EUROPEAN RESPIRATORY *journal*

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

Early View

Original article

Novel airway smooth muscle-mast cell interactions and a role for the TRPV4-ATP axis in non-atopic asthma

Sara J. Bonvini, Mark A. Birrell, Eric Dubuis, John J. Adcock, Michael A. Wortley, Pauline Flajolet, Peter Bradding, Maria G. Belvisi

Please cite this article as: Bonvini SJ, Birrell MA, Dubuis E, *et al*. Novel airway smooth muscle-mast cell interactions and a role for the TRPV4-ATP axis in non-atopic asthma. *Eur Respir J* 2020; in press (https://doi.org/10.1183/13993003.01458-2019).

Novel airway smooth muscle-mast cell interactions and a role for the TRPV4-ATP axis in non-atopic asthma

*Sara J. Bonvini PhD[1], *Mark A. Birrell PhD[1,2], Eric Dubuis PhD[1], John J. Adcock PhD[1,2], Michael A. Wortley PhD[1], Pauline Flajolet PharmD[1], Peter Bradding PhD[3], #Maria G. Belvisi PhD[1,2].

Affiliations:

[1]Respiratory Pharmacology Group, Airway Disease, National Heart & Lung Institute, Imperial College London, Exhibition Road, London SW7 2AZ, UK; [2] Research & Early Development, Respiratory, Inflammation Autoimmunity (RIA) BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden; [3]Department of Infection, Immunity and Inflammation, University of Leicester University, Institute for Lung Health, Glenfield Hospital, Leicester, LE3 9QP, UK.

* Contributed equally;

Correspondence to: Professor Maria G. Belvisi: m.belvisi@imperial.ac.uk

Author Contributions: Conception and design: MAB, MGB, SJB; data analysis and interpretation: MGB, MAB, SJB, ED, JJA, MAW, PB; writing the paper: SJB, MAB, MGB.

Funding: SJB and ED were funded by a Medical Research Council (MRC, UK) MICA award (MR/K020293/1) and Wellcome Trust (207504/B/17/Z). MAW was funded by the North-West Lung Centre Charity. The human vagus experiments in this study were undertaken with the support of the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust. Work in Leicester was supported by the National Institute for Health Research Leicester Biomedical Research Centre - Respiratory.

The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Take Home Message:

Using a technique not previously applied to respiratory research we have uncovered important IgE-independent mechanisms involved in human mast cell-airway smooth muscle interactions which may be responsible for the bronchospasm associated with non-atopic asthma.

Abstract

Rationale: Mast cell-airway smooth muscle (ASM) interactions play a major role in the IgE-dependent bronchoconstriction seen in asthma but less is known about IgE-independent mechanisms of mast cell activation. TRPV4 activation causes contraction of human ASM via the release of cysteinyl leukotrienes (cysLTs) but the mechanism is unknown.

Objective: To investigate a role for IgE-independent, mast cell-ASM interaction in TRPV4-induced bronchospasm.

Methods: Bronchoconstriction was measured in anaesthetised guinea-pigs and contraction of human and guinea-pig airway tissue assessed using isometric tension measurements. Increases in intracellular $[Ca^{2+}]_I$ were imaged using the Ca^{2+}-sensitive dye FURA2, and time-lapse ptychography was utilised as a surrogate for contraction of ASM cells.

Results: The TRPV4 agonist GSK1016790A caused contraction *in vivo* in the guinea-pig, and in human and guinea-pig tracheal tissue, which was inhibited by the TRPV4 antagonist GSK2193874. GSK1016790A increased $[Ca^{2+}]_i$ and released ATP in human ASM cells without causing contraction. TRPV4 and ATP evoked contraction in isolated tracheal tissue but co-culture experiments indicated a requirement for human lung mast cells . Expression profiling and pharmacological studies demonstrated that mast cell activation was dependent upon ATP activating the P2X4 receptor. Trypsin was shown to evoke contraction of tracheal tissue via activation of PAR-2-TRPV4-ATP-cystLT axis indicating the potential disease relevance of this signalling pathway.

Conclusions: TRPV4 activation increases $[Ca^{2+}]_i$ and releases ATP from ASM cells triggering P2X4-dependent release of cysLTs from mast cells resulting in ASM contraction. This study delineates a novel mast cell-ASM interaction and TRPV4 as a driver of IgE-independent mast cell-dependent bronchospasm. (249 words).

INTRODUCTION

Asthma is a chronic airway inflammatory disease characterised by airflow obstruction and symptoms such as chest tightness, wheezing and cough[1-3]. Most asthmatics with early onset disease are atopic and demonstrate an allergic inflammatory response. This is referred to as T2 asthma and is driven by CD4+ T-helper 2 (Th2) lymphocytes, mast cells and possibly innate lymphoid cells (ILC2) which secrete IL-4, IL-5 and IL-13 leading to eosinophilic inflammation and production of IgE from B cells[4,5]. In atopic asthma mast cells are sensitised by the binding of IgE to high affinity IgE receptors (FCεR1). When allergen-specific IgE is cross-linked by the relevant allergen, receptor aggregation triggers mast cell mediator release, with the release of preformed granule-derived mediators, prostanoids, cysteinyl leukotrienes (cysLT) and cytokines thought responsible for the early and late bronchospasm seen following allergen exposure[5]. Although atopic asthma and the T2 inflammatory response are often suppressed in patients treated with inhaled corticosteroids (ICS) subgroups of patients with both atopic and non-atopic asthma have persistent symptoms despite high doses of ICS[6] and so novel approaches to treatment are required.

Mast cells are likely key effectors in the pathogenesis of asthma through their association with the airway smooth muscle (ASM). In asthma, mast cells infiltrate the ASM bundles[7,8], where they interact with ASM cells[9–12]. Human lung mast cells, *in situ* and when in co-culture with HASM, are in a continuously 'activated' state with evidence of ongoing degranulation [8,10,12] and the expression of T2 cytokines[13] which can lead to increased inflammation and bronchoconstriction. However, it is recognised that there are multiple IgE-independent mechanisms of mast cell activation which may also play a role in asthma although less is known about the mechanisms involved. Here we have studied the mast-cell ASM

interactions that are involved in IgE independent, bronchospasm elicited by transient receptor potential cation channel, subfamily V, member 4 (TRPV4) and by disease relevant mediators such as trypsin that can evoke contraction via PAR2 induced gating of TRPV4.

TRPV4 is a Ca^{2+} permeable polymodally gated ion channel[14–16] which is expressed by and causes Ca^{2+} flux in human ASM cells (HASMCs)[17]. Recently, TRPV4 was shown to cause contraction of isolated guinea pig and human tracheal tissue via release of cysLTs[18]. In other cellular systems in the lung, TRPV4 activation evokes ATP release and initiates downstream functional consequences via activation of P2X receptors[19,20]. ATP levels are increased in the bronchoalveolar lavage fluid (BALF) of asthmatics and in murine models of asthma[21]. Further, ATP induces mediator release in rodent mast cells independently of IgE [22,23], and activation of purinoceptors present on rodent mast cells can lead to degranulation, cytokine secretion, chemotaxis and apoptosis[23]. Several P2X receptors are expressed on human lung mast cells (HLMCs)[24], and so we hypothesised that the TRPV4-ATP axis plays a role in human ASM-HLMC crosstalk, evoking mast cell-dependent bronchospasm which may play a key role in asthma pathophysiology.

METHODS

Further details of methods used can be found in the Supplementary Information

Animals

Male Dunkin-Hartley guinea-pigs (300--800g) were purchased from B&K (Hull, U.K.), and housed in temperature-controlled (21°C) rooms with food and water freely available for at least 1 week before commencing experimentation. Experiments were performed in accordance with the U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986 and the ARRIVE guidelines[25].

In vivo measurement of bronchoconstriction

Guinea pigs were anaesthetized with urethane (1.5g/kg) and the trachea cannulated with a short length of Perspex tubing and animals artificially ventilated as previously described[19].

Human tissue

Human airway samples (trachea, major bronchus, secondary bronchi) surplus to transplant requirement were obtained from the International Institute of Advancement of Medicine (IIAM, Edison, New Jersey, USA). We receive human tracheal and lung samples that are unsuitable for transplant, and therefore we often receive donor tissue that is not defined as 'normal'. This is one of the limitations of this study but to avoid erroneous results we have used 13 donors and a range of techniques all of which confirm the hypothesis presented. We have been transparent about the provenance of the tissue by noting the lung diseases and smoking history of the donors in table 1. In all cases consent was granted for use in scientific research and ethical approval was obtained from the Royal Brompton & Harefield Trust

(Ethics Number: REC 09/H0708/72, active from Dec 2009). See Table 1 for the patient demographic details made available to us.

Cell culture

HASMCs were harvested as previously described[26]. Human lung mast cells (HLMCs) from the University of Leicester were purified from macroscopically normal lung resections from 3 patients as described previously[27] with a final purity greater than 99%. The purified HLMCs were cultured in DMEM/HEPES containing Glutamax I and 10% heat inactivated fetal calf serum, 100ng/ml SCF, 50ng/ml interleukin-6 and 10ng/ml interleukin-10 with half the medium replaced every 7 days[28].

***In vitro* measurement of contraction: Organ Bath**

Human tracheal and bronchial tissue (approximately 3-4mm in width) and guinea-pig trachea (approximately 2 cartilage rings in width) was cut longitudinally by cutting through the cartilage directly opposite to the smooth muscle layer and transverse sections cut to produce strips and connected to force transducers as previously described[29].

Calcium imaging in HASMCs

HASMCs harvested from donor tissue were grown in 35mm glass bottom fluorodishes in DMEM supplemented with 10% FCS, and serum starved 24 hours prior to experimental use. On the day of the experiment, DMEM was removed from the cells and replaced with sterile extracellular solution (ECS) and allowed to equilibrate for 30 minutes at 37^{o}C. The cells were then loaded with the intracellular calcium $[Ca^{2+}]_i$ dye Fura-2-AM (12μM, supplemented with

1% PowerLoad + Probenicid (1mM)) for 1h and $[Ca^{2+}]_i$ responses monitored using a Widefield inverted microscope.

Assessment of single HASM cell contraction: Ptychography

A novel imaging technique, ptychography, was utilised as a surrogate for cellular contraction of HASMCs[30]. Ptychography is a label free, high contrast microscopy technique which yields contrast similar to fluorescent imaging with minimal cell manipulation. The dish containing the cells and a low intensity near infrared laser (635nm) are moved over each other to create a sequential array of overlapping illuminated areas, where the light scattered is captured as an array of diffraction pattern on a detector. This diffraction pattern array is then processed using Virtual Lens© algorithm to calculate a quantitative measure of the light absorbed, the scattering and the phase delay introduced into the illumination as it passes through the specimen, which enables analysis of the 3D characteristics of the sample[30]. This technique has yielded contractile responses in HASMCs (for example in response to ACh) matching $[Ca^{2+}]_i$ elevation recorded in the same cells and similar to contractile responses found using whole tissue[31].

Data Analysis

Data was expressed as mean ± SEM of n observations. Statistical significance was determined using students t-test or one-way ANOVA with an appropriate post hoc test. Statistical significance was set at $P<0.05$ and all treatments were compared to the appropriate vehicle control and n numbers refer to the number of different donor tissues/cells used

RESULTS

Effect of TRPV4 on contraction in vivo

Aerosolised GSK1016790A 100ng/ml (153nM for 15s) caused a significant and sustained increase in tracheal pressure in anaesthetised guinea pigs (Figure 1A-C). In all cases, both vagal nerves were cut, therefore this effect was due to a direct effect on the smooth muscle rather than a parasympathetic reflex. Increases in tracheal pressure induced by a submaximal concentration 100ng/ml (153nM for 15s) of the TRPV4 agonist GSK1016790A were significantly inhibited 1 hour following i.p. administration of the TRPV4 antagonist GSK2193874 (300mg/kg; 6% cyclodextrin in saline) (Figure 1C) but remained unaffected following vehicle (Figure 1B).

Effect of TRPV4 agonists on isolated airway tissue in vitro

GSK1016790A caused a concentration-dependent contraction in isolated guinea pig trachea. Due to the long-lasting contraction induced by the agonist, only one concentration was tested on each piece of tissue (Figure 2A). Contraction was significantly inhibited following 30 minutes preincubation with GSK2193874 (10µM) (Figure 2B). A similar effect was seen in human tissue (Figure 2C, D). A recent publication indicated that TRPV4-induced contraction of ASM is dependent upon the release of cysLTs[18]. Consistent with this observation, GSK1016790A (100nM)-induced contraction was inhibited following preincubation with a cystLT1 antagonist (montelukast 10µM) and a 5-LO inhibitor (zileuton 10µM) in human tissue, confirming that cysLTs are responsible for the contraction induced by the TRPV4 agonist in human ASM (Figure 2E,F). Monteleukast (10µM) and zileuton (10µM) alone had no effect on airway tone *per se*.

The role of mast cells in TRPV4 induced contraction

The TRPV4 agonist GSK1016790A caused a concentration-dependent increase in intracellular Ca^{2+} in HASMCs as assessed by fluorescent FURA2 imaging (Figure 3A,B). To assess the effect of GSK1016790A on contraction of primary HASMCs we used a novel imaging technique known as ptychography. Using this technique, GSK1016790A incubated with HASMCs alone did not cause contraction, however the positive control ACh (10µM) did, as shown by an increase in optical density (Figure 3C, D, E). Mast cells are a major source of cysLTs and in co-cultures of HASMCs with primary HLMCs, GSK1016790A caused contraction of the HASMCs as demonstrated using ptychography. However, mast cell-free media had no effect, indicating the requirement for HLMCs for TRPV4 mediated contraction of HASMCs (Figure 3F and G; a video showing a HASM cell contracting in the presence of HLMCs and GSK1016790A can be seen here:

HASM-MastCells video.mp4

Role of ATP in TRPV4 induced contraction

The data suggested that TRPV4 agonists activate the TRPV4 ion channel present on HASMCs to increase $[Ca^{2+}]_i$ causing the release of an unknown mediator that would cause the release of cysLTs from mast cells. HASM cells, but not HLMCs, expressed TRPV4 at the mRNA level (Figure 4A), suggesting that activation of TRPV4 does not have a direct effect on mast cells. Previous work has shown that activation of TRPV4 on macrophages and airway sensory nerves induces the release of ATP[19,20], suggesting that ATP may be the previously unknown mediator. GSK1016790A caused ATP release from cultured human ASM cells, which was inhibited following administration of GSK2193874 (10µM) (Figure 4B). ATP (1mM) was

shown to cause contraction of human isolated smooth muscle in the organ bath, which was inhibited by both montelukast and zileuton, indicating a similar pharmacological profile to GSK1016790A (Figure 4C). Furthermore, ATP caused the release of cysteinyl leukotrienes from human donor lung mast cells but did not contract HASM cells that were cultured alone and in the absence of HLMCs. (Supplementary Figure E1 A, B).

Mechanism of TRPV4 induced contraction of Human ASM

ATP activates both ionotropic P2X purinoceptors and metabotropic P2Y receptors. Mast cells have been shown to express a number of purinoceptor ion channels[24,32] and P2X1, P2X4, P2X5, P2X6 and P2X7 were shown to be expressed at the mRNA level on primary HLMCs (Figure 5A). Using ptychography, contraction of the human ASM cells induced by GSK1016790A (100nM) in the presence of HLMCs was only significantly inhibited following incubation with the P2X4 antagonist 5BDBD (50 μM) but not following incubation with a P2X1 (Ip5I 10 μM) or a P2X7 antagonist (AZ11645373 10μM) (Figure 5B). This result translated in human airway tissue, where contraction induced by both GSK1016790A (100nM; Figure 5C, D) and ATP (1mM; Figure 5E, F) was inhibited following incubation with 5BDBD (50 μM).

In the search for an endogenous TRPV4 ligand, we speculated that PAR2 activation may play a role. The GPCR PAR2 has been shown to be functionally coupled to TRPV4 and cause activation of the ion channel[33,34]. Using RTPCR, similarly to TRPV4, PAR2 was shown to be consistently expressed on HASMs, but not HLMCs, where it was only expressed in 1/3 patients (Supplementary Figure E2, A). We then utilised the endogenous PAR2 ligand trypsin (1000U/ml), which caused contraction of isolated human tracheal strips (Figure 5G) as

previously described[35]. This contraction was inhibited by the same antagonists that blocked TRPV4 induced contraction, including GSK2193874, montelukast and 5BDBD, along with the serine protease inhibitor camostat mesylate (Figure 5G). These data indicate that asthma 'disease-relevant' mediators such as mast-cell proteases (eg. PAR2 activators such as tryptase) can activate this pathway. This mechanism was shown to be TRPV4 contraction-specific as neither GSK2193874 (10μM), montelukast (10μM) nor 5BDBD (50μM) had any effect on contraction induced by the common contractile stimuli histamine and ACh (Supplementary Figure E2 B,C).

In this series of experiments, we have demonstrated that activation of TRPV4 on human ASM induces the release of ATP which activates P2X4 receptors on HLMCs in close proximity to the ASM, in turn inducing the release of cysLTs which causes cystLT1-dependent contraction of ASM. This mechanism is outlined as a schematic in Figure 6.

DISCUSSION

HLMC-ASM interactions are thought to play a fundamental role in the pathogenesis of atopic asthma due to the IgE-dependent release of bronchoconstrictor mediators and cytokines which contribute to the early and late phase responses following allergen exposure. However, it is now recognised that there are many IgE-independent mechanisms of mast cell activation which may play a role in both atopic and non-atopic asthma and across other chronic lung diseases[5]. Here we investigate the role that ASM-mast cell interactions and non IgE-dependent mechanisms play in contractile responses to 'disease-relevant' asthma mediators.

Mast cell proteases are believed to play an important role in the development of asthma and the continuing symptoms that are seen with persistent disease[5]. Many of the proteases associated with allergens or released from mast cells are serine proteases (trypsin-like, chymotrypsin-like or neutrophil elastase-like) that can activate the G protein-coupled receptor (GPCR), proteinase-activated receptor-2 (PAR2). The effects of PAR2 in both allergic and also nonallergic asthma have been reported to be complex, with Nichols *et al* demonstrating that PAR2 can act via several independent signalling pathways; proinflammatory effects are thought to be mediated through β-arrestin and protective effects are mediated through GPCR activation and increases in intracellular calcium[35] However, it is not clear how PAR2 activation leads to functional consequences such as bronchospasm[36]. Signals that originate from the GPCR superfamily activation, including PAR2, have been shown to converge on certain transient receptor potential (TRP) family members, including TRPV4, leading to channel activation and sensitization which amplify pain, itch and neurogenic inflammation[33,37]. TRPV4 activation contributes to a variety of effects within the lung, including sensory nerve activation, intracellular Ca^{2+} flux in ASM[17–19] and cysLT_1-dependent contraction of both guinea pig and human airway tissue[18].

Here we show for the first time the ability of the TRPV4 agonist GSK1016790A to cause slow onset, sustained ASM contraction *in vivo* in the guinea pig which was abolished in the presence of a TRPV4 antagonist. *In vitro* this contraction was also slow in onset and long lasting and was inhibited by the TRPV4 inhibitor GSK2193874. As previously shown, we confirmed that this contraction was inhibited by the cysLT1 receptor antagonist montelukast, and the 5-LO inhibitor, zileuton, in both guinea pig and human tissue[18]. Since

cysLTs are known to be synthesised by activated mast cells[18,38], it was hypothesised that mast cell-ASM interactions were central to this process.

To investigate this interaction further, we initially investigated whether TRPV4 activation could evoke increases in $[Ca^{2+}]i$ in isolated HASMCs as this assessment is often used as a surrogate measure of contraction[39]. The TRPV4 agonist GSK1016790A induced an increase in $[Ca^{2+}]i$ in HASMCs as previously shown[17,18] but did not cause contraction of ASM cells in culture as assessed by a novel technique; ptychography[31], which utilises changes in optical density as a surrogate for changes in contractile status. An increase in calcium is often suggestive of contraction of ASM cells, however other stimuli have been shown to have a strong increase in intracellular calcium in ASM but do not initiate contraction, including bitter taste receptor (TASR) agonists[40], which instead induce relaxation. Mast cells are known to be resident within ASM bundles in both healthy, non-atopic patients[41] and those with asthma where they play a key role in contraction through the release of various mediators including cysLTs[7]. Therefore, HASMCs were co-cultured with primary HLMCs to assess the mast cell-dependent nature of the TRPV4-induced contraction. Using ptychography we demonstrated that GSK1016790A was only able to increase optical density indicative of contraction when HASMCs were in the presence of HLMCs. This was unlikely a direct effect on HLMCs, as RT-PCR indicated that TRPV4 mRNA was expressed on HASMCs but not on HLMCs.

Since TRPV4 was expressed on HASMCs, but not on HLMCs, and because of the delayed contractile response we hypothesised the involvement of a secondary messenger. TRPV4 induces ATP release in several different cellular systems which can act on a number of

purinoceptors that have a variety of downstream effects in the lung[19,20]. Similarly, we found that the addition of the TRPV4 agonist to isolated HASMCs induced ATP release. ATP is a ubiquitous molecule found in every cell of the body, and directly activates animal mast cells [21–23.] We demonstrated that, consistent with previous studies, a number of purinoceptors were expressed on the cell surface of HLMCs[24]. In addition, we have demonstrated that contraction of airway tissue in response to ATP can be abolished in the presence of a P2X4 inhibitor, indicating that it is likely to be the P2X4 receptor present on HLMCs which is activated by ATP, and induces the release of cysLTs. P2X4 is the most widely expressed of the purinergic P2X receptors[42] and previous work has linked P2X4 with asthma, as the P2X4 inhibitor 5BDBD attenuated the response to antigen in an OVA "asthma" model in BALBc mice[43,44]. P2X4 is a Ca^{2+} permeable ion channel, and Ca^{2+} signalling plays a major role in regulating secretion from HLMCs[45]. This mechanism has also been linked with P2X7 activation which can cause degranulation of the LAD2 human mast cell line[32]. Elevated levels of ATP and its breakdown products have been found in the BAL fluid of asthmatic patients[21] and ATP can cause bronchoconstriction and dyspnea[46], indicating that this may be a relevant pathway for mast cell activation and cysLT release in the asthmatic lung.

Identification of the PAR2-TRPV4-ATP axis in evoking bronchospasm illustrates the complex role that mast cells may have in driving the pathophysiology of structural cells and symptoms in both atopic and non-atopic asthma. These and similar mechanisms may be important in the generation of asthma symptoms in response to various stimuli including for example exercise induced bronchospasm (EIB). CysLTs are thought to be key mediators of EIB; they are released from activated mast cells, and asthmatics which exhibit EIB have been shown to have increased levels of cystLTs in both sputum and exhaled breath condensate[47].

Furthermore, a single oral dose of montelukast, which we have shown to inhibit TRPV4 mediated contraction, has been shown to exert significant protection against EIB at 2, 12 and 24 hours post exercise[48]. The symptoms of EIB are thought to be evoked by the osmotic and thermal effects of increased respiration[47] which are known to activate TRPV4[49]. In addition, tryptase has also been shown to be elevated in asthmatic patients with EIB[50] providing further evidence of a role for this axis in exercise-induced bronchospasm.

Mast cells appear to be key effector cells in the pathogenesis of asthma through their close association with the ASM. However, the functional consequences of this close association are not fully understood. In this study we have implicated the PAR2-TRPV4-ATP-cysLT axis in mast cell-dependent bronchospasm. TRPV4 activation is linked to the GPCR, protein activated receptor 2 (PAR2), where activation of PAR2 has been shown to both sensitise[37] and also directly gate TRPV4[33,51]. In support of this hypothesis we have shown that trypsin can cause contraction of human tracheal strips via activation of PAR2-TRPV4-ATP-cysLT axis, as the contraction was inhibited by the serine protease inhibitor camostat mesylate[52], the TRPV4 antagonist GSK2193874, the P2X4 inhibitor 5BDBD and also montelukast.

In conclusion, we have described a novel mast cell-ASM interaction involving the ion channel TRPV4. We suggest that TRPV4 is activated on ASM by endogenous ligands such as arachidonic acid derivatives or proteases, leading to the release of ATP, potentially through the pannexin ion pore, which activates P2X4. This mechanism may play an important role in IgE-independent mast cell mediator release in asthma, for example during EIB, and in non-atopic asthma where common aeroallergens do not appear to contribute but levels of endogenous indirect activators of TRPV4 such as tryptase are elevated[50] and also where

cysLt1 receptor antagonists such as montelukast have been shown to be effective[53]. Furthermore, this mechanism may also be relevant in other lung diseases where increased lung tissue mast cells have been identified and linked with disease pathophysiology[54].

COMPETING INTERESTS

MGB and MAB are employed by AstraZeneca and are non-executive directors of an Imperial College spinout contract research company engaged in respiratory pre-clinical work. MGB has been a consultant for Ario Pharma, Aboca, Patara, NeRRe, MedImmune, Boehringer Ingelheim.

ACKNOWLEDGEMENTS

We would like to acknowledge Mr. James Bolaji, Ms. Xue Chen, and Dr Nadja Kobold (Imperial College) for assistance with some of the organ bath, RTPCR and ptychography experiments.

REFERENCES

1. Lemanske RF, Busse WW. 6. Asthma. J Allergy Clin Immunol. 2003; 111: S502–19.

2. Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, et al. Global strategy for asthma management and prevention: GINA executive summary. Eur Respir J. 2008; 31: 143–78.

3. Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, et al. Global strategy for asthma management and prevention: GINA executive summary. Eur Respir J Off J Eur Soc Clin Respir Physiol. 2008; 31: 143–78.

4. Barnes PJ. Cellular and molecular mechanisms of asthma and COPD. Clin Sci. 2017; 131:1541–58.

5. Bradding P, Arthur G. Mast cells in asthma -- state of the art. Clin Exp Allergy. 2016; 46; 194-263

6. Samitas K, Zervas E, Gaga M. T2-low asthma: current approach to diagnosis and therapy. Curr Opin Pulm Med. 2017;23: 48–55.

7. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. N Engl J Med. 2002; 346: 1699–705.

8. Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. Thorax. 2007; 62: 8–15.

9. Yang W, Wardlaw AJ, Bradding P. Attenuation of human lung mast cell degranulation by bronchial epithelium. Allergy. 2006; 61: 569–75.

10. Hollins F, Kaur D, Yang W, Cruse G, Saunders R, Sutcliffe A, et al. Human airway smooth muscle promotes human lung mast cell survival, proliferation, and constitutive activation: cooperative roles for CADM1, stem cell factor, and IL-6. J Immunol. 2008; 181: 2772–80.

11. Woodman L, Siddiqui S, Cruse G, Sutcliffe A, Saunders R, Kaur D, et al. Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1. J Immunol. 2008; 181: 5001–7.

12. Lewis RJ, Chachi L, Newby C, Amrani Y, Bradding P. Bidirectional Counterregulation of Human Lung Mast Cell and Airway Smooth Muscle β2 Adrenoceptors. J Immunol. 2016; 196: 55–63.

13. Brightling CE, Symon FA, Holgate ST, WARDLAW aj, Pavord ID, Bradding P. Interleukin-4 and -13 expression in co-localized to mast cells within the airway smooth muscle in asthma. Clin Exp Allergy 2003; 33: 1711-6.

14. Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. Nat Cell Biol. 2000; 2: 695–702.

15. Liedtke W, Friedman JM. Abnormal osmotic regulation in trpv4-/- mice. Proc Natl Acad Sci USA. 2003; 100: 13698–703.

16. Mizuno A, Matsumoto N, Imai M, Suzuki M. Impaired osmotic sensation in mice lacking TRPV4. Am J Physiol Cell Physiol. 2003; 285: C96-101. Available from:

17. Jia Y, Wang X, Varty L, Rizzo CA, Yang R, Correll CC, et al. Functional TRPV4 channels are expressed in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol [Internet]. 2004; 287: L272-8.

18. McAlexander MA, Luttmann MA, Hunsberger GE, Undem BJ. Transient receptor potential vanilloid 4 activation constricts the human bronchus via the release of cysteinyl leukotrienes. J Pharmacol Exp Ther. 2014; 349: 118–25.

19. Bonvini SJ, Birrell MA, Grace MS, Maher SA, Adcock JJ, Wortley MA, et al. Transient receptor potential cation channel, subfamily V, member 4 and airway sensory afferent activation: Role of adenosine triphosphate. J Allergy Clin Immunol. 2016; 138: 249–261.e12.

20. Baxter M, Eltom S, Dekkak B, Yew-Booth L, Dubuis ED, Maher SA, et al. Role of transient receptor potential and pannexin channels in cigarette smoke-triggered ATP release in the lung. Thorax. 2014; 69: 1080–9.

21. Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MAM, Muskens F, et al. Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. Nat Med. 2007; 13: 913–9.

22. Burnstock G, Boeynaems J-M. Purinergic signalling and immune cells. Purinergic Signal. 2014; 10: 529–64.

23. Bulanova E, Bulfone-Paus S. P2 receptor-mediated signaling in mast cell biology. Purinergic Signal. 2010; 6: 3–17.

24. Wareham K, Vial C, Wykes RCE, Bradding P, Seward EP. Functional evidence for the expression of P2X1, P2X4 and P2X7 receptors in human lung mast cells. Br J Pharmacol. 2009; 157: 1215–24.

25. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PloS Biol. 2010; 8: e1000412.

26. Clarke DL, Belvisi MG, Catley MC, Yacoub MH, Newton R, Giembycz MA. Identification in human airways smooth muscle cells of the prostanoid receptor and signalling pathway through which PGE2 inhibits the release of GM-CSF. Br J Pharmacol. 2004; 141: 1141–50.

27. Sanmugalingam D, Wardlaw AJ, Bradding P. Adhesion of human lung mast cells to bronchial epithelium: evidence for a novel carbohydrate-mediated mechanism. J Leukoc Biol. 2000; 68: 38–46.

28. Duffy SM, Lawley WJ, Kaur D, Yang W, Bradding P. Inhibition of human mast cell proliferation and survival by tamoxifen in association with ion channel modulation. J Allergy Clin Immunol. 2003; 112: 965–72.

29. Buckley J, Birrell MA, Maher SA, Nials AT, Clarke DL, Belvisi MG. EP4 receptor as a new target for bronchodilator therapy. Thorax. 2011; 66: 1029–35.

30. Marrison J, Räty L, Marriott P, O'Toole P. Ptychography--a label free, high-contrast imaging technique for live cells using quantitative phase information. Sci Rep. 2013; 3: 2369.

31. Dubuis ED, Belvisi MG, Poushpas S, Flajolet P, Langley K, Birrell MA. A Novel Method For Measuring Contraction Of Primary Human Airway Smooth Muscle Cells. In: Am J Respir Crit Care Med. 2015. p. A1224.

32. Wareham KJ, Seward EP. P2X7 receptors induce degranulation in human mast cells. Purinergic Signal. 2016; 12: 235–46.

33. Poole DP, Amadesi S, Veldhuis NA, Abogadie FC, Lieu T, Darby W, et al. Protease-activated receptor 2 (PAR2) protein and transient receptor potential vanilloid 4 (TRPV4) protein coupling is required for sustained inflammatory signaling. J Biol Chem. 2013; 288: 5790–802.

34. Grace MS, Lieu T, Darby B, Abogadie FC, Veldhuis N, Bunnett NW, et al. The tyrosine kinase inhibitor bafetinib inhibits PAR2-induced activation of TRPV4 channels in vitro and pain in vivo. Br J Pharmacol. 2014; 171: 3881–94.

35. Nichols HL, Saffeddine M, Theriot BS, Hegde A, Polley D, El Mays T, Vliagoftis H, Hollenburg MD, Wilson EH, Walker JK, Defea KA. β-Arrestin-2 mediates the proinflammatory effects of proteinase-activated-receptor-2 in the airway. Proc Natl Acad Sci USA. 2012; 16660-5

36. Schmidlin F, Amadesi S, Vidil R, Trevisani M, Martinet N, Caughey G, et al. Expression and function of proteinase-activated receptor 2 in human bronchial smooth muscle. Am J Respir Crit Care Med. 2001; 164: 1276–81.

37. Grant AD, Cottrell GS, Amadesi S, Trevisani M, Nicoletti P, Materazzi S, et al. Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. J Physiol. 2007; 578: 715–33.

38. Gauvreau GM, El-Gammal AI, O'Byrne PM. Allergen-induced airway responses. Eur Respir J. 2015; 46: 819–31.

39. Bergner A, Sanderson MJ. Acetylcholine-induced calcium signalling and contraction of airway smooth muscle cells in lung slices. J Gen Physiol. 2002; 119: 187–98.

40: Robinett KS, Koziol-White CJ, Akoluk A, An SS, Panettieri RA Jr, Liggett SB. Bitter taste receptor function in asthmatic and nonasthmatic human airway smooth muscle cells. Am J Respir Cell Mol Biol. 2014: 678-83

41: Andersson CK, Mori M, Bjermer L, Lofdahl CG, Erjefalt JS. Novel site specific mast cell subpopulations in the human lung. Thorax. 2009: 297-305

42. Tanaka J, Murate M, Wang CZ, Seino S, Iwanaga T. Cellular distribution of the P2X4 ATP receptor mRNA in the brain and non-neuronal organs of rats. Arch Histol Cytol. 1996; 59: 485–90.

43. Chen H, Xia Q, Feng X, Cao F, Yu H, Song Y, et al. Effect of P2X4R on airway inflammation and airway remodeling in allergic airway challenge in mice. Mol Med Rep. 2016; 13: 697–704.

44. Zech A, Wiesler B, Ayata CK, Schlaich T, Dürk T, Hoßfeld M, et al. P2rx4 deficiency in mice alleviates allergen-induced airway inflammation. Oncotarget. 2016; 7: 80288–97.

45. Vig M, Kinet J-P. Calcium signaling in immune cells. Nat Immunol. 2009; 10: 21–7.

46. Basoglu OK, Pelleg A, Essilfie-Quaye S, Brindicci C, Barnes PJ, Kharitonov SA. Effects of aerosolized adenosine 5'-triphosphate vs adenosine 5'-monophosphate on dyspnea

and airway caliber in healthy nonsmokers and patients with asthma. Chest. 2005; 128: 1905–9.

47. Hallstrand TS, Henderson WR. Role of leukotrienes in exercise-induced bronchoconstriction. Curr Allergy Asthma Rep. 2009;9:18–25.

48. Pearlman DS, van Adelsberg J, Philip G, Tilles SA, Busse W, Hendeles L, et al. Onset and duration of protection against exercise-induced bronchoconstriction by a single oral dose of montelukast. Ann Allergy Asthma Immunol. 2006; 97: 98–104.

49. Vriens J, Watanabe H, Janssens a, Droogmans G, Voets T, Nilius B. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. Proc Natl Acad Sci. 2004; 101: 396–401.

50. Lai Y, Altemeier WA, Vandree J, Piliponsky AM, Johnson B, Appel CL, et al. Increased density of intraepithelial mast cells in patients with exercise-induced bronchoconstriction regulated through epithelially derived thymic stromal lymphopoietin and IL-33. J Allergy Clin Immunol. 2014; 133: 1448–55.

51. Grace MS, Lieu T, Darby B, Abogadie FC, Veldhuis N, Bunnett NW, et al. The Tyrosine Kinase Inhibitor Bafetinib Inhibits PAR2 -induced Activation of TRPV4 In Vitro and Pain In Vivo. Br J Pharmacol. 2014; 61.

52. Gibo J, Ito T, Kawabe K, Hisano T, Inoue M, Fujimori N, et al. Camostat mesilate attenuates pancreatic fibrosis via inhibition of monocytes and pancreatic stellate cells activity. Lab Investig. 2005; 85: 75–89.

53. Leff JA, Busse WW, Pearlman D, Bronsky EA, Kemp J, Hendeles L, et al. Montelukast, a leukotriene-receptor antagonist, for the treatment of mild asthma and exercise-induced bronchoconstriction. N Engl J Med. 1998; 339: 147–52.

54. Bradding P, Pieler G. The controversial role of mast cells in fibrosis. Immunological Review. 2018; 282: 198-231

FIGURE LEGENDS

Figure 1. (**A**): Example trace of a GSK1016790A 100ng/ml (153 nM for 15s) mediated response. Contraction was unchanged following vehicle (6% cyclodextrin in saline) administration (**B**) but significantly inhibited following administration of GSK2193874 (300mg/kg, i.p.) (**C**). Mean ± SEM of n=3. * indicates statistical significance ($p<0.05$), paired t test comparing responses before and after antagonist/vehicle administration in the same animal.

Figure 2: **(A)**: GSK1016790A caused contraction in isolated guinea pig trachea, which was inhibited by GSK2193874 (10µM) (n=4-6) (**B**). (**C**): Response to GSK1016790A (100nM) plus vehicle (top panel) or GSK2193874 (10µM) (bottom panel) in human tissue. (**D**) GSK2193874 (10µM) inhibited the response in human tissue (n=3). (**F**): Preincubation with either montelukast (10µM) (example trace **E**, middle panel), or zileuton (10µM) (example trace **E**, bottom panel) significantly inhibited GSK1016790A induced contraction of human tissue (n=3). Mean ± SEM * indicates statistical significance ($p<0.05$) using a one way ANOVA with Dunnetts Multiple comparison test comparing responses to vehicle (2A, F) or a t-test comparing responses with antagonist to the vehicle control (2B, D).

Figure 3: (**A, B**): Example image, trace and graph of the GSK1016790A-induced increase in calcium in HASMcs. Example ptychography reconstituted images (top panel) and traces (bottom panel) from (**C**) GSK1016790A (100nM) treated and **(D)** ACh (10 µM) treated HASMCs (n=2 donors). (**E**): Ptychography data indicating no contractile effect to GSK1016790a in human HASMCs alone. ACh was used as a positive control (n=2 donors). (**F**): Reconstituted ptychographic images; Top two panels show HASMCs alone. Bottom two

panels show HASM co-cultured with mast cells, where the addition of GSK1016790A (100nM) led to an increase in optical density. (**G**): In the presence of mast cells GSK1016790A (100nM) caused contraction of HASMCs (n=2 donors for HASMCs, n=3 donors for HLMCs). Data shown as Mean ± SEM (n=8-56 readings;). Size bar indicates 100μm. * indicates statistical significance ($p<0.05$) using a Kruskall Wallis test with Dunns post comparison test comparing responses to vehicle (3B) or a t test comparing responses to vehicle control (3G).

Figure 4: (**A**): TRPV4 mRNA is expressed on HASMCs, but not on HLMCs. **(B):** GSK1016790A (100nM) induced ATP release from HASMCs which was inhibited by GSK2193874 (10μM). (**C**) ATP contraction of isolated human tracheal strips was inhibited by montelukast and zileuton (10μM). Data shown as Mean ± SEM HASMCs were isolated from 4 patients, tracheal strips from 3 donors lungs. * indicates statistical significance ($p<0.05$); t-test comparing responses to relevant control (4B) or using a one-way ANOVA with Dunnetts Multiple comparison test comparing responses to vehicle (4C).

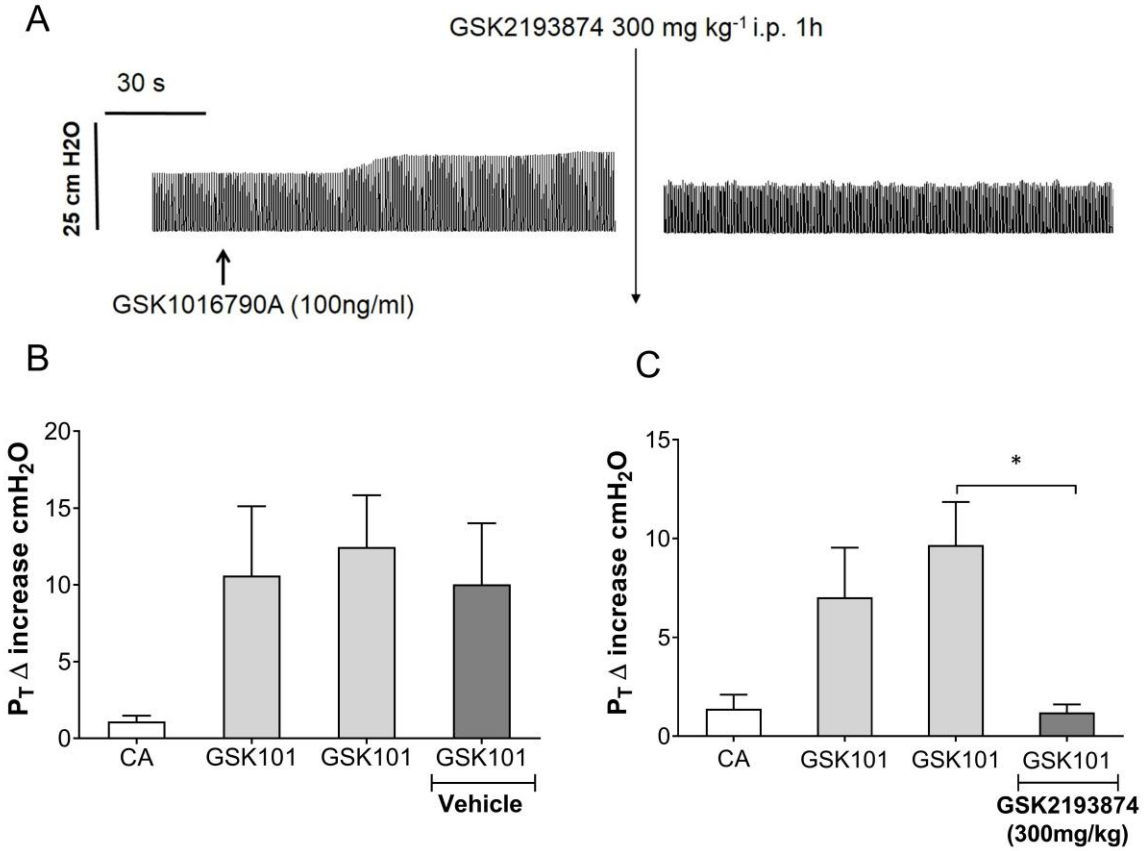
Figure 5: (**A**) Expression of P2X receptor mRNA on HLMCs (n=3 donors). (**B**) Effect of P2X receptor antagonist -P2X1 (Ip51, 10μM), P2X4 (5BDBD, 50μM) and P2X7 (AZ11645373, 10 μM) on contraction of HASMCs treated with GSK101679A (100nM). Effect of the P2X4 inhibitor (5BDBD, 50μM) on GSK101679A (100nM) (**C,D**) and ATP (1mM) (**E,F**) -induced contraction of human tissue (n=3). (**G**): Trypsin induced contraction of human tracheal strips inhibited by camostat mesylate, GSK2193874, montelukast and 5BDBD (n=2-3). Mean ± SEM. * indicates statistical significance using a t-test ($p<0.05$) compared to relevant control

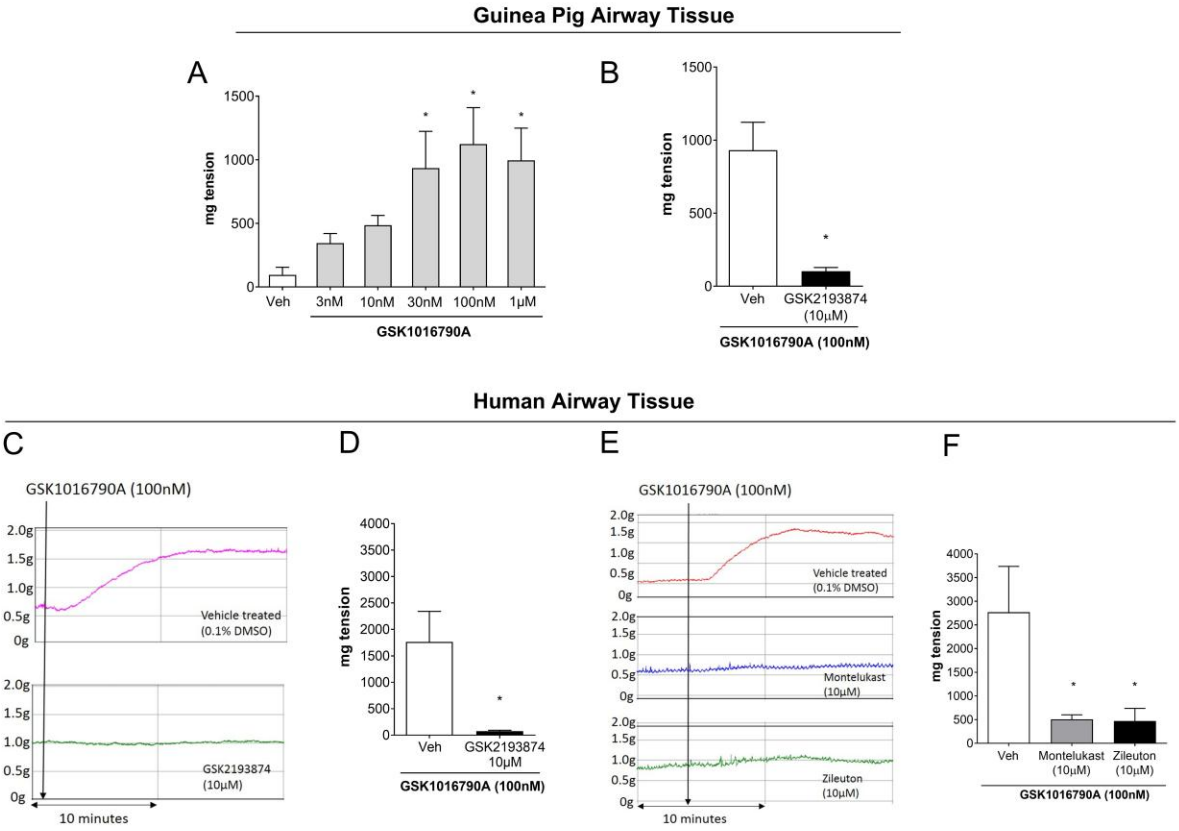
(Figure 5B) or using a one way ANOVA with Dunnetts post test ($p<0.05$) comparing antagonist to vehicle control (Figure 5G).

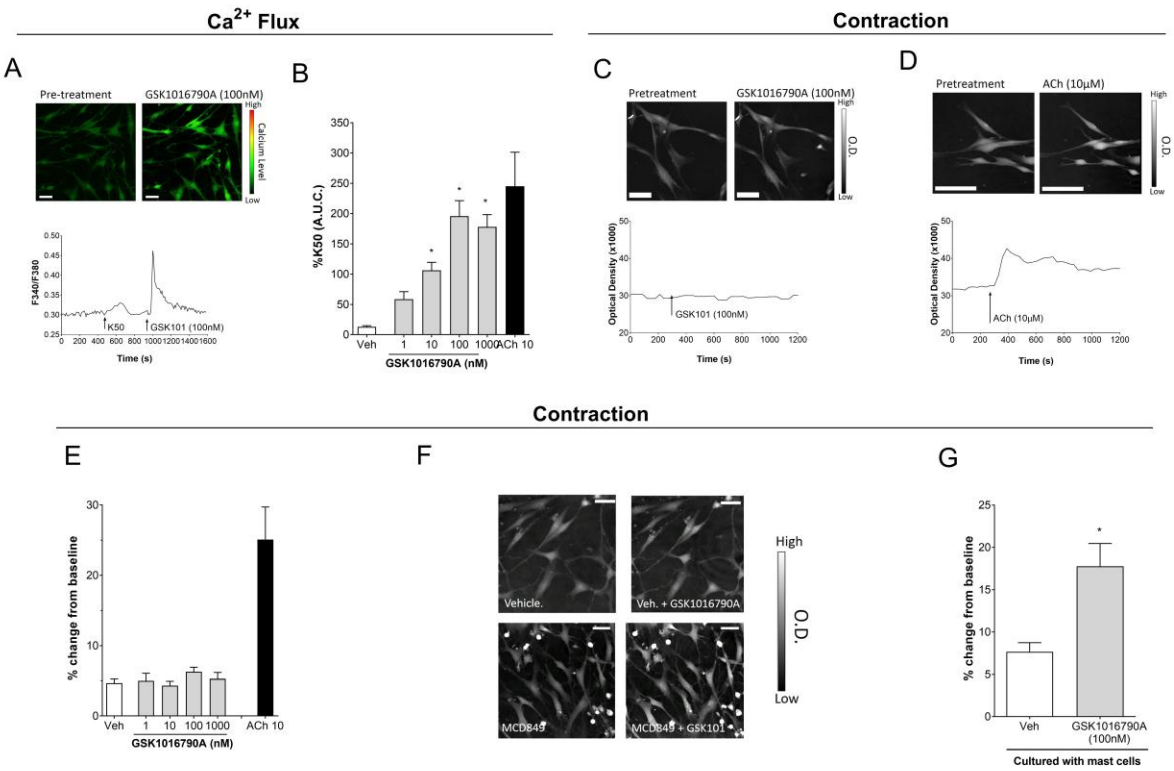
Figure 6: Schematic diagram which illustrates the mast cell dependent contraction of human ASM evoked by TRPV4. In this series of experiments, we have demonstrated that activation of TRPV4 on human ASM induces the release of ATP which activates P2X4 receptors on mast cells, near and within the ASM bundles, to induce the release of cysteinyl leukotrienes which causes cystLT1-dependent contraction of ASM.

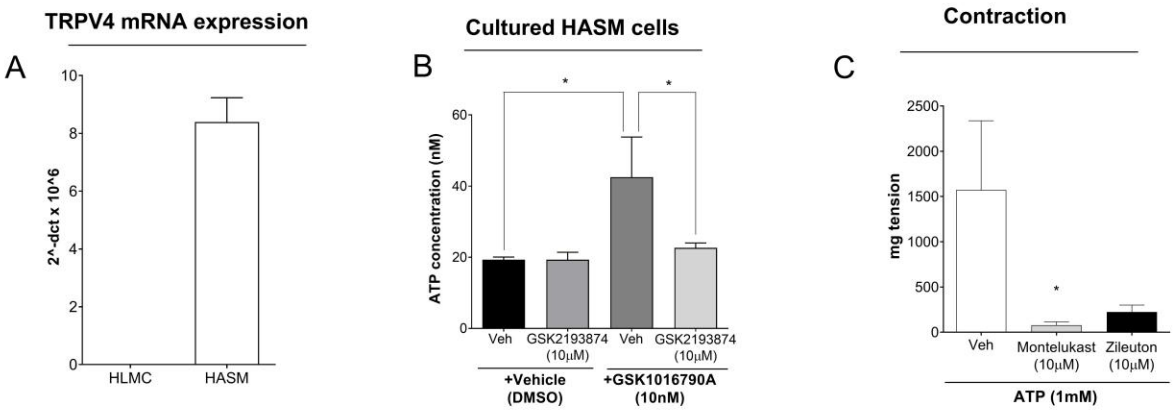
Table 1: Human tissue demographics for the donor lungs from which the tracheal and bronchi strips were sourced.

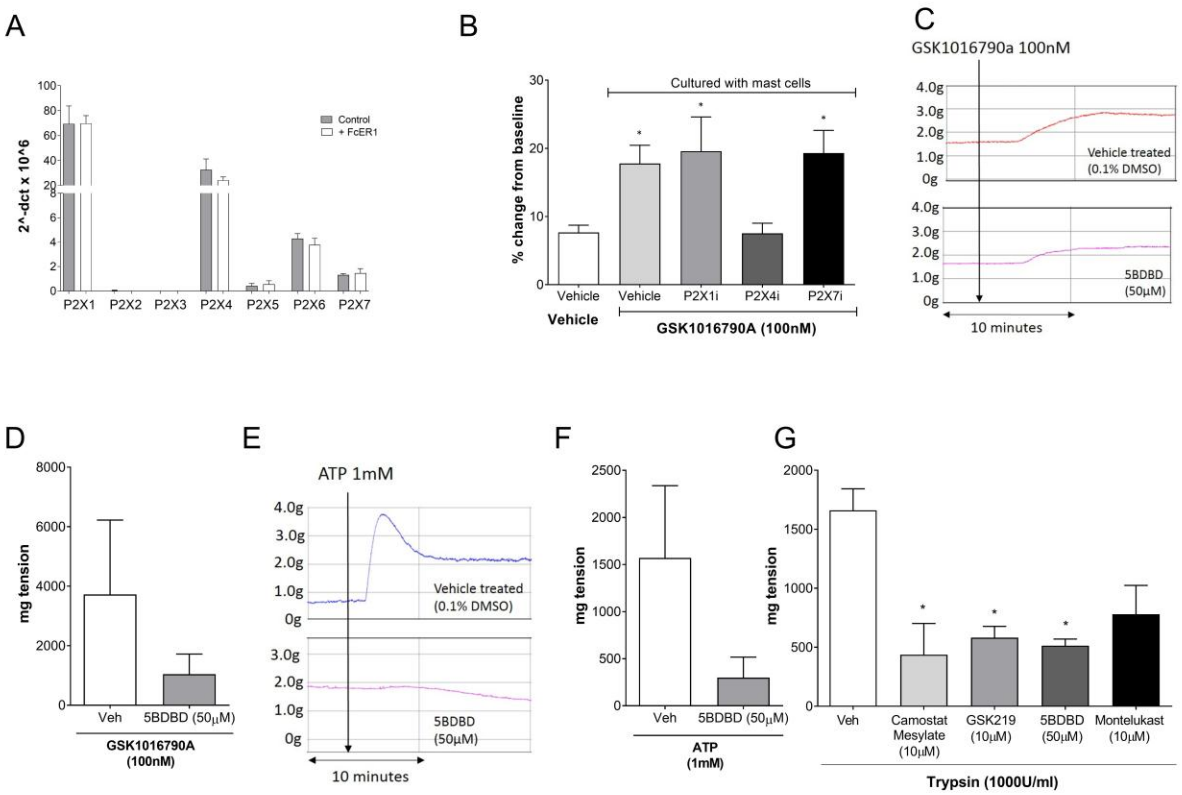
	Sex	**Age**	**Respiratory conditions**
1.	Female	31	Mild Asthma
2.	Male	48	Ex Smoker
3.	Female	68	N/A
4.	Female	73	N/A
5.	Female	70	Smoker
6.	Male	56	N/A
7.	Female	75	Asthma
8.	Female	59	Bronchitis
9.	Male	43	Smoker
10.	Female	38	Asthma, bronchitis, smoker
11.	Female	39	Asthma
12.	Male	57	Smoker
13	Female	73	Ex Smoker

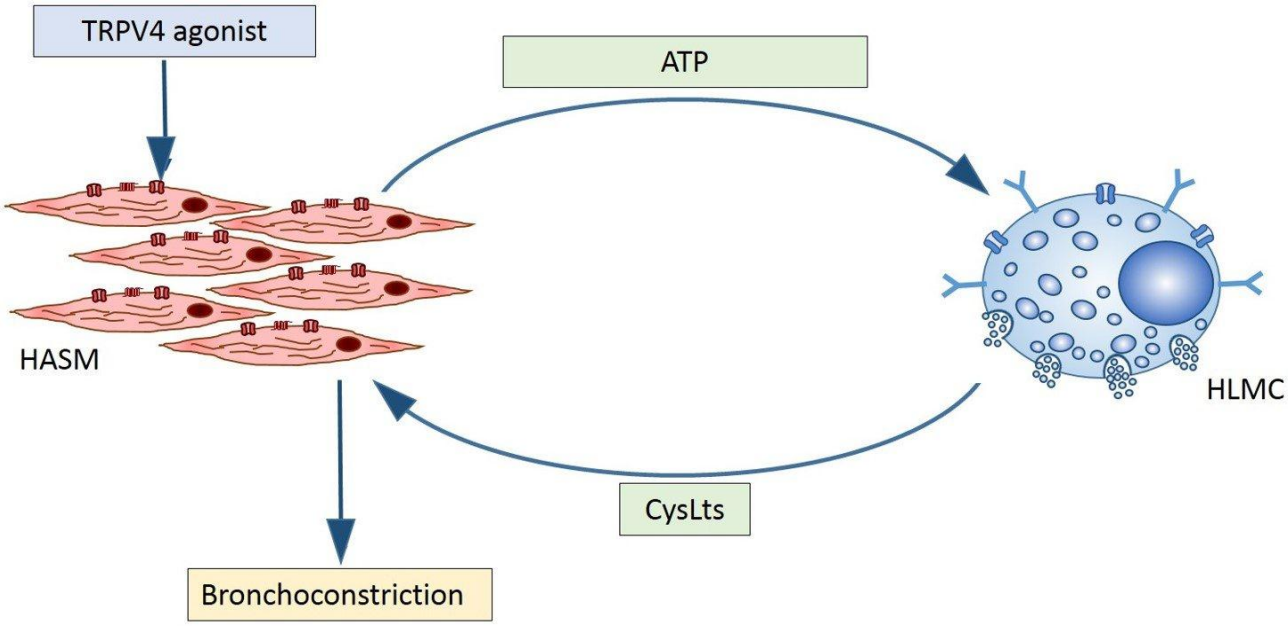

A
GSK2193874 300 mg kg-1 i.p. 1h
30 s
25 cm H2O
GSK1016790A (100ng/ml)
B
PT Δ increase cmH2O
20
15
10
5
0
CA
GSK101
GSK101
GSK101
Vehicle
C
PT Δ increase cmH2O
15
10
5
0
*
CA
GSK101
GSK101
GSK101
GSK2193874
(300mg/kg)


Guinea Pig Airway Tissue
A
mg tension
1500
1000
500
0
Veh
3nM
10nM
30nM
100nM
1μM
GSK1016790A
B
mg tension
1500
1000
500
0
Veh
GSK2193874
(10μM)
GSK1016790A (100nM)
Human Airway Tissue
C
GSK1016790A (100nM)
2.0g
1.5g
1.0g
0.5g
0g
Vehicle treated
(0.1% DMSO)
GSK2193874
(10μM)
10 minutes
D
mg tension
4000
3500
3000
2500
2000
1500
1000
500
0
Veh
GSK2193874
10μM
GSK1016790A (100nM)
E
GSK1016790A (100nM)
Vehicle treated
(0.1% DMSO)
Montelukast
(10μM)
Zileuton
(10μM)
10 minutes
F
mg tension
Veh
Montelukast
(10μM)
Zileuton
(10μM)
GSK1016790A (100nM)


Ca2+ Flux
Contraction
A
Pre-treatment
GSK1016790A (100nM)
High
Low
Calcium Level
F340/F380
K50
GSK101 (100nM)
Time (s)
B
%K50 (A.U.C.)
Veh
1
10
100
1000
ACh 10
GSK1016790A (nM)
C
Pretreatment
GSK1016790A (100nM)
O.D.
Optical Density (x1000)
GSK101 (100nM)
Time (s)
D
Pretreatment
ACh (10μM)
O.D.
Optical Density (x1000)
ACh (10μM)
Time (s)
Contraction
E
% change from baseline
Veh
1
10
100
1000
ACh 10
GSK1016790A (nM)
F
Vehicle.
Veh. + GSK1016790A
MCD849
MCD849 + GSK101
High
O.D.
Low
G
% change from baseline
Veh
GSK1016790A (100nM)
Cultured with mast cells


TRPV4 mRNA expression
A
2^-dct x 10^6
10
8
6
4
2
0
HLMC
HASM
Cultured HASM cells
B
ATP concentration (nM)
60
40
20
0
*
*
Veh
GSK2193874 (10μM)
Veh
GSK2193874 (10μM)
+Vehicle (DMSO)
+GSK1016790A (10nM)
Contraction
C
mg tension
2500
2000
1500
1000
500
0
*
Veh
Montelukast (10μM)
Zileuton (10μM)
ATP (1mM)


A
2^-dct x 10^6
Control
+ FcER1
P2X1
P2X2
P2X3
P2X4
P2X5
P2X6
P2X7
B
% change from baseline
Cultured with mast cells
Vehicle
Vehicle
P2X1i
P2X4i
P2X7i
Vehicle
GSK1016790A (100nM)
C
GSK1016790a 100nM
Vehicle treated
(0.1% DMSO)
5BDBD
(50μM)
10 minutes
D
mg tension
Veh
5BDBD (50μM)
GSK1016790A
(100nM)
E
ATP 1mM
Vehicle treated
(0.1% DMSO)
5BDBD
(50μM)
10 minutes
F
mg tension
Veh
5BDBD (50μM)
ATP
(1mM)
G
mg tension
Veh
Camostat
Mesylate
(10μM)
GSK219
(10μM)
5BDBD
(50μM)
Montelukast
(10μM)
Trypsin (1000U/ml)


TRPV4 agonist
ATP
HASM
HLMC
CysLts
Bronchoconstriction

ONLINE REPOSITORY: Novel airway smooth muscle-mast cell interactions and a role for the TRPV4-ATP axis

*Sara J. Bonvini PhD[1], *Mark A. Birrell PhD[1,2], Eric Dubuis PhD[1], John J. Adcock PhD[1], Pauline Flajolet PharmD[1], Michael A. Wortley PhD[1], Peter Bradding[3], #Maria G. Belvisi PhD[1,2].

Affiliations:

[1]Respiratory Pharmacology Group, Airway Disease, National Heart & Lung Institute, Imperial College London, Exhibition Road, London SW7 2AZ, UK; [2] Research & Early Development, Respiratory, Inflammation Autoimmunity (RIA) BioPharmaceuticals R&D , AstraZeneca, Gothenburg, Sweden, [3]Department of Infection, Immunity and Inflammation, University of Leicester University, Institute for Lung Health, Glenfield Hospital, Leicester, LE3 9QP, UK.

* Contributed equally

Correspondence to: Professor Maria G. Belvisi

Author Contributions: Conception and design: MAB, MGB, SJB; data analysis and interpretation: MGB, MAB, SJB, ED, JJA, MAW, PF, PB; writing the paper: SJB, MAB, MGB.

Funding: SJB and ED was funded by a Medical Research Council (MRC, UK) MICA award (MR/K020293/1) and Wellcome Trust (207504/B/17/Z). MAW was funded by the North-West Lung Centre Charity. The human vagus experiments in this study were undertaken with the support of the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust. Work in Leicester was supported by the National Institute for Health Research Leicester Biomedical Research Centre - Respiratory. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

METHODS

In vivo measurement of bronchoconstriction

Guinea pigs were anaesthetized with urethane (1.5g/kg) and the trachea cannulated with a short length of Perspex tubing and animals artificially ventilated as previously described[1]. The right jugular vein and carotid artery (passed to the ascending aorta/aortic arch) were also cannulated for drug administration and measurement of systemic arterial blood pressure. Animals were paralysed with vecuronium bromide (0.1mg/kg i.v. initially then 0.05mg/kg i.v. every 20 minutes to maintain paralysis). The cervical vagal nerves were located and both cut at the central end[19]. Tracheal pressure was measured with an air pressure transducer (SenSym647) connected to a side arm of the tracheal cannula.

GSK1016790A (100ng/ml, 153nM for 15s) was aerosolised into the airways and changes in bronchoconstriction indicated by an increase in tracheal pressure which were monitored for 60 minutes or until responses returned to baseline levels. In antagonist studies, GSK2193874 (300mg/kg, i.p.) or vehicle (6% cyclodextrin in saline, i.p) were administered 30 minutes prior to aerosolization of GSK1016790A (100ng/ml).

Cell culture

Primary HASMCs were harvested as previously described[2]. Once extracted, cells were cultured in Dulbecco Modified Eagles medium (DMEM) containing L-glutamine (2mM), sodium pyruvate (1mM), penicillin (100U/mL), streptomycin (100µg/mL), amphotericin B (5µg/ml), nonessential amino acids (1%) and supplemented with 10% (v/v) foetal bovine serum (FBS). The HASMCs were cultured in 35mm glass bottom fluorodishes in air containing 5% carbon dioxide in a humidified chamber at 37°C, until cells had grown to 50% confluence. Prior to treatment, culture media was aspirated, and replaced with serum-starved media (DMEM containing L-glutamine (2mM), sodium pyruvate (1mM),

penicillin (100U/mL), streptomycin (100µg/mL), amphotericin B (5µg/ml), nonessential amino acids (1%), and Bovine Serum Albumin (1.3%) for 24h to induce growth to arrest.

Expression profiles in HASMCs and HLMCs

As a number of TRPV channels have previously been shown to be expressed in both guinea pig and human ASM, including TRPV1 and TRPV2 alongside TRPV4[3,4], expression of TRPV4 on ASM was confirmed using Quantitative Real time PCR. Assays for the TRP and P2X ion channels and the PAR2 GPCR were purchased from Applied Biosystems and validated using cDNA from human tissue/organs that expressed the target ion channel at high levels. Expression levels of TRP and P2X ion channels along with PAR2 were determined using the validated assays, with real time RT-PCR performed as described previously[5].

***In vitro* measurement of contraction: Organ Bath**

Human tracheal and bronchial tissue (approximately 3-4mm in width) and guinea-pig trachea (approximately 2 cartilage rings in width) was cut longitudinally by cutting through the cartilage directly opposite to the smooth muscle layer and transverse sections cut to produce tracheal strips. These were sutured and attached to a steel hook at the bottom end and to force-displacement transducers on the other end in 10ml organ baths containing Krebs-Hensleitt solution kept at 37^{o}C, bubbled with 95% O2/5% CO_2. Changes in force were measured isometrically using force-displacement transducers connected to a data acquisition system as previously described[5]. In all cases the epithelium was left intact, and indomethacin (10 µM), a non- selective COX inhibitor was present throughout to prevent the production of endogenous prostanoids which are known to influence airway smooth muscle tone. Once the tissues had equilibrated they were challenged with a supramaximal concentration of ACh (1 mM) three times to confirm viability and to standardise responses. All experiments were carried out under basal levels of tension. Following the final ACh response, single concentrations of the TRPV4 agonist GSK1016790A (1 nM-1 µM), or ATP (100 µM-3

mM) were added to separate baths and left to incubate for 40 minutes, or until responses returned to baseline, after which a final stimulation with ACh was added to the baths to ensure tissue viability. For antagonist experiments, the tissue was preincubated with the selective antagonists (TRPV4: GSK2193874 (10 μM); CysLt1: Montelukast (10 μM); 5LO: Zileuton (10 μM); P2X4: 5BDBD (50 μM)) in separate baths for 30 minutes. A submaximal concentration of GSK1016790A (100 nM) or ATP (1 mM) was then added to the baths and left to incubate for 40 minutes, or until responses returned to baseline. A final stimulation with ACh was added following this to ensure tissue viability.

Calcium imaging in HASMCs

HASMCs harvested from donor tissue were grown in 35mm glass bottom fluorodishes in DMEM supplemented with 10% FCS. Twenty-four hours prior to experimental use, culture media was replaced with serum-starved media. On the day of the experiment, DMEM was removed from the cells and replaced with sterile extracellular solution (ECS) and allowed to equilibrate for 30 minutes at 37^{o}C. The cells were then washed twice with ECS at 25^{o}C and loaded with the intracellular calcium $[Ca^{2+}]_i$ dye Fura-2-AM (12μM, supplemented with 1% PowerLoad + Probenicid (1mM)) for 1h in the dark at RT. The cells were then washed twice with ECS and left to de-esterify in the dark at room temperature for 1h prior to imaging analysis. After washing, a single fluorodish was placed in a full incubation chamber mounted on a stage of a Widefield inverted microscope and imaged at excitation and emission fluorescence wavelengths of approximately λ=360/380 nm and λ=650 nm, respectively to measure $[Ca^{2+}]_i$ in response to agonist. K50 (50mM potassium solution, as opposed to 5.4mM used in ECS) was applied at the start of each experiment for 30s to assess cell viability and normalise responses. Once responses were obtained the cells were washed to return to baseline, and then treated with vehicle (0.1% DMSO) for ten minutes, then washed, following which a single concentration of the TRPV4 agonist (1-1000nM) was added to the dish and incubated for ten minutes and $[Ca^{2+}]_i$ responses monitored.

Assessment of single human airway smooth muscle cell contraction: Ptychography

A novel imaging technique, ptychography, was utilised as a surrogate for cellular contraction of HASMCs[7]. Ptychography is a label free, high contrast microscopy technique which yields contrast similar to fluorescent imaging with minimal cell manipulation[7]. The dish containing the cells and a low intensity near infrared laser (635nm) are moved over each other to create a sequential array of overlapping illuminated areas, where the light scattered is captured as an array of diffraction pattern on a detector. This diffraction pattern array is then processed using Virtual Lens© algorithm to calculate a quantitative measure of the light absorbed, the scattering and the phase delay introduced into the illumination as it passes through the specimen, which enables analysis of the 3D characteristics of the sample[7]. The parameter extracted for this study was the optical density which measures the thickness of the cells as it changes during isotonic contraction. This technique has yielded contractile responses in HASMCs (for example in response to ACh) matching $[Ca^{2+}]_i$ elevation recorded in the same cells and similar to contractile responses found using whole tissue[8].

In order to determine the role of mast cells in TRPV4 induced contraction, HASMCs were cultured with HLMCs, or mast cell media (DMEM/HEPES containing Glutamax I and 10% heat inactivated fetal calf serum, 100ng/ml SCF, 50ng/ml interleukin-6 and 10ng/ml interleukin-10). Cultured primary HASMCs were growth arrested for 24 h, following which they were co-cultured with HLMCs +3% FBS at a 4:1 ASM to mast cell ratio, or mast cell media, in triplicate in 35mm glass bottomed fluorodishes. One hour later, cells were treated with either vehicle or P2X antagonist and incubated at 37^{o}C for 1 hour. Cells were placed in the ptychography system (VL21, Phase Focus UK) and then stimulated with GSK1016790A (100nM) or vehicle (0.1% DMSO). The effect of GSK1016790A on contraction of HASMCs alone and HASMCs co-cultured with HLMCs was then assessed using time lapse ptychography. A control experiment conducted in parallel was HASMCs cultured in the same conditions without HLMCs, and treated with either GSK1016790A or ATP.

ATP release assay

Human ASM cells from four patients were seeded onto separate 24 well plates and grown to 90% confluence. 24 hours following growth arrest, cells were incubated with vehicle or GSK2193874 (10μM) at 37^{o}C for 30 minutes. The cells were then incubated with GSK1016790A (10nM) or vehicle for 1 hour at 37^{o}C, following which the supernatant was aspirated and frozen for later analysis. ATP levels were assessed using an ATPlite assay (Perkin Elmer, Cambridge, UK) according to pack instructions.

Cysteinyl Leukotriene ELISA

Primary mast cells were isolated from a macroscopically normal lung biopsy (n=1) as described previously[9]. Cells were cultured for 4 weeks in DMEM GlutaMAX containing 10%FCS, hSCF (100ng/mL), hIL-6 (50ng/mL) and hIL-10 (10ng/mL) prior to any experiment. Final purity was >98% assessed by metachromatic staining with modified Wright-Giemsa stain.

Cells were seeded onto a 96 well plate at a density of 10,000 cells/well in 100μL DMEM GlutaMAX containing 10%FCS, SCF (100ng/mL), IL-6 (50ng/mL) and IL-10 (10ng/mL). 24 hours later, cells were washed twice and equilibrated for 10min at 37^{o}C in 90uL HEPES containing 0.04% BSA SCF (100ng/mL), IL-6 (50ng/mL) and IL-10 (10ng/mL) prior to challenge with vehicle (1% dH_20), ATP (1mM), ATP (3mM), or the positive control positive control, PMA (10nM)/ionomycin (1μM) in duplicate. Following a 30-minute incubation, the supernatant was aspirated and frozen for later analysis. Total Cysteinyl Leukotriene (LTC_4, LTD_4 and LTE_4) levels were assessed using a Cysteinyl Leukotriene Express ELISA kit (Cayman Chemical) according to pack instructions. Viability of the cells was not affected, with >90% in all treatment groups.

Compounds and materials

The selective TRPV4 agonist and antagonist GSK1016790A and GSK2193874 were chosen as the ligands to probe the role of the TRPV4 receptor as they have been shown to be potent activators and

inhibitors both *in vivo* and *in vitro*, with limited off target effects[10,11]. These were purchased from Sigma Aldrich (Poole, UK) with montelukast, zileuton, AZ11645373 and DMSO. 5-BDBD, and Ip5I were purchased from Tocris (UK). Drugs were made up in stock solutions using DMSO, or dH_20 (ATP) with the final concentration of DMSO or dH_20 kept at 0.1% for experiments.

FIGURE LEGENDS

Supplementary Figure E1

(A) The effect of ATP on contraction of primary HASM cells alone. ATP at 0.3, 1mM or 3mM did not cause contraction of the cells analysed using ptychography, in some cases there was relaxation. The contractile stimulus ACh (10μM) caused contraction of the cells as expected. Data shown as % change from baseline (resting state), N=2 patients, n=17-27 cells.

(B) The effect of vehicle, ATP at 1mM and 3mM and PMA (10nM) ionomycin (1μM) on total cysteinyl leukotriene release from primary human mast cells. Addition of ATP (1 or 3nM) caused a concentration dependent increase in total cysteinyl leukotriene release compared to vehicle control. Data shown is from mast cells isolated from 1 (73-year-old female donor).

Supplementary Figure E2

(A) PAR2 mRNA was expressed in HASMCs from 6 patients, but not in HLMCs of two patients, and expressed at very low levels in the third. The effect of vehicle GSK2193874, montelukast and 5BDBD on contraction induced by the common contractile stimuli histamine (B) and acetylcholine (C) in isolated guinea pig trachea. No antagonist had any effect on contraction different to that of vehicle. Mean± SEM. of n=6 (HASMs), 3 (HLMCs), 3-4 (trachea).

REFERENCES

E1: Bonvini SJ, Birrell MA, Grace MS, Maher SA, Adcock JJ, Wortley MA, et al. Transient receptor potential cation channel, subfamily V, member 4 and airway sensory afferent activation: Role of adenosine triphosphate. J Allergy Clin Immunol. 2016;138:249–261.e12.

E2: Clarke DL, Belvisi MG, Catley MC, Yacoub MH, Newton R, Giembycz MA. Identification in human airways smooth muscle cells of the prostanoid receptor and signalling pathway through which PGE2 inhibits the release of GM-CSF. Br J Pharmacol. 2004;141:1141–50.

E3: Jia Y, Wang X, Varty L, Rizzo CA, Yang R, Correll CC, et al. Functional TRPV4 channels are expressed in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol [Internet]. 2004; 287: L272-8.

E4: Rousseau E, Cloutier M, Morin C, Proteau S. Capsazepine, a vanilloid antagonist, abolishes tonic responses induced by 20-HETE on guinea pig airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2005; 288:L460–L470

E5: Baxter M, Eltom S, Dekkak B, Yew-Booth L, Dubuis ED, Maher SA, et al. Role of transient receptor potential and pannexin channels in cigarette smoke-triggered ATP release in the lung. Thorax. 2014;69:1080–9.

E6: Buckley J, Birrell MA, Maher SA, Nials AT, Clarke DL, Belvisi MG. EP4 receptor as a new target for bronchodilator therapy. Thorax. 2011;66:1029–35.

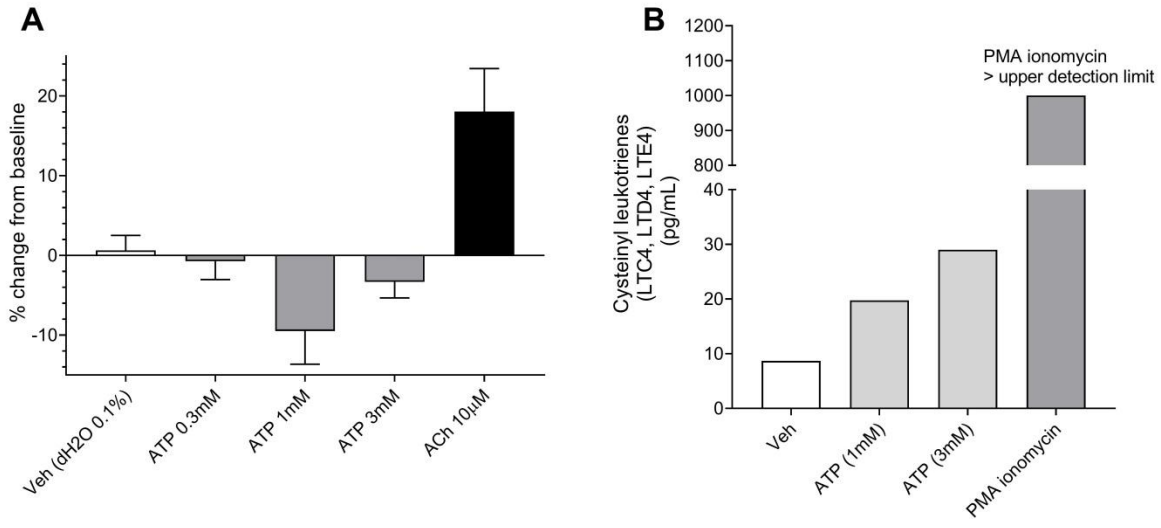
E7: Marrison J, Räty L, Marriott P, O'Toole P. Ptychography--a label free, high-contrast imaging technique for live cells using quantitative phase information. Sci Rep. 2013;3:2369.

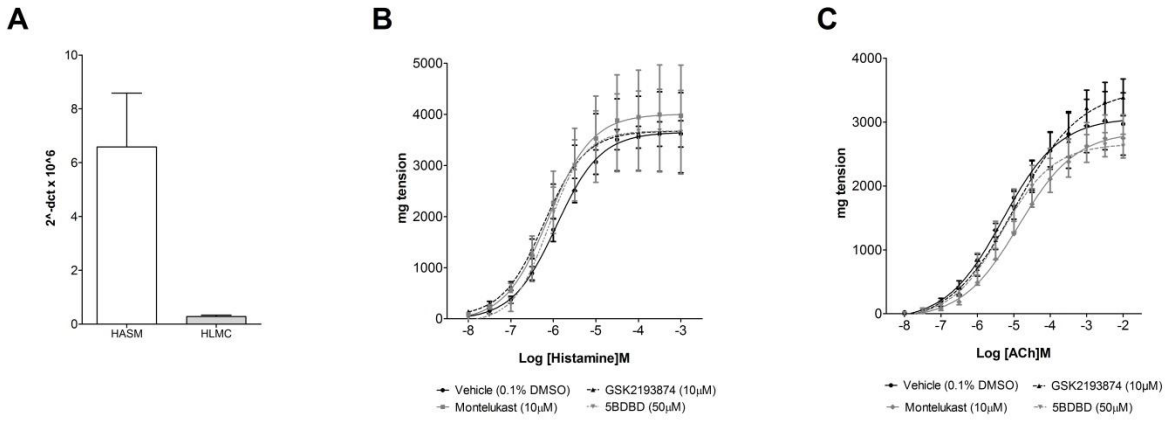
E8: Dubuis ED, Belvisi MG, Poushpas S, Flajolet P, Langley K, Birrell MA. A Novel Method For Measuring Contraction Of Primary Human Airway Smooth Muscle Cells. In: Am J Respir Crit Care Med. 2015. p. A1224.

E9: Sanmugalingam D, Wardlaw A, Bradding P. Adhesion of human lung mast cells to bronchial epithelium: evidence for a novel carbohydrate mediated mechanism. 2000. Journal of Leukocyte Biology. 68(1):38-46.

E10: Thorneloe KS, Sulpizio AC, Lin Z, Figuero DJ, Clouse AK, McCafferty GP, Chendrimada TP, Lashinger ES, Girdon E, Evans L, Misajet BA, Demarini DJ, Nation JH, Casillas LN, Marquis RW, Votta BJ, Sheardown SA, Xu X, Brooks DP, Laping NJ, Westfall TD. N-((1S)-1-{[4-((2S)-2-{[(2,4-dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (**GSK1016790A**), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I. 2011. J. Pharmacol Exp Ther. 338(1):410

E11: Cheung M, Bao W, Behm DJ, Brooks CA, Bury MA, Dowdell SE, Eiden HS, Fox RM, Goodman KB, Holt DA, Lee D, Roethke TJ, Willette RN, Xu X, Ye G, Thorneloe KS. Discovery of GSK2193874: An Orally Active, Potent and Selective Blocked of Transient Receptor Potential 4. ACS Med Chem Lett. 2017. 549-554.


A
% change from baseline
20
10
0
-10
Veh (dH2O 0.1%)
ATP 0.3mM
ATP 1mM
ATP 3mM
ACh 10µM
B
Cysteinyl leukotrienes
(LTC4, LTD4, LTE4)
(pg/mL)
1200
1100
1000
900
800
40
30
20
10
0
PMA ionomycin
> upper detection limit
Veh
ATP (1mM)
ATP (3mM)
PMA ionomycin


A
B
C
2^-dct x 10^6
HASM
HLMC
mg tension
Log [Histamine]M
Log [ACh]M
Vehicle (0.1% DMSO)
GSK2193874 (10μM)
Montelukast (10μM)
5BDBD (50μM)