Whole-body plethysmography:** Non-invasive barometric plethysmography (Buxco Research Systems) was carried out in unrestrained, conscious mice as described previously (31). Following establishment of baseline enhanced pause (Penh) (49) standard nebulized methacholine challenge was performed (0.1-100 mg/ml in saline; 3 min recording/dose; Pulmostar nebulizer, Sunrise medical), and Penh values calculated and expressed as percentage change ($\Delta$ Penh, %). While the physiological data provided by a Penh-based approach differs from that using the forced-oscillation technique of the FlexiVent system, the non-invasive approach allowed for longitudinal measurements of baseline and chronic drug effects in the same animals (as below).

**Polycation-induced AHR:** 24 hours following baseline measurements of Penh with MCh challenge, mice were exposed to aerosolized PLA (3 µM), NPS89636 (3 µM), PLA + NPS89636 or vehicle (0.3% DMSO, v/v) for 1 h and MCh challenge repeated. For experiments with PLA + NPS89636 or vehicle, mice were pre-treated for 30 min with NPS89636 (or vehicle) and then co-treated 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 collected and analyzed for serum Ca$^{2+}$. 
**OVA–induced AHR:** Male BalbC mice were sensitized on day 0 and 5 by i.p. injection of 100 μg/mouse OVA and 50 mg/mouse aluminum hydroxide 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 μM, or 0.03% DMSO vehicle) by inhalation, 4 hours apart. 29 hours after the first OVA inhalation, Penh was again recorded during MCh challenge.

**BALF analysis:** Bronchoalveolar lavage was performed after the terminal experiment and cells were isolated by CytoSpin centrifugation (Thermo Scientific) and total and differential cell counts performed following Leishman’s staining. ELISA (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 Calhex231 was purchased from Tocris. All other chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Primary antibodies used were: anti-SM22α (Abcam); anti-CaSR (AnaSpec, or Abcam); anti-phospho ERK, anti-phospho Akt and anti-p38 MAPK (Cell Signalling). Secondary antibodies used were: Alexa Fluor 488, and Alexa Fluor 594 or Alexa Fluor 647 (Life Technologies). Nuclei were counterstained using Hoechst. Omission of the primary antibodies acted as negative control.

**Supplementary Materials**

Materials and Methods

Fig. S1. Negative controls and original western blots for Fig. 1

Fig. S2. Polycations increase \([Ca^{2+}]_i\) by acting on the human CaSR

Fig. S3. Calcilytics prevent CaSR activation in human asthmatic ASM

Fig. S4. Technical replicates of data presented in Fig. 2F

Fig. S5. Phenotypic characterization of the SM22α-CaSRΔflox/Δflox mouse

Fig. S6. Validation of the mixed allergen asthma model

Fig. S7. CaSR expression in human eosinophils

Database S1. Source data for Fig. 1 to 5 and Fig. S1 to S5 (provided as Excel file)
Statistics
Statistical significance was determined using Graph Pad Prism 6 software. Student’s two-sided, unpaired or paired t-test was used to compare a group of two data sets, one-way or two-way ANOVA with Bonferroni post hoc test, or non-parametric (Friedman) with Dunn post hoc test, as stated in figure legends, used to compare three or more data sets. Where applicable, statistical comparisons were made between non-normalized data groups, but normalized data are presented in the figures.

Author contribution
PLY, AS, VS, RDB, MAT, APPL, MF, BA, SCB, MS, TD, SY, ZC, KR, MEBK, and DTW performed the experiments. WC generated the SM22α LoxP CaSR mice. YSP, HK, PLY, PJK and DR designed and analyzed the experiments and performed the statistical analyses. EJK, WRF and KJR developed the OVA-sensitized mouse asthma model, CMP developed the MA mouse asthma model. CJC, JPTW, PJK, YSP, PLY and DR wrote the manuscript.

Conflict of interests
DR, PJK, CJC and JPTW are co-inventors on a patent (WO2014049351) claiming the use of CaSR antagonists for the treatment of inflammatory lung disorders. The other authors declare no competing interests.

References


Acknowledgements

This work was supported by grants from Asthma UK (11/056, to CJC, JPTW, DR and PJK), the Cardiff Partnership Fund (to DR, PJK, EJK and WRF), a Marie Curie Initial Training Network “Multifaceted CaSR” (to DR and PJK), the BBSRC (BB/D01591X to DR and PJK), and grants from the National Institutes of Health of the US (HL056470, HL088029 (to YSP) and HL090595 (to CMP)). The authors also acknowledge support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy’s & King’s College London and King’s College Hospital NHS Foundation Trust, Dr DH Edwards for access to wire myography equipment, Dr David Wright for his help with human ASM cells, Dr David Richards for the human eosinophil cytospins, and Dr E.F. Nemeth for the gift of NPS89636.
Figure 1. CaSR immunolocalizes to human and mouse airways and is over-expressed in asthma

(A) Human airway biopsy (upper panels) or mouse intralobular bronchi (lower panels) stained with CaSR antibody (red) and SM22α (green) show immunoreactivity in both smooth muscle and epithelium (scale bar = 10 μm). (B) Human and mouse ASM cells stained with anti-SM22α antibody and showing CaSR immunoreactivity (bar = 100 μm). (C) Quantitative RT-PCR shows higher CaSR expression in moderate asthmatics than in healthy subjects (N = 4 patients per group, fold change vs. healthy, mean (line) ± 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 TNFα (20 ng/ml) or IL-13 (50 ng/ml) for 48h 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 following induction of airways inflammation with mixed allergens in comparison to unsensitized mice (N = 4 mice per group; 10 airways/mouse, fold change vs. unsensitized, mean (line) ± SD (box). Statistical comparisons were performed (on ΔΔCt values for C and F) by two-tailed, unpaired Student’s t-tests (C, D and F) and one-way ANOVA with Bonferroni post hoc test (E). *P < 0.05, **P < 0.01, ***P < 0.001 vs. healthy, control or unsensitized, as shown. Source data, details of the statistical analysis and P values are given in the Supplementary Materials.

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

(A) ECPs (N = 7), poly-L-arginine (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) or Calhex 231 (N = 5) also prevented spermine-induced CaSR activation. (B) In human ASM cells, exposure to at 2 mM \([Ca^{2+}]_o\) increased \([Ca^{2+}]_i\) in asthmatics, but not healthy ASM (B, left), an effect prevented by the calcilytic, NPS2143 (B, right) (N = 3 each). (C, 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 \([Ca^{2+}]_i\), which was greater in asthmatic ASM cells. This effect was prevented by NPS2143. (E, F) Western analysis (exemplar gel (E) and summary data (F)) of healthy ASM cell lysates show the effects of 5 mM Ca\(^{2+}\)_o in the absence or presence of NPS2143 on Akt, p38 MAPK and ERK phosphorylation (n = 17-19 independent
experiments from cells isolated from N = 2 non-asthmatic patients). Statistical significance was determined by one-way ANOVA, Bonferroni post hoc test (A), two-way ANOVA, Bonferroni post hoc test (B-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-D), or from 5 mM Ca\(^{2+}\) (B). Source data, details of the statistical analysis and P values are given in the Supplementary Excel spreadsheet.

Figure 3. CaSR ablation from airway smooth muscle cells blunts polycation-induced bronchoconstriction

Tension measurements in intralobular bronchi from mice with targeted CaSR ablation from ASM cells (\(^{SM22\alpha}CaSR^{\Delta f l o x/\Delta f l o x}\), KO) and from SM22\(\alpha\)-Cre mice (WT) (A-F). (A) In 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). 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 7 experiments; summary in H; N = 7), while there was no spermine potentiation or calcilytic effect in KO mice (G, lower panels, representative of 4 experiments; summary in I; N = 4). Scale bar = 100 µm. Statistical comparisons made by two-way ANOVA (A-F, between curves and for WT vs. KO for identical agonist concentrations) or two-tailed, paired Student’s t test (H, I, performed on the non-normalized 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.

Figure 4. Activation of the airways 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) Mixed allergens (MA)-sensitized mice showed enhanced response to MCh. Pre-exposure to the calcimimetic resulted in a further increase in R\(_L\) whilst the calcilytic NPS 2143 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.

**Figure 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 (ΔPenh, %), in MCh-challenged mice (N = 6 mice per condition). 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 TNFα (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, 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.
Figure 1

A

SM2α | CaSR | Overlay

Human

Mouse

B

Human | Mouse

C

Human

D

Human

E

Human Healthy

F

Mouse

CaSR mRNA (fold change)

CaSR protein (AU)

CaSR protein (AU)

CaSR mRNA (fold change)
Figure 2

(A) Bar graph showing the effects of different treatments on cell permeability (Rるのは؟)

(B) Line graphs comparing control vs. calciolytic conditions in different groups:

1. Asthmatic
2. Healthy

(C) Bar graph showing the effect of ACh on intracellular calcium ([Ca^{2+}]_i) in different groups:

1. Healthy
2. Asthmatic
3. Calciolytic

(D) Bar graph showing the effect of histamine on intracellular calcium ([Ca^{2+}]_i) in different groups:

1. Healthy
2. Asthmatic
3. Calciolytic

(E) Western blot analysis showing the expression levels of various proteins:

1. pAkt
2. p38^{MAPK}
3. pERK
4. β-actin

(F) Bar graph showing the effect of different calcium concentrations and timolol on protein phosphorylation.

- 0.5 mM Ca^{2+}
- 5 mM Ca^{2+}
- 5 mM Ca^{2+} + calcilytic
Figure 3

(A) WT ○ ○ KO ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○○
Figure 4
Figure 5