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AIRWAY SMOOTH MUSCLE AS A TARGET IN ASTHMA: NEW INSIGHTS INTO BRONCHORELAXATION AND HYPERREACTIVITY

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Cover: Human bronchial biopsy, in which the airway smooth muscle layer is stained in pink using an antibody against α-actin (photo taken by Anna James)

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ABSTRACT

Patients with asthma suffer from reversible airway obstruction accompanied by airway hyperresponsiveness (AHR). Airway smooth muscle (ASM) is central to bronchoconstriction and AHR and is therefore the target of existing and potential new therapies for asthma.

Activation of bitter taste sensing type 2 receptors (TAS2Rs) on ASM was recently suggested to induce bronchorelaxation by a novel mechanism. Therefore, the first aim of this thesis was to determine the potential of TAS2Rs (and their respective agonists) as possible therapeutic targets on ASM. A secondary aim of this thesis was to assess whether inflammation induced by IL-13 can alter ASM function in intact human airways.

The methodology used in this thesis mainly involved the study of isometric contractions and relaxations in smooth muscle preparations from guinea-pigs and humans. The measurement of intracellular Ca^{2+} and analyses of mRNA expression following interventions (cytokines/siRNA) was performed in cultured ASM cells.

Defined TAS2R agonists including chloroquine, denatonium, dextromethorphan, noscapine and quinine were found to strongly relax guinea pig trachea, pre-contracted by muscarinic agonists. Although the TAS2R agonists were less potent bronchorelaxants than β_2-adrenoceptor agonists, they were equally (or more) efficacious following stronger pre-contractions. Chloroquine relaxed antigen-induced contractions, whereas denatonium failed to relax other types of pre-contractions. TAS2R agonists were shown to induce relaxation via distinct mechanisms that do not involve conventional pathways.

Human pulmonary arteries, expressing TAS2R mRNA, were relaxed by chloroquine, dextromethorphan and noscapine, indicating that TAS2R agonists also affect vascular smooth muscle function. In guinea pig aorta, TAS2R agonists elicited relaxations following noradrenergic pre-contractions, independently of the endothelium, BK or L-type Ca2+ channels. Again, responses towards TAS2R agonists were shown to be dependent on the agents used for pre-contraction. Studies in guinea pig taenia coli demonstrated antagonism by denatonium and quinine at α-adrenoceptors.

In human ASM cells, TAS2R mRNA expression was decreased by TNFα and glucocorticoids, and increased following 24-48 hours of serum-deprivation. Chloroquine, dextromethorphan, noscapine and quercetin induced relaxation of intact human airways, but differentially inhibited histamine-induced intracellular Ca^{2+} mobilisation. The siRNA-induced decrease of TAS2R14 mRNA could not prevent quercetin-mediated inhibitions in human ASM cells.

In human small airways, IL-13 induced a 2.5-3.1-fold increase in the potency of carbachol and histamine contractions, without affecting their amplitude. In addition, IL-13 impaired relaxant responses towards salbutamol, but not those towards formoterol, chloroquine and noscapine. IL-13 increased both the potency and the amplitude of histamine-induced intracellular Ca^{2+} mobilisation in human ASM cells. The IL-13-induced hyperreactivity of human bronchi and ASM cells was not prevented by the glucocorticoid dexamethasone.

In summary, although TAS2R agonists exert powerful relaxations in smooth muscle, their effects in ASM are not mediated by common pathways. As a consequence, the TAS2Rs may be questioned as a target for bronchorelaxation in asthma. On the other hand, IL-13 induces functional remodelling of the ASM in human airways that can contribute to AHR.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications:

I. Pulkkinen V, **Manson ML**, Säfholm J, Adner M, Dahlén S-E.
The bitter taste receptor (TAS2R) agonists denatonium and chloroquine display distinct patterns of relaxation of the guinea pig trachea
Am J Physiol Lung Cell Mol Physiol. 2012 Dec 1;303(11):L956-66.

II. **Manson ML**, Säfholm J, Al-Ameri M, Bergman P, Orre AC, Swärd K, James A, Sven-Erik Dahlén, Mikael Adner
Bitter taste receptor agonists mediate relaxation of human and rodent vascular smooth muscle
Eur J Pharmacol 2014, 2014 Oct 5;740:302-11

III. **Manson ML**[*], James A[*], van Rheenen M, Jager M, Zuccaro L, Pulkkinen V, Dahlén S-E, Adner M
Effects of bitter tastants on human airway smooth muscle in relation to TAS2R expression
Manuscript
* These authors contributed equally

IV. **Manson ML**, James A, Säfholm J, Al-Ameri M, Bergman P, Orre AC, Dahlén S-E, Adner M
IL-13 induces glucocorticoid-insensitive hyperreactivity of human small airways
Manuscript submitted

During my time at Karolinska Institutet, I contributed to two additional research studies that were not included in this thesis

Säfholm J, **Manson ML**, Bood J, Delin I, Orre AC, Bergman P, Al-Ameri M, Dahlén SE and Adner M

Prostaglandin E_2 inhibits mast cell-dependent bronchoconstriction in human small airways via the EP_2 receptor

J Allergy Clin Immunol, accepted for publication

Larsson OJ, **Manson ML**, Starkhammar M, Fuchs B, Adner M, Kumlien Georén S, Cardell LO

TLR7 agonists relax guinea pig airways independently of TLR7: a possible role for quinoline moieties

Manuscript submitted

CONTENT

LIST OF ABBREVIATIONS

5-HT	5-Hydroxytryptamine
AHR	Airway hyperresponsiveness
cADPR	Cyclic adenosine diphosphate ribose
cAMP	Cyclic 3´-5´- adenosine monophosphate
cGMP	Cyclic guanine monophosphate
CysLT	Cysteinyl-leukotrienes
ELISA	Enzyme linked immunosorbent assay
HASM	Human airway smooth muscle
HEK	Human embryonic kidney
HPA	Human pulmonary artery
IgE	Immunoglobulin E
IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
KCl	Potassium chloride
LABA	Long-acting β_2-adrenoceptor agonists
MLCP	Myosin light chain phosphatases
mN	MilliNewton
PCLS	Precision cut lung slices
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin F_2
PKA	Protein kinase A
PKC	Protein kinase C
ROCK	Rho-associated, coiled-coil-containing protein kinase
SABA	Short-acting β_2-adrenoceptor agonists
SR	Sarcoplasmic reticulum
STAT-6	Signal transducer and activator of transcription 6
TAS2R	Taste sensing type 2 receptors
T_h2	T-helper 2
Treg	Regulatory T cell
TNFα	Tumor necrosis factor α
TP	Thromboxane receptor
TRP	Transient receptor potential cation channel
TSLP	Thymic stromal lymphopietin

BACKGROUND

1.1 ASTHMA

Asthma is a common chronic and heterogeneous disease of the airways that with a wide prevalence, depending on the population examined (1-18%), affects more than 300 million people worldwide [1]. The prevalence of asthma continues to increase as a consequence of societies adopting Western lifestyles and becoming urbanized [2, 3]. The lifetime prevalence for developing asthma (around 35%) is similar to other chronic diseases, such as cancer, diabetes and coronary heart disease [4]. However, in comparison to other chronic diseases, asthma develops much earlier in life, often during childhood, which causes life-long suffering and despite a relatively low mortality, makes it a substantial socioeconomic burden [1, 4, 5].

Individuals with asthma suffer from recurrent episodes of expiratory airflow limitation (exacerbations), accompanied by airway hyperresponsiveness. These periods of airway obstruction result in symptoms of wheeze, shortness of breath, chest tightness and/or cough, that resolve either spontaneously or following pharmacological treatment [1]. Airway inflammation and airway remodelling drive the pathophysiology of the disease by inducing airway smooth muscle contraction, increasing mucus production, airway oedema and airway wall thickening [6]. Exacerbations represent acute worsening of asthma that can lead to hospitalization. These exacerbations are predominantly triggered by viral infections, but dependent on the phenotype of the asthma, also by allergens, pollution and pregnancy [7, 8].

The guidelines for the treatment of asthma are described in a five-step protocol by the Global Initiative for Asthma consortium and focus on the control of symptoms, the prevention of exacerbations and decline in lung function, as well as minimizing the risk of side-effects [1]. Asthma management is achieved through the use of controller medications that affect the airway inflammation, and reliever therapies that reverse airway obstruction and can be used when symptoms of asthma worsen.

The first step of the treatment regime for asthma includes the as-needed use of short-acting β-adrenoceptor agonists (SABAs). However, as SABAs only facilitate symptom control and do not treat inflammation, patients with mild asthma generally receive low-doses of inhaled glucocorticosteroids (step 2). Leukotriene synthesis inhibitors or receptor antagonists can be considered as alternative first-line or add-on therapy when side-effects develop towards glucocorticoids, or when patients prefer oral medications. In case of moderate asthma, long-acting β_2-adrenoceptor agonists (LABAs) can be added to the regime (step 3), together with an increased dose of inhaled glucocorticoids (step 4). Add-on therapy by tiotropium, oral glucocorticoids or monoclonal antibodies against IgE can be used in patients with severe asthma that experience symptoms despite using high doses of inhaled glucocorticoids and LABA agonists (step 5). Moreover, improving adherence to therapy and instruction of inhalation technique, can promote clinical outcome [9, 10].

1.2 PATHOPHYSIOLOGY

1.2.1 Heterogeneous disease

Asthma is often considered to be an allergic, T-helper 2 (T_h2)-type dependent inflammation, as bronchial biopsies and/or bronchoalveolar fluid from asthmatics were characterized by higher numbers of $CD4^+$ lymphocytes, eosinophils and mast-cells [11-13]. This has encouraged studies of T_h2 inflammation and has provided us with a reasonable understanding of how T_h2 inflammation can contribute to the features of allergic asthma (**paragraph 1.2.3**).

Nonetheless, asthma is a heterogeneous disease that includes a large population of patients that do not display a typical T_h2-type or eosinophilic inflammation [14]. Ongoing studies have started to define other specific phenotypes (e.g. neutrophilic, late-onset eosinophilic) on the basis of physiological, clinical and biological features [15-17]. However the mechanisms of their contribution to asthma remain poorly defined and are therefore not discussed in this thesis.

1.2.2 Epithelium

The respiratory epithelium consists of specialized columnar ciliated epithelial cells, mucus-producing goblet cells and surfactant-secreting Clara cells, that through tight junctions form an impermeable physical barrier [18, 19]. This barrier is the first line of defence of the airways against potential threats, such as microbes, viruses, pollutants and allergens that are present within our environment. Disruption of this barrier function has been suggested as a cause of asthma, as this would increase the exposure of the immune system to potential threats [20].

Epithelial cells can also modulate immune responses, as they express pathogen-recognition receptors and produce several lipid mediators, growth-factors, chemokines and cytokines [21]. Epithelial cells can also provide co-stimulatory signals for dendritic cell activation, thereby increasing allergen sensitization [18, 22]. Moreover, epithelial damage, as a consequence of viral infections and/or pollutants, induces the production of TSLP, IL-25 and IL-33. These cytokines can drive asthma pathology in the absence of allergens, as they can directly activate the adaptive immune system via innate lymphoid cells [23, 24].

1.2.3 Airway inflammation – T_h2 type

Naïve T-cells differentiate, following antigen presentation by dendritic cells, into T_h2 cells that start to produce the typical T_h2 cytokines; IL-4, IL-5 and IL-13. IL-4 and IL-13 induce the immunoglobulin class-switching of B-cells resulting in the synthesis of IgE, whereas IL-5 contributes to the recruitment and survival of eosinophils. In addition, T_h2 cytokines facilitate the recruitment and maturation of mast cells. Re-exposure to allergen can activate mast cells via cross-linking with allergen-specific-IgE bound to the FcγR1 receptor on the cell membrane. The subsequent release of spasmogenic (e.g. histamine, cysteinyl-leukotrienes and prostanoids) and inflammatory mediators (TNFα and IL-13) from mast cells, induces bronchoconstriction, increased vascular permeability, mucus hypersecretion and further recruitment of inflammatory cells [6].

Chronic inflammation can develop as a consequence of continuous or repetitive allergen exposure. Ultimately, this leads to an extensive inflammatory environment, in which different types of adaptive immune cells (T_h2, T_h17, Treg), granulocytes (eosinophils, mastcells, neutrophils) and structural cells (epithelial cells, fibroblasts, airway smooth muscle cells) interact with each other, thereby inducing structural damage of the airways [6].

1.2.4 Airway remodelling

Airway remodelling describes the structural changes of the airways that develop upon tissue damage and repair. The structural changes that can be observed in asthma include; a loss of epithelial integrity, goblet cell metaplasia, subepithelial fibrosis due to matrix deposition, increased vascularity and as a consequence of hyperplasia/hypertrophy, increased airway smooth muscle mass [25, 26]. This airway remodelling results in increased mucus production and as a consequence of increased airway smooth muscle mass, in a thicker airway wall that presumably increases airway narrowing [27]. Airway remodelling was thought to be the consequence of chronic inflammation, however studies have shown that the induction of bronchoconstriction by itself, in the absence of inflammation can also induce airway remodelling [28].

1.3 AIRWAY HYPERRESPONSIVENESS

Airway hyperresponsiveness (AHR) is a key feature of asthma that is defined by excessive airway narrowing following physical (cold air, exercise), chemical (pollutants, tobacco smoke) or pharmacological (methacholine, histamine) stimuli that normally have little, or no, effect in healthy individuals [29, 30].

AHR can be clinically assessed by bronchial provocations with direct or indirect stimuli [29, 31]. Direct stimuli (e.g. histamine, cysteinyl leukotrienes and methacholine) induce a decline in lung function via an immediate constriction of the airway smooth muscle. This form of hyperresponsiveness is characterized by both an increased sensitivity (potency) and a greater maximal response (amplitude) to bronchoconstrictors [31]. On the other hand, indirect stimuli (e.g. mannitol, allergen or exercise) constrict the airways through intermediate pathways; most often via the release of spasmogens from mast cells, but some indirect stimuli may also cause neuronal activation [29].

It is apparent that AHR includes both a variable and a persistent component [29, 32]. The variable component of AHR is inducible and is considered to reflect the acute degree of airway inflammation, which is therefore better represented by bronchoprovocations to indirect stimuli [33, 34]. On the other hand, AHR to direct stimuli persists in the absence of inflammation and despite the improvement achieved by the long-term use of glucocorticoids [35-37]. This persistent component of AHR is therefore thought to be a consequence of airway remodelling, but the precise mechanisms remain undefined [29, 32]. Nonetheless, as AHR to direct stimuli reflects constriction of the airway smooth muscle, it speaks for the importance of a decreased airway lumen diameter, increased airway smooth muscle mass and altered intrinsic/mechanic properties of the airway smooth muscle itself [29].

1.4 SMOOTH MUSCLE

Smooth muscle consists of spindle-shaped, single-nuclear, non-striated cells that cannot be voluntary controlled. Smooth muscle can be found throughout the body in the walls of hollow organs, such as airways, blood vessels and intestines, where its primary function is to regulate the flow of air, circulation of blood or peristaltic movement via contraction and relaxation [38].

Airway and vascular smooth muscle are organized as multi-unit (visceral) smooth muscles in which each smooth muscle cell is separately innervated. On the other hand, single-unit muscles, such as the intestinal and detrusor smooth muscle, contain more gap-junctions and less neuronal innervations, which promotes fluctuations of membrane potentials and myogenic activity [39]. Furthermore, variations in excitation-contraction coupling as well as heterogeneity in the distribution of ion-channels and receptors, causes the response to a certain hormone, neurotransmitter or pharmacological agent to differ between smooth muscles [40].

1.5 AIRWAY SMOOTH MUSCLE

In humans, the entire respiratory tree from the trachea to the smaller airways is circumscribed by airway smooth muscle. Historically, the airway smooth muscle has solely been considered an effector cell-type in asthma, which causes airway narrowing as a consequence of spasmogen release from mast cells and nerves, and an impaired release of epithelium-derived relaxing factors. However, the introduction of pharmacological strategies that can prevent either the actions of spasmogens (e.g. cysteinyl leukotrienes) or facilitate bronchorelaxation (β_2-adrenoceptor agonists) has proven the importance of the airway smooth muscle as a therapeutic target for asthma.

This traditional view has been changing and the airway smooth muscle is becoming, in addition to its contractile function, acknowledged for its active contribution to the development of asthma [41, 42]. Accordingly, the airway smooth muscle can produce a variety of growth factors, chemokines and cytokines [43] that can promote the recruitment and survival of inflammatory cells, such as mast cells [44]. Moreover, airway smooth muscle cells from asthmatics exhibit increased synthetic functions [45], as a consequence of an altered extracellular matrix [46, 47] and the variety of mediators that are released during inflammation [48, 49]. These increased synthetic functions may contribute to hyperplasia and hypertrophy, and the increased airway smooth muscle mass that are observed in patients with asthma [50, 51].

The airway smooth muscle has, because of the increased sensitivity of the airways to direct constrictors of the airway smooth muscle, also logically been linked to AHR [30, 42, 52]. However, attempts intended to define whether the mechanical and/or intrinsic properties are changed in asthmatic airway smooth muscle has provided conflicting results [30, 52-54]. On one hand, experimental observations suggest that inflammatory mediators (e.g. TNFα and IL-13) can directly alter the intrinsic properties of the airway smooth muscle and therefore induce hyperreactivity [55, 56]. These investigations have however been primarily conducted in airway smooth muscle cell cultures and in airways from animals. The importance of the modulation of airway smooth muscle function by inflammatory mediators remains to be confirmed in intact human airways, which will be part of the scope of this thesis.

1.6 AIRWAY SMOOTH MUSCLE CONTRACTION

Airway contraction is induced via a consecutive series of signalling events that encompass the excitation-contraction coupling of the airway smooth muscle, which is initiated by the binding of spasmogens (e.g. histamine and carbachol) to their respective G-protein coupled receptor ($G\alpha_q\beta\gamma$) on the cell-membrane. This protein complex is activated upon agonist binding which, via GTP, results in a conformational change of the receptor that causes the dissociation of the $G\alpha_q$ and $G\beta\gamma$ subunits. The $G\alpha_q$ unit activates membrane-bound phospholipase-Cβ (PLCβ) that promotes the hydrolysis of phosphatidylinositol 4,5-biophosphate (PIP_2) and results in the formation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG activates protein kinase C (PKC), whereas IP_3 induces Ca^{2+} release from the sarcoplasmic reticulum (SR) via the IP_3 receptor [57, 58].

The release of Ca^{2+} into the cytosol triggers the opening of other IP_3 and ryanodine (RYR) channels on the SR resulting in a further increase in intracellular Ca^{2+}. The levels of intracellular Ca^{2+} can also be raised by entry of extracellular Ca^{2+} via the opening of receptor-operated channels, store-operated Ca^{2+} channels or voltage-dependent Ca^{2+} channels [57, 59, 60]. Together, these processes induce oscillations of intracellular Ca^{2+} that via calmodulin, facilitate the activation of myosin light chain kinase, resulting in phosphorylation of myosin light chains. This enables cross-bridge cycling between actin and myosin ultimately resulting in contraction of the airway smooth muscle [57, 58, 61].

In addition, contractile agonists can increase the sensitivity of airway smooth muscle towards Ca^{2+}. This process of Ca^{2+} sensitization is suggested to be regulated by PKC and Rho kinase (ROCK), that decrease the reversal of myosin light chain phosphorylation by inhibiting myosin light chain phosphates [57, 58, 61]. Importantly, each contractile agonist induces a unique contraction of the airway smooth muscle, as contractile agonists have specific and distinct effects on Ca^{2+} mobilisation and sensitisation. In this context, the signalling of an agonist is determined by several factors, including the binding properties (e.g. duration of onset), receptor regulation (e.g. uncoupling or internalization), the G-protein (different α and βγ subunits) and compartmentalization [58].

1.7 AIRWAY SMOOTH MUSCLE RELAXATION

Reversal of bronchoconstriction through airway smooth muscle relaxation is an important strategy for the treatment of acute symptoms of asthma, and the mechanism of action of reliever therapies such as β_2-adrenoceptor agonists. Agonists (e.g. salbutamol, formoterol and salmeterol) of β_2 adrenoceptors, as well as prostaglandin E_2 via EP_2 and EP_4 receptors, activate the G_s family of G-protein-coupled receptors. Agonist binding to the $G\alpha_s\beta\gamma$-complex results in the dissociation of the $G\alpha_s$ and $G\beta\gamma$ subunits. The $G\alpha_s$ subunit induces membrane-bound adenylyl cyclase to catalyse the formation of cyclic 3´-5´- adenosine monophosphate (cAMP), which can activate protein kinase A (PKA) and exchange protein activated by cAMP (Epac) [57, 62].

These down-stream effectors of cAMP reduce Ca^{2+} oscillations through inhibition of IP_3 signalling and a decrease in Ca^{2+} sensitization. These effects reduce the phosphorylation of myosin light chains and thus facilitate airway smooth muscle relaxation [57, 62]. The individual contributions of the down-stream effectors of cAMP remain incompletely understood. Recent studies have demonstrated that the acute relaxations by β_2 adrenoceptor agonists are dependent on PKA and not Epac activation [63], implying other physiological functions for Epac [64].

Airway smooth muscle relaxation can also be achieved through other pathways. Nitric oxide (NO), enters the cytosol and induces via guanylyl cyclase the formation of cyclic guanine monophosphate (cGMP), which exerts its effects through protein kinase G [57, 65]. However, NO, is in comparison to its effects in vascular smooth muscle, a relatively weak relaxant of airway smooth muscle [66]. Phosphodiesterase inhibitors like theophylline induce relaxation of the airway smooth muscle by preventing the breakdown of the cyclic nucleotides (cAMP and cGMP). Relaxation of airway smooth muscle can also be achieved via interaction with ion channels [65]. Blockade of voltage-dependent Ca^{2+} channels (e.g. L-type) prevents the entry of extracellular Ca^{2+}, thereby limiting the availability of Ca^{2+} for contraction. On the other hand, potassium channels, like large-conductance Ca^{2+} activated potassium (BK) channels, control membrane potential and upon opening, cause relaxation through hyperpolarization [65, 67, 68].

1.8 TASTE SENSING TYPE 2 RECEPTORS

1.8.1 General

Taste perception in the gustatory system is based on the recognition and distinguishment of five unique taste qualities; bitter, sweet, salt, sour and umami. Bitter taste is detected in the taste bud regions of the tongue via taste sensing type 2 receptors (TAS2Rs), which belong to the family of G-protein coupled receptors [69-72]. The signal transduction of TAS2Rs is considered to involve the taste-specific Gα subunit gustducin [73]. Upon activation, gustducin induces the induction of intracellular Ca^{2+} via IP_3, which is suggested to trigger the influx of cations via TRPM5 channels [74]. However, transduction of bitter taste is not entirely dependent on these pathways [73, 75].

TAS2R function is considered to be important in evolution as this class of receptors prevents us from the intake of potentially harmful substances, which commonly have a bitter taste [76]. The human genome contains 25 different TAS2Rs, a number that differs between 3 and 50 among other species [77, 78]. However, TAS2Rs can, despite their relatively low number, recognize an enormous range (> 1000) of structurally diverse bitter compounds [76].

Structural screenings in TAS2R-overexpressing human embryonic kidney (HEK) cells using bitter compounds demonstrated that TAS2R agonists display a relatively low affinity for the TAS2Rs, as high micromolar concentrations were needed to activate the TAS2Rs [78]. However, most TAS2R agonists could activate multiple TAS2Rs, suggesting that the perception of a bitter taste by the gustatory system is determined by a complex interaction/activation profile of the TAS2R agonists.

1.8.2 Extra-oral functions

In addition to their function in the oral cavity, TAS2Rs have been implicated in several extra-oral functions within other organs of the human body, including the respiratory [67, 79-82], gastro-intestinal [83, 84], cardiovascular [85, 86] and reproductive system [87].

In the airways, TAS2R expression was first described in respiratory epithelial cells, where they sense and facilitate clearance of chemical and bacterial irritants by increasing ciliary beat frequency [79-81, 88]. The involvement of TAS2Rs in such a role was confirmed in patients with polymorphisms for TAS2R38, a receptor known to be activated by acyl-homoserine lactone quorum-sensing molecules from gram-negative bacteria, as these patients were more susceptible to infections with *Pseudomonas Aeruginosa* [80]. In relation to respiratory disease, a transcriptome analysis on blood leukocytes revealed that TAS2R expression was increased in children with severe asthma, which suggests a possible role for TAS2Rs in asthma [82].

Interestingly, TAS2Rs were also found to be expressed on airway smooth muscle and implicated in bronchorelaxation, as TAS2R agonists induced strong relaxations of airway smooth muscle preparations [67]. Moreover, TAS2R agonists were shown to be more protective than β_2-adrenoceptor agonists in an *in vivo* mouse model of allergic asthma [67]. This first study proposed a new concept for airway smooth muscle relaxation, as TAS2R agonists were suggested to induce an opening of BK channels through localized Ca^{2+} signalling, that resulted in hyperpolarization of the cell membrane [67]. These findings have encouraged both ourselves and others to define the effects of TAS2Rs and TAS2R agonists in airway smooth muscle. Thus, the effects and mechanisms of these agonists is a central theme in this thesis.

1.8.3 TAS2R agonists

Most studies that have described extra-oral functions of TAS2Rs base their conclusions, in the absence of antagonists and gene-deficient animals, on the expression of TAS2Rs and the observed effects of TAS2R agonists in a certain tissue. Although TAS2R agonists exhibit well-defined activity at TAS2Rs [78], their use at relatively high concentrations increases the possibility for interactions with other targets, such as receptors and ion-channels. A summary of reported non-TAS2R interactions for the different TAS2R agonists used in this study is presented in **Table 1**.

TAS2R agonists	Pathway of interaction	Actions	Ref
Chloroquine	α-adrenoceptors	Antagonist	[89]
	Muscarinic receptors	Antagonist	[90, 91]
Denatonium	Delayed rectifier K^+ channels	Blocker	[92]
Dextromethorphan	Opioid receptors	Agonist	[93, 94]
	NMDA receptors	Antagonist	[95, 96]
	Sigma receptors	Agonist	[97]
	Voltage-dependent Ca^{2+} channels	Blocker	[98]
Noscapine	Opioid receptors	Agonist	[99]
Quinine	α-adrenoceptors	Antagonist	[100, 101]
	Voltage-gated K^+ channels	Blocker	[102, 103]

Table 1: Summary of possible interactions between TAS2R agonists and TAS2R-independent pathways as reported in the literature.

1.9 IL-13

IL-13 is a typical T_h2 type cytokine, which in addition to being produced by T_h2 cells, is produced by many other inflammatory cells including mast cells, eosinophils and innate lymphoid cells. IL-13 plays a prominent role in the development of allergic inflammation, as it has been shown to induce eosinophilic inflammation, mucus hypersecretion and AHR [104-106]. IL-13 can bind to the IL-4Rα, IL-13Rα1 and IL-13Rα2 receptors [107]. The binding to a heterodimer of the IL-13Rα1 and IL-4Rα chains seems to be critical for the majority of effects mediated by IL-13 [105, 107, 108]. Activation of the IL-13Rα1/IL-4Rα dimer receptor complex induces the phosphorylation of STAT-6 via activation of JAK, which then translocates to the nucleus and promotes the transcription of IL-4/IL-13 responsive genes [107, 109].

The IL-13Rα2 receptor, which exhibits higher affinity for IL-13, was initially thought to be a decoy receptor, as it lacked a transmembrane signalling domain [107]. However, this concept has been questioned [110], and the contribution made by IL-13Rα2 to allergic inflammation remains therefore unclear.

Interestingly, from initial mouse studies, it became apparent that IL-13 caused the induction of AHR independently of IgE and eosinophilia, which suggested that IL-13 induced AHR via a direct effect on the airway smooth muscle [104, 111]. Indeed, airway smooth muscle cells were found to express IL-13 receptors and display STAT-6 activation upon IL-13 stimulation [112]. Functionally, IL-13 has been shown to impair responsiveness towards β_2-adrenoceptor agonists in both airway smooth muscle cells [112] and isolated animal airways [113]. Moreover, IL-13 increases the amplitude of airway smooth muscle contractions in mouse and rabbit airways [113-117]. These effects suggest that IL-13 can affect airway smooth muscle function and therefore has the potential to contribute to the development of airway hyperresponsiveness.

Currently, several new strategies attempt to block the effects of IL-13 in asthma [118-120]. However, in the context of airway smooth muscle, the effects of IL-13 have only sparsely been investigated in intact human airways [121].

2 MATERIALS AND METHODS

This section will provide a summary of the methods used in this thesis. Detailed descriptions of these methods can be found in the material and method sections of the different publications and manuscripts.

2.1.1 General

The initial studies in this thesis were conducted using isolated airway or vascular smooth muscle preparations from guinea pigs (**Paper I and II**), mice (**Paper II**) and humans (**Paper II, III and IV**). Further studies designed to investigate the regulation of TAS2Rs (**Paper III**) and the mechanisms underlying the effects of TAS2R agonists (**Paper III**) and IL-13 (**Paper IV**) were conducted using human airway smooth muscle cells.

2.1.2 Animals

All studies including animals (**Paper I and II**) were conducted in accordance with the ethical permits provided by the Swedish animal experimentation ethical review board. Guinea pigs (Dunkin-Hartley) and mice (C57BL/6) were obtained from Harlan and housed according a 12 hour light/dark cycle and were given food and water ad libitum. Guinea pigs were sacrificed by an overdose of pentobarbital or CO_2, whereas mice were killed by cervical dislocation. The heart-lung package was immediately removed, along with eventual other smooth muscles, and placed in ice-cold Krebs-Henseleit solution until further dissection.

2.1.3 Human lung tissue

The use of human lung tissue in **paper II, III and IV** was approved by the Stockholm ethical review board 2010/181-31/2 and all patients provided written informed consent beforehand. Macroscopically healthy human lung tissues were obtained from patients undergoing lobectomy for adenocarcinoma and other indications, and after surgery were immediately placed into ice-cold Krebs Henseleit buffer.

Human airways and pulmonary arteries (inner diameter < 2 mm) were identified in the lung specimens and distinguished from each other by their visual appearance under the microscope. Human bronchi were identified based on the presence of mucus and their transparent colour, as the airway wall is much thinner and less white than the vessel wall. Airways and arteries were then dissected out with the help of a thin smooth fishing line that was used to determine the direction of the airways and arteries within the tissue (**Figure 1**).

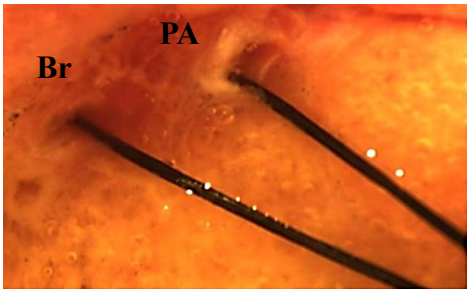

Figure 1: Human lung tissue demonstrating the identification of the human small bronchus (Br) and the pulmonary artery (PA). The black fishing line has a diameter of 0.25 mm.

2.1.4 Organ culture

Human bronchi that are used immediately after isolation from human lung tissue exhibit a lot of spontaneous activity, which makes these preparations difficult to work with. A common way to overcome this is by storing the isolated bronchi overnight at 4°C in Krebs Henseleit buffer. Although this procedure removes spontaneous tone, the rate of survival, particularly of the smaller human bronchi was relatively low.

To increase utility of this tissue, isolated human bronchial segments were transferred to culture plates containing DMEM medium supplemented with penicillin and streptomycin and placed into organ culture at 37°C in a humidified incubator at 95% CO_2 and 5% O_2 [122]. Every day, segments were transferred to fresh DMEM medium. The viability of human small bronchi segments was greatly improved by this methodology and the most consistent results were obtained from human bronchi that were maintained in this way for 2-4 days.

This methodology allowed investigations of the effects of inflammatory mediators on airway smooth muscle function, a method that was developed earlier to study the influence of inflammatory mediators on mouse tracheal smooth muscle [123]. Thus, IL-13 or a vehicle control were administrated daily to the culture. For specific purposes, bronchial segments could also be re-cultured after an initial assessment of their contractile function (**Figure 2**).

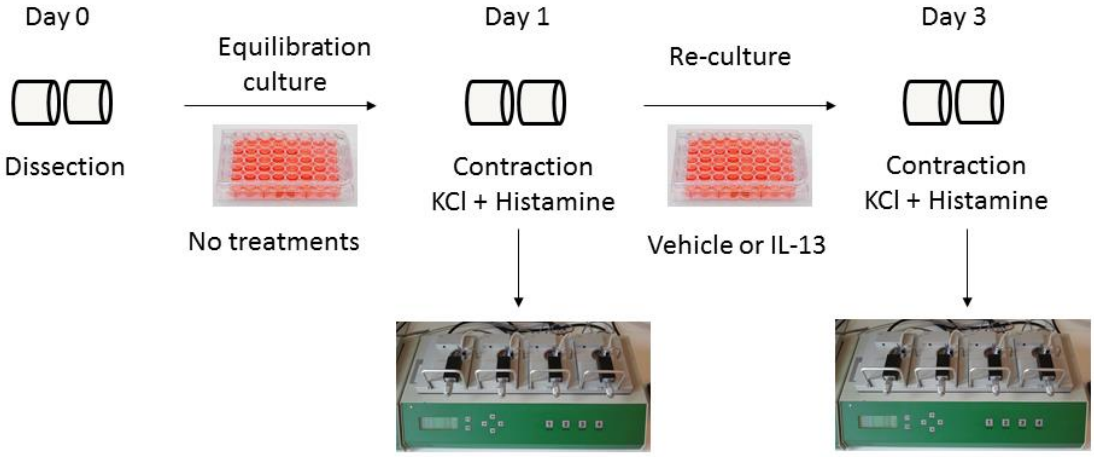

Figure 2: Schematic scheme of the re-culture assay.

2.1.5 Organ bath – *in vitro* pharmacology

The main methodology used in this thesis involves the measurement of contractions and relaxations in isolated smooth muscle preparations in the presence of different pharmacological interventions. Smooth muscle preparations were isolated from guinea pig, mice and humans and included airway smooth muscle (trachea or bronchi), vascular smooth muscle (aorta and pulmonary artery), gastrointestinal smooth muscle (taenia coli and ileum) and detrusor smooth muscle. Airway and vascular smooth muscle preparations were used as rings, whereas taenia coli [124], longitudinal ileum smooth muscle [125] and detrusor smooth were prepared as strips.

Following dissection, smooth muscle preparations were mounted in 5-10 ml organ baths or myographs filled with Krebs Henseleit buffer that was maintained at a temperature of 37 ºC and continuously bubbled with 5% CO_2 in O_2 to maintain a pH of 7.4. Changes in smooth muscle force were detected using an isometric force-displacement transducer linked to a Grass polygraph. Responses were displayed using LabChart 7 software.

Smooth muscle preparations were stretched and equilibrated for (1-2 hours) before the assessment of the viability of the smooth muscle preparations using either histamine or 60 mM of potassium chloride (KCl). The detailed experimental protocols for the different research studies can be found in the individual papers.

2.1.6 Airway smooth muscle cell culture

Human airway smooth muscle (HASM) cells were commercially purchased from Lonza or Promocell and grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in a humidified incubator. Confluent HASM cells were used for gene expression studies or measurements of intracellular Ca^{2+}. Stimulation experiments were conducted on HASM cells that were growth-arrested for 24-hours in serum-deprived medium containing 0.3% FBS. Detailed descriptions of the different protocols used can be found in the methods sections of studies III and IV.

2.1.7 RNA/cDNA isolation and synthesis

RNA from lysed HASM cells and isolated smooth muscle tissue preserved in RNAlater®, was extracted according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen, Hilden, Germany). To prevent genomic DNA contamination, a DNase step was always included. Concentration and purity were assessed using a Nanodrop 3300 spectrophotometer-

RNA (320-1000 ng) was transcribed to cDNA using Quantitect® Reverse Transcription kit (Qiagen) or High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

2.1.8 Real-time polymerase chain reaction (PCR)

Real-time PCR was performed using Taqman® primers. All samples were analysed in duplicate and in the presence of relevant negative controls (-RT and water). In experiments where the levels of expression were reported, gene expression levels were normalized against the endogenous control β-actin.

Changes in mRNA expression in intervention studies (cytokines, siRNA and glucose/serum deprivation) were calculated using the $^{\Delta\Delta}$Ct method. Normalization in these studies was conducted using the average of three endogenous controls that were individually chosen for each experimental set-up.

2.1.9 Small interfering RNA (siRNA)

Our aim was to develop an efficient siRNA transfection protocol in HASM cells that could be used to down-regulate the expression of TAS2Rs.

Airway smooth muscle cells were seeded on day 1 in 6-wells plate at a density of 10^5 cells per well and grown in normal DMEM culture medium. On day 2, the medium was replaced with 900 µl DMEM growth medium. Commercially available siRNAs (Qiagen) were mixed with 6-12 µl HiPerfect (Qiagen) in Opti-MEM® medium (Life technologies) in a total volume of 100 µL. This mixture was vortexed and incubated for 10 minutes at room temperature and then added to the cells in a drop-wise fashion. The same procedure was repeated on day 3 and cells were harvested for evaluation of mRNA expression on day 4. Functional experiments using measurements of intracellular Ca^{2+} mobilisation were conducted on day 5.

The transfection uptake of siRNA of HASM cells following different amounts of transfection reagent (6 and 12 µL) was evaluated using an immunofluorescence technique (**Figure 3**). These studies demonstrated that the uptake of siRNA was improved by increasing the concentration of HiPerfect® transfection reagent. The viability and growth of the HASM cells was not affected by the transfection reagent.

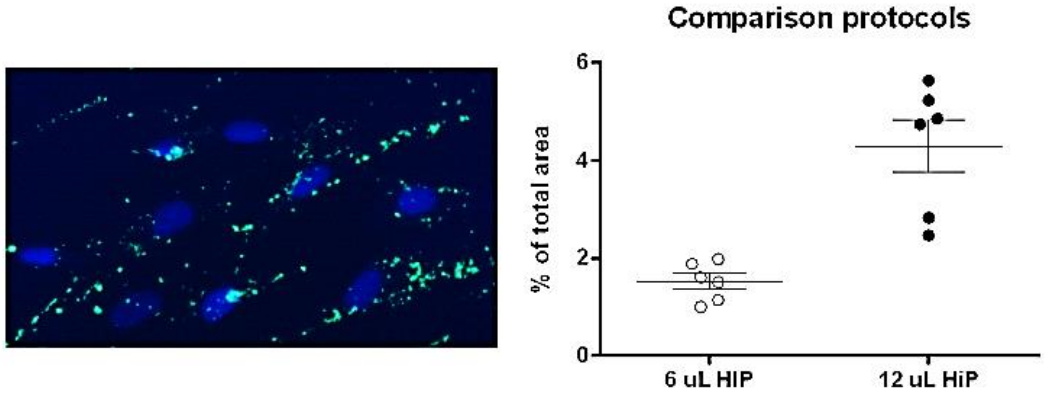

Figure 3: Relative immunofluorescence 24-hours after transfection of HASM cells transfected with an AlexaFluor 488-labeled siRNA.

The knock-down efficacy of the different transfection protocols was evaluated by examining the down-regulation of MAPK1 by RT-PCR, as the positive control siRNA in our experiments was directed against MAPK1. Changes in mRNA were compared to negative siRNA, which exhibited a similar expression pattern compared to those cells treated with transfection reagent only. Repeated transfection using 12 µL of transfection reagent resulted in a robust decrease in MAPK1 mRNA (79%; **Figure 4**). The observed siRNA-induced reduction in mRNA is in line with previous reports of similar procedures in HASM cells [126]. No attempts were made to further investigate whether this improved efficacy was a consequence of the time or the 2nd transfection. For the actual experiments, HASM cells were transfected with 40 nM siRNA (Negative) or 4x10 nM (TAS2R14) and 12 µL of transfection reagent on two consecutive days.

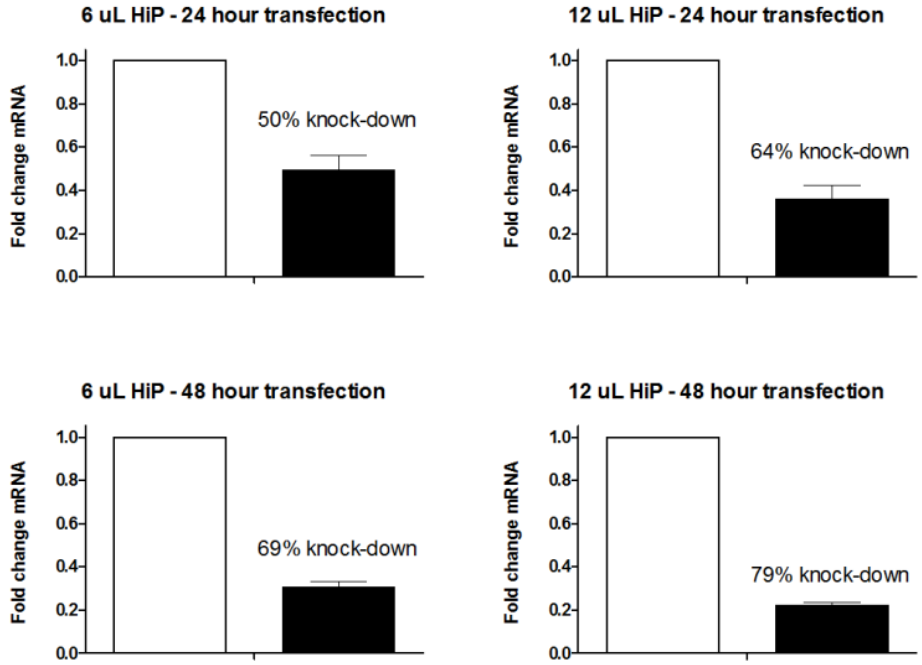

Figure 4: Evaluation of MAPK1 knock-down after different siRNA transfection protocols. Changes in MAPK1 mRNA expression were compared between HASM cells treated with Allstars-negative or MAPK1 siRNA (40 nM) using the comparative C_t ($2^{-\Delta\Delta Ct}$) method. Data are presented as means ± SEM.

2.1.10 Measurements of mobilisation intracellular Ca^{2+}

Mobilisation of intracellular Ca^{2+} in HASM cells was measured in studies III and IV for different purposes. A detailed description of the dye-loading protocol can be found in the method section of study IV. Briefly, HASM cells were detached as usual using trypsin/EDTA, incubated at room temperature for thirty minutes in PBS containing 3 µM Fluo-4-AM, 0.02% pluronic F-127 and 2.5 µM probenecid. Following a wash with probenecid, HASM cells were re-suspended at a concentration of 10^6 cells per mL in Dulbecco's PBS (DPBS) containing Ca^{2+} Mg^{2+}, 10 mM glucose and 0.1% BSA. Pluronic F-127 was used to enhance solubilisation of the dye Fluo-4-AM, whereas probenecid was included in the protocol to block the anion transporter and thereby prevent leakage of Fluo-4 from the cell. Ca^{2+} fluxes were analysed by flow cytometry (LSR Fortessa®).

In both studies, the baseline mean fluorescence intensity (MFI) was measured for thirty seconds, which was followed by the administration of histamine and a subsequent recording of ninety seconds. In **paper III**, HASM cells were pre-incubated in the dark for 5-minutes with vehicle or TAS2R agonists before measurements of baseline MFI and the induction of intracellular Ca^{2+} by histamine (100 µM). In **paper IV**, the mobilisation of intracellular Ca^{2+} following the non-cumulative administration of histamine (0.1-100 µM) was evaluated in HASM cells that were cultured for 24-hours in the presence of vehicle, IL-13, dexamethasone or dexamethasone and IL-13.

Data was analysed using FlowJo X software (Ashland, OR, USA). Histamine-induced induction of intracellular Ca^{2+} was presented as a percentile increase from baseline MFI (**Paper III and IV**). In addition, in order to determine potential shifts in potency in **paper IV**, the increases for the individual concentrations of histamine were normalized against the maximal induction of Ca^{2+} (100 µM of histamine) within each individual group.

2.1.11 Enzyme-linked immunosorbent assay (ELISA)

The production of cAMP and release of PGE_2 in the guinea pig trachea upon the administration of chloroquine and denatonium, was measured by ELISA in study I. As cAMP is an intracellular second messenger with a rapid turnover rate, levels were measured in liquid nitrogen snap-frozen guinea pig tracheal rings that were pre-treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine [127]. PGE_2 levels were determined in the organ bath fluid that was collected during different time-points of the relaxant responses. Salbutamol (cAMP) and bradykinin (PGE_2) were used as positive controls for these studies. PGE_2 levels were measured using Cayman Chemical's "Prostaglandin E_2 EIA kit – Monoclonal" (cat nr 514010.1). The detection limit was 15 pg/ml with cross-reactivity's towards PGE_3 (43.0%), 8-iso PGE_2 (37.4%), PGE_1 (18.7%) and 6-keto PGF_1 (1%), 8-iso $PGF_{2\alpha}$ (0.25%) and 13,14-dihydro-15-keto PGE_2 (0.02%). cAMP levels were measured by Cayman Chemical's "Cyclic AMP EIA kit" (cat nr 581002). The detection limit was 3 pmol/ml with a cross-reactivity towards cGMP (0.69-1.5%), but not towards AMP, ATP and adenosine (<0.01%).

2.1.12 Calculations and statistics

In organ bath experiments, contractions were expressed in relation to the tissues own maximal response or a reference contraction and for specific purposes in **paper IV** as absolute contraction in mN. Relaxations were presented as a percentile change from pre-contraction and related to maximal relaxations within the tissue. A non-linear regression analysis with a variable slope was used to calculate pEC_{50} from concentration-response curves.

Statistics and data analysis were performed using GraphPad Prism® software and all data in the different studies are presented as mean ± SEM. For comparisons between two groups, a Mann-Whitney or paired-t test was used. One-way or two-way ANOVAs were used to compare between 3 or more groups, single or two-independent variables respectively. To correct for multiple comparisons, Dunnett's post-hoc test was used for many-to any comparisons and otherwise a Bonferroni's post-hoc test was used. The exact test used for specific comparisons is described in the different manuscripts.

3 AIMS OF THE THESIS

The overall aim of this thesis was to investigate whether the effects of TAS2R agonists and IL-13 on airway smooth muscle could be targets for the development of future asthma therapies.

Specific aims

- To characterize the effects of TAS2R agonists on the airway smooth muscle function in cells and intact rodent and human airways.

- To investigate whether the effects of TAS2R agonists are specific for airway smooth muscle, by examining the effects of bitter tastants in vascular and other smooth muscle preparations.

- To define the possible mechanism(s) via which TAS2R agonists elicit bronchorelaxation.

- To assess the involvement of TAS2Rs in the bronchorelaxation by TAS2R agonists.

- To determine the effect of IL-13 on airway smooth muscle function in relation to airway hyperreactivity.

4 RESULTS AND DISCUSSION

4.1 CHARACTERISATION OF EFFECTS TAS2R AGONISTS

Initial functional experiments demonstrating that airway smooth muscle can be relaxed by TAS2R agonists, were primarily conducted in mouse airway smooth muscle [67]. To further increase our understanding of the relaxant activities of TAS2R agonists, investigations with a broader panel of TAS2R agonists were performed in the guinea pig trachea, a model that resembles human airway pharmacology better than murine models [128]

4.1.1 TAS2R agonist selection

Guinea pig orthologues for TAS2R1, TAS2R3, TAS2R4, TAS2R10, TAS2R14, TAS2R16, TAS2R41 and TAS2R42 were identified using the Ensembl database (ensembl.org). For functional characterization of these receptors, TAS2R agonists were selected on the basis of their reported activities in TAS2R-overexpressing HEK cells [78] (**Table 2**). Chloroquine was considered to be a specific agonist for TAS2R3, as 1000-fold higher concentrations were needed to elicit a response in TAS2R10-overexpressing HEK cells (**Table 2**). Similarly, denatonium was deliberated to selectively activate TAS2R10, as its activity at this receptor was 100-fold greater than at TAS2R4, and no orthologues were found for the other TAS2Rs that denatonium can activate in HEK cells.

TAS2R agonist	TAS2R1	TAS2R3	TAS2R4	TAS2R10	TAS2R14	TAS2R16	TAS2R40	TAS2R41
Chloroquine	-	10 µM	-	10000 µM	-	-	-	-
Denatonium	-	-	300 µM	3 µM	-	-	-	-
Dextromethorphan	10 µM	-	-	10 µM	-	-	-	-
Noscapine	-	-	-		10 µM	-	-	-
Quinine	-	-	10 µM	10 µM	10 µM	-	10 µM	-
Thiamine	1000 µM	-	-	-	-	-	-	-
Saccharine	-	-	-	-	-	-	-	-
Quercetin[#]	nd.	nd.	nd.	nd.	1 µM	nd.	nd.	nd.

Table 2: Minimal concentrations needed to elicit a positive Ca^{2+} signal in TAS2R-overexpressing HEK cells. This information is based on the work from Meyerhof et al [78]. [#]Quercetin was recently identified as a new agonists for TAS2R14 in the same cellular system, however its activity at other TAS2Rs has not yet been determined (nd.).

4.1.2 Airway smooth muscle

4.1.2.1 Guinea pig trachea

To study relaxations, guinea pig tracheal rings were pre-contracted with the muscarinic agonist carbachol, followed by the cumulative administration of the TAS2R agonists thiamine (TAS2R1), chloroquine (TAS2R3), denatonium (TAS2R10), noscapine (TAS2R14), dextromethorphan (TAS2R1&10) or quinine (TAS2R4, 10 & 14). Chloroquine, denatonium, dextromethorphan, noscapine, quinine and thiamine all induced relaxation following muscarinic pre-contraction of guinea pig trachea (**Figure 5**). These TAS2R agonist-mediated relaxations are in line with observations made in the mouse, where

chloroquine, denatonium and quinine were shown to relax methacholine-induced contractions [129, 130]. On the other hand, saccharine, an agonist for TAS2R31 that is known to relax human bronchi [67], did not elicit relaxations in the guinea pig trachea (**Paper I, figure 1G**). This finding supports the possibility that these agonists exert their effects via TAS2Rs, as the receptor for saccharine (TAS2R31) was lacking in the guinea pig genome.

To further evaluate the potential of TAS2R agonists in settings of relevance to asthma, the effects of chloroquine and denatonium were examined following antigen-induced contractions of the guinea pig trachea. This contraction is similar to indirect bronchoconstriction in humans and is caused by the release of spasmogenic mast cell mediators [131]. Denatonium could not reverse antigen-induced contractions in the guinea pig trachea (**Paper I, figure 4A**). Neither could denatonium relax pre-contractions induced by the individual components of the antigen response including histamine, cysteinyl-leukotrienes and U-46619 (TP-receptor agonist) (**Paper I, figure 4B-D**), indicating that the effects of this TAS2R10 agonist are dependent on the type of pre-contraction. Two other agonists for TAS2R10, dextromethorphan and quinine, were also unable to relax TP receptor-mediated contractions (**Figure 5**). On the other hand, chloroquine completely relaxed antigen-induced contractions (**Paper 1, figure 4A),** strengthening its potential as an anti-asthmatic intervention.

Furthermore, chloroquine, denatonium, dextromethorphan, noscapine and quinine did not induce baseline contractions in guinea pig tracheal rings. These experiments were conducted in the presence of the cyclooxygenase inhibitor indomethacin, to prevent the development of spontaneous tone as a consequence of PGE_2 production.

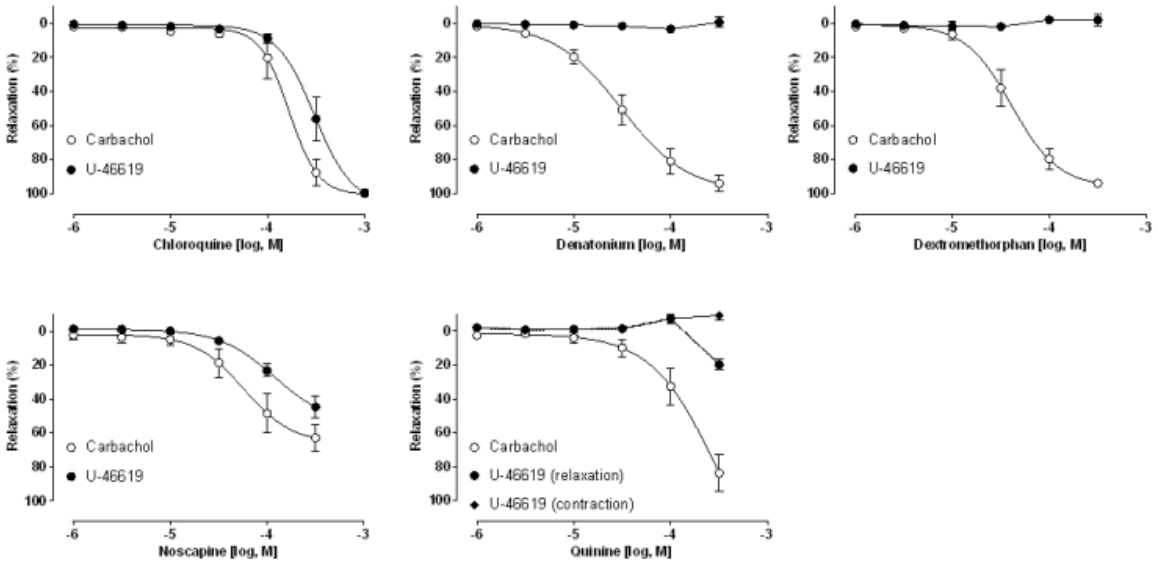

Figure 5: Cumulative-concentration responses for the TAS2R agonists chloroquine, denatonium, dextromethorphan, noscapine and quinine in guinea pig tracheal rings pre-contracted by 100 nM carbachol (○) or 100 nM U-46619 (●). Data are presented as means ± SEM (n: 4-8).

4.1.2.2 Mouse trachea

In the mouse trachea, chloroquine, denatonium and noscapine elicited relaxations following pre-contractions induced by carbachol. These relaxations were also more potent than in guinea pig trachea. However, the efficacy and potency of chloroquine- and denatonium-mediated relaxations were markedly lower following pre-contractions with U-46619 (**Figure 5**). Conversely, the potency of noscapine was increased following pre-contraction with U-46619 in mouse trachea (**Figure 6**).

The impact of the type of pre-contraction on TAS2R-agonist mediated relaxations, was also supported by studies by Tan and colleagues, who showed that the potency of chloroquine-mediated relaxations were reduced when mouse airways were pre-contracted by 5-HT [130]. In addition, the first study reporting the actions of denatonium, only showed relaxations following muscarinic pre-contractions [67].

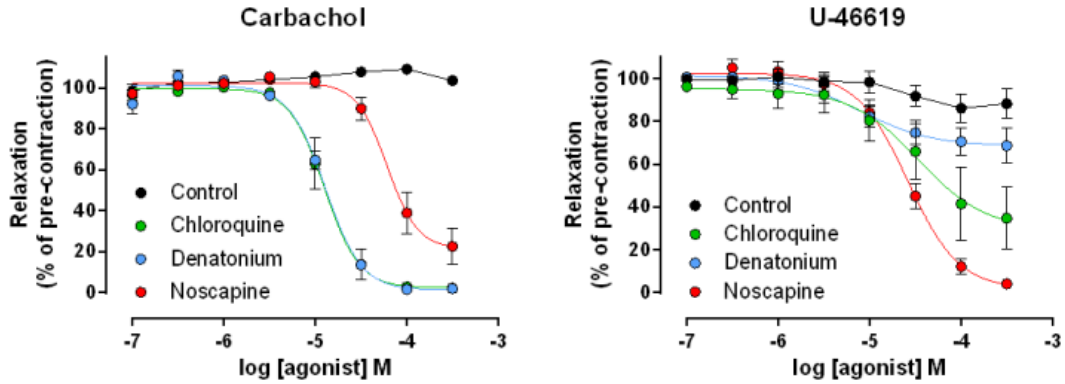

Figure 6: Cumulative-concentration responses for the TAS2R agonists chloroquine, denatonium and noscapine in mouse trachea pre-contracted by carbachol or the TP-receptor agonist U-46619.

4.1.2.3 Human bronchi

Investigations in intact human airways demonstrated that the TAS2R agonists chloroquine, dextromethorphan, noscapine and the recently identified TAS2R14 agonist quercetin, exerted relaxations of both histamine- and carbachol-induced pre-contractions (**Paper III, figure 3 and Paper IV, figure 3**). The potency of chloroquine-induced relaxations was almost 10 fold-greater in human airways compared to the guinea pig trachea, whereas the potency and efficacy of dextromethorphan and noscapine were similar. Furthermore, the capacity of these agonists to relax pre-contractions other than those caused by muscarinic stimuli, excludes the possibility that these TAS2R agonists induce relaxation via antagonism of muscarinic receptors.

4.1.2.4 Summary

Taken together, our findings in guinea-pig, mouse and human airways clearly indicate that TAS2R agonists induce relaxation of airway smooth muscle by distinct mechanisms that are probably linked to different components of excitation-contraction coupling in the airway smooth muscle.

4.1.3 Vascular smooth muscle

4.1.3.1 Guinea pig aorta

To assess whether relaxations induced by TAS2R agonists were specific to airway smooth muscle, the effects of TAS2R agonists on vascular smooth muscle function were first examined in the guinea pig aorta.

Chloroquine, denatonium, dextromethorphan, noscapine and quinine induced complete relaxations in aortic rings pre-contracted with the α_1-adrenoceptor agonist phenylephrine. The potencies of the relaxations were similar for the different TAS2R agonists tested in the guinea pig aorta. Moreover, the potencies of chloroquine-, noscapine- and quinine-mediated relaxations were greater in the aorta compared to the guinea pig trachea. The relaxations were unaffected by denudation of the endothelium, indicating that TAS2R agonists exert their effects directly on the smooth muscle and not via the release of endothelium-derived mediators.

Similar to the airway smooth muscle, TAS2R agonist relaxations in vascular smooth muscle were shown to be dependent on the agonists used for pre-contraction. Thus, denatonium, dextromethorphan and quinine were unable to relax contractions caused by U-46619 or $PGF_{2\alpha}$ (**Paper II, figure 1**), which corresponds with their inability to relax contractions induced by the TP-receptor agonist U-46619 in guinea pig trachea (**Figure 5**).

In order to examine the effects of TAS2R agonists on contractions that occur independently of G-protein-coupled receptor activation, guinea pig aortic rings were pre-contracted by potassium chloride. All TAS2R agonists elicited slow and relatively minor relaxations of the depolarisation-induced contractions evoked by potassium chloride (**Paper II, figure 1**).

4.1.3.2 Human pulmonary artery

In order to translate the guinea pig findings to human vascular smooth muscle, the responses induced by TAS2R agonists were examined in human pulmonary arteries (HPA). Human pulmonary arteries expressed mRNA for TAS2R3, TAS2R10 and TAS2R14, suggesting that the receptors for chloroquine, dextromethorphan and noscapine are expressed in HPA (**Paper II, figure 7**). Functional experiments demonstrated that chloroquine, dextromethorphan and noscapine induced strong relaxations of phenylephrine-induced contractions. However, when HPA rings were pre-contracted by U-46619, the relaxations induced by chloroquine were impaired, the effects of dextromethorphan were negligible, whereas the responses to noscapine remained equally as strong (**Paper II, figure 6**). These observations were similar to the findings in the guinea pig aorta, indicating that the effects of TAS2R agonists in vascular smooth muscle are similar between species.

4.1.4 Gastrointestinal smooth muscle

Contractions in the airway and vascular smooth muscle are primarily induced by the IP_3-driven mobilisation of Ca^{2+} from intracellular stores [132]. On the other hand, the excitation-contraction coupling in the smooth muscle of the gastrointestinal or urogenital tract is different and depends to a greater extent on other Ca^{2+} signalling mechanisms [40, 132]. Therefore, the effects of TAS2R agonists were studied in the ileum and the detrusor smooth muscle. This was done in an attempt to provide new information on the mechanism of action of the TAS2R agonists.

Due to the phasic nature of the contractions in ileum and detrusor smooth muscle, the ability of TAS2R agonists to inhibit the development of contractions in these preparations was examined. Pre-incubation for five minutes with the selected TAS2R agonists chloroquine, denatonium, dextromethorphan, noscapine and quinine prevented the development of contractions caused by both histamine and acetylcholine in guinea pig ileum longitudinal smooth muscle (**Figure 7**). The TAS2R agonist-mediated inhibitions were, with the exception of denatonium-treated segments, reversible after a wash-out period (**Figure 7**). The effects of denatonium in the guinea pig ileum differ from our observations for denatonium in the guinea pig trachea, where denatonium did not affect histamine-induced contractions and responses were reversible after wash-out. This raises the possibility that denatonium exerts its effects in the guinea pig ileum via another pathway.

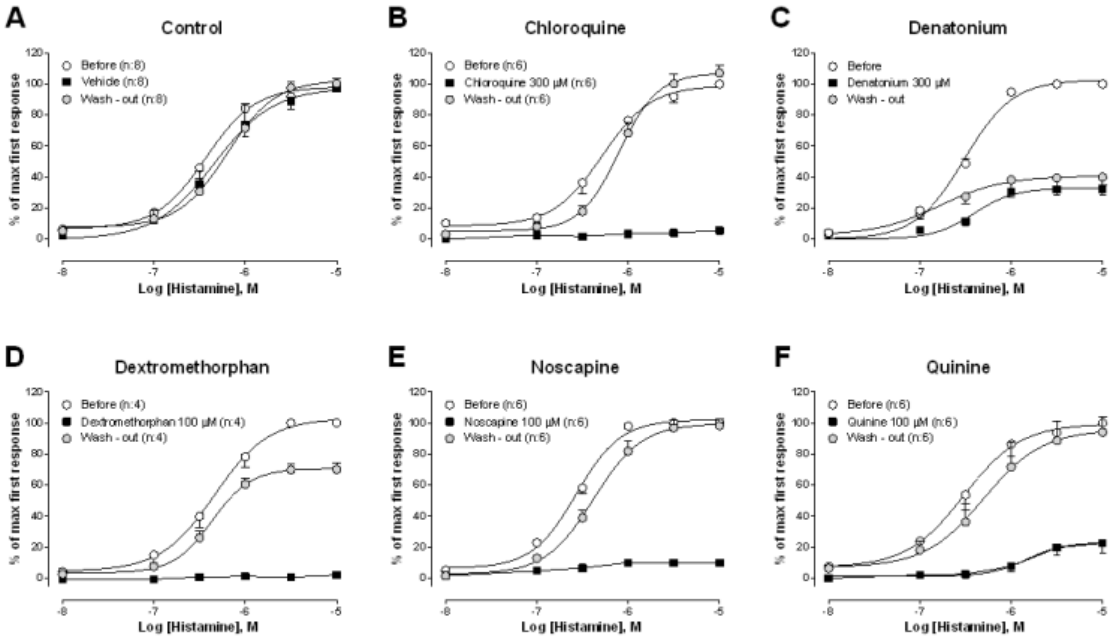

Figure 7: Cumulative concentration- responses to histamine in guinea pig longitudinal smooth muscle preparations before, after 5-min pre-stimulation with the different TAS2R agonists and after wash-out. Data are presented as mean ± SEM.

Other patterns of inhibitions were observed when contractions were mediated by non-cumulative administrations of PGE_2. Thus, the PGE_2-induced contractions were inhibited by dextromethorphan, but not by chloroquine, noscapine and quinine (**Figure 8**). In line with previous experiments, our experiments with the selective EP_1 receptor antagonist ONO-8130 demonstrated that contractions induced by PGE_2 in the guinea pig ileum were mediated by the EP_1 receptor [133]. However, dextromethorphan-mediated inhibitions were not a consequence of EP_1 receptor antagonism, as contractions induced by the EP_1 receptor agonist 17-trinor-phenyl-PGE_2 in the guinea pig trachea were unaffected by dextromethorphan.

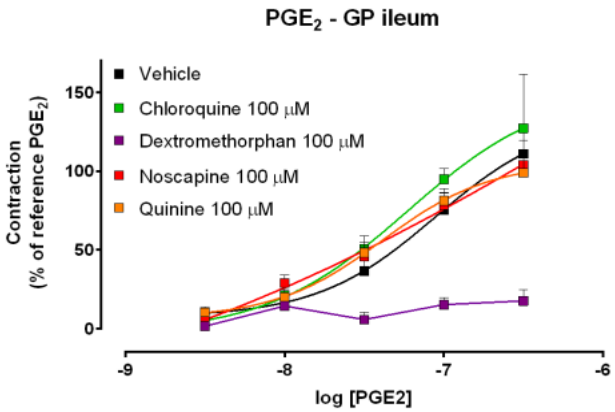

Figure 8: TAS2R agonist-mediated inhibitions of contractions by PGE_2 in guinea pig ileum

4.1.5 Detrusor smooth muscle

The detrusor smooth muscle is a unique smooth muscle preparation in which contractions are induced primarily via membrane depolarization as a result of the opening and closure of Ca^{2+} and K^+ channels [132, 134]. The effects of TAS2R agonists were evaluated in detrusor smooth muscle, because these channels have been implicated in the relaxations caused by TAS2R agonists [67, 135],

All selected TAS2R agonists (100 μM) enhanced the amplitude of the spontaneous baseline activity in guinea pig detrusor smooth muscle (**Figure 9A-B**). This increase in spontaneous activity was, again with the exception of denatonium, completely reversible. These findings regarding the myogenic tone are of particular interest due to the recent suggestion that bitter tastants (e.g. denatonium) trigger bladder reflexes via the release of acetylcholine [136]. However, our study demonstrated that the TAS2R agonist-induced increases in spontaneous activity were resistant to atropine, excluding the possibility that the effect was caused by acetylcholine release. Instead, our data suggest that the effects of the TAS2R agonists reflect an interaction with a certain family of ion channels, in particular potassium channels [137].

More distinct effects of the different TAS2R agonists were observed following muscarinic-mediated contractions, as dextromethorphan profoundly inhibited carbachol-induced contractions, chloroquine and noscapine exerted smaller inhibitions, and denatonium and quinine were without effect (**Figure 9C**). On the other hand, the maximal response of contractions induced by the purinergic P_2X agonist α,β-methylene ATP, were not affected by any of the TAS2R agonists **(Figure 9D**). However, the return to baseline of contractions induced by α,β-methylene ATP was more rapid in the presence of dextromethorphan.

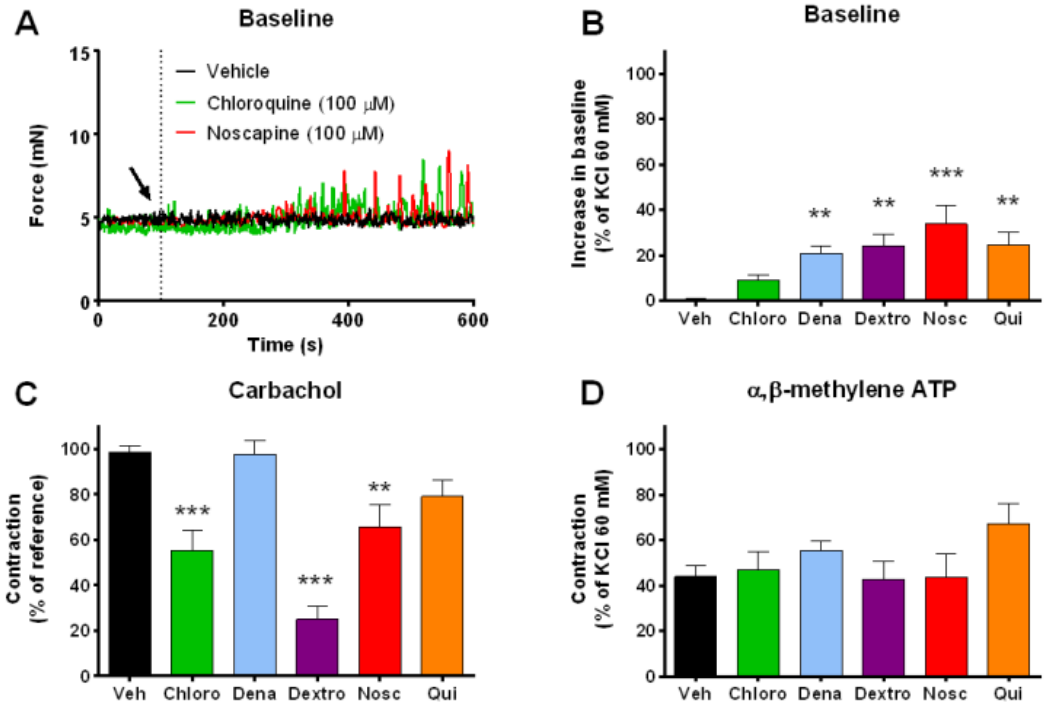

Figure 9: Effects of TAS2R agonists in detrusor smooth muscle. **A**) Experimental traces of the increased spontaneous activity that develops after the administration of TAS2R agonists. **B**) Compiled data on the TAS2R agonist- induced increases in the amplitude of baseline responses TAS2R-agonist mediated inhibitions of contractions by **C**) carbachol- and **D**) α,β-methylene-ATP. TAS2R agonists (100μM) were administrated 15-minutes before the evaluations of contractile responses. Data are presented as mean ± SEM.

Complementary studies in detrusor smooth muscle confirmed that contractions induced by carbachol and α,β-methylene ATP were largely dependent on the influx of extracellular Ca^{2+} via L-type Ca^{2+} channels, as these contractions were abolished in the presence of 10 μM nifedipine [137, 138]. Moreover, nifedipine prevented the TAS2R agonist-induced increases in myogenic activity.

The data in detrusor smooth muscle indicate that TAS2R agonists interact with at least two different signalling pathways. The TAS2R agonist-mediated inhibitions of muscarinic contractions probably reflect the same mechanism(s) that are responsible for the relaxation in airway and vascular smooth muscle. In addition, TAS2R agonists were shown to enhance myogenic activity, which highlights an interaction with another pathway that controls membrane depolarisation.

4.1.6 Summary

The scheme below provides a summary of the effects of the TAS2R agonists that were observed following the different pre-contractions in smooth muscle.

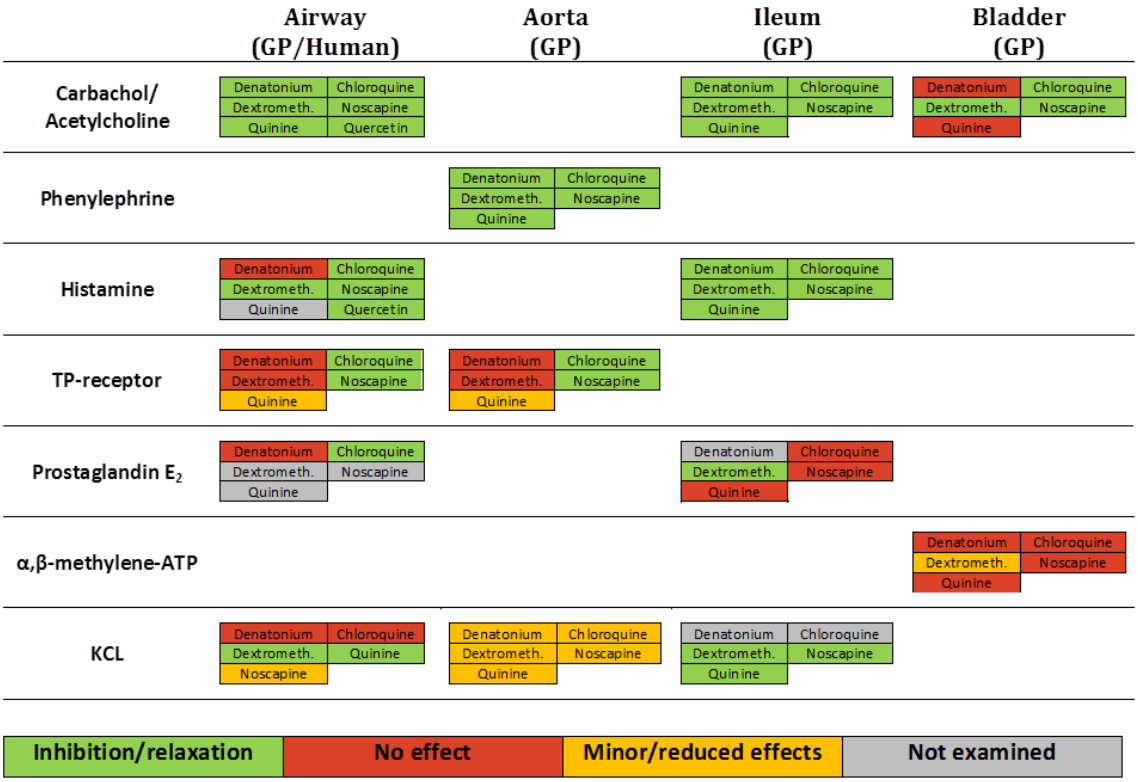

Figure 10: Overview of the effects of TAS2R agonists on the different pre-contractions in smooth muscle. Green indicates a profound inhibition of contractions or a strong relaxation. Red indicates no inhibitory effect. Yellow indicates a reduced relaxation/inhibition in relation to the inhibitions seen by the same TAS2R agonists within that particular smooth muscle tissue.

4.2 MECHANISMS UNDERLYING SMOOTH MUSCLE RELAXATION

Different pathways have been proposed to underlie the mechanism(s) of TAS2R agonist-induced bronchorelaxation. These mechanisms are presented in **Figure 12** and discussed below in relation to our own observations and those made by others.

4.2.1 Cyclic nucleotides (cAMP/cGMP)

Asthma therapies, such as β_2-adrenoceptor agonists or theophylline, induce bronchodilation by increasing the production of cAMP or reducing the degradation of cyclic nucleotides (cAMP and cGMP). As TAS2R agonists have been suggested to exert their effects via modulation of cyclic nucleotides [139, 140], possible interactions between TAS2R agonists and cyclic nucleotides were evaluated in the airway smooth muscle.

Denatonium-induced relaxations were examined in the presence of blockers of protein kinase A and G. The responses to denatonium were not affected by PKA or PKG blockers indicating that the downstream effector pathways of cAMP and cGMP are not required for the relaxations (**Paper I, figure 6D**). A similar finding has been observed for chloroquine, which failed to activate PKA in HASM cells [67]. In addition, we measured the production of cAMP in guinea pig trachea using an ELISA. Administration of the β_2-adrenoceptor agonist salbutamol as a positive control strongly increased cAMP levels in the trachea. In contrast, denatonium and chloroquine failed to raise cAMP levels **(Paper I, figure 6E**). Taken together, these findings indicate that TAS2R agonists elicit bronchorelaxation independently of cyclic nucleotides.

4.2.2 Hyperpolarization via BK channels

Deshpande and colleagues hypothesised that hyperpolarisation via opening of large conductance Ca^{2+} activated K^+ (BK) channels could induce relaxation of the airway smooth muscle via localised Ca^{2+} release [67].

To study the involvement of BK channels in TAS2R-agonist mediated relaxations, sections of guinea pig trachea were pre-treated with pharmacological blockers of BK channels; iberiotoxin, charybdotoxin and paxillin. Chloroquine-induced relaxations were not affected by the presence of BK blockers. On the other hand, relaxations induced by denatonium, as well as salbutamol, were decreased in potency following treatment with BK blockers (**Paper I, figure 6A-C**). These initial experiments suggested that BK channels were required in part for relaxations occurring due to denatonium and salbutamol. However, we also observed that the blockade of BK channels resulted in stronger pre-contractions [141]. Additional experiments demonstrated that stronger pre-contractions alone can decrease the potency of denatonium and salbutamol in the guinea pig trachea (**Paper I, figure 7**). This indicates that the apparent inhibition of denatonium-induced relaxations by BK channel blockers was a consequence of the increased level of pre-contraction, rather than an effect of the BK channel itself. We and others have therefore concluded that BK channels are not critical for the relaxation induced by TAS2R agonists [142-145].

4.2.3 Localised induction of intracellular Ca^{2+}

In their initial study, Deshpande et al showed that millimolar concentrations of TAS2R agonists were able to increase intracellular Ca^{2+} levels in HASM cells to a similar degree as common spasmogens such as bradykinin and histamine [67]. However, others could detect only minor, or no increases at all, in intracellular Ca^{2+} following the administration of millimolar concentrations of TAS2R agonists in HASM cells or mouse precision-cut lung slices (PCLS) [130, 135]. Moreover, although relatively high concentrations (10-100 µM) of TAS2R agonists were required to achieve complete relaxations of human and animal airways, these concentrations were still markedly lower than the millimolar concentrations that were required to increase intracellular Ca^{2+} levels in HASM cells. Therefore it is unlikely that the observed bronchorelaxation due to TAS2R agonists is the result of an induction of intracellular Ca^{2+}.

4.2.4 Inhibition of bronchoconstrictor-induced Ca^{2+} induction

As mobilisation of intracellular Ca^{2+} is critical for the induction and maintenance of smooth muscle contraction in general, it was considered plausible that TAS2R agonists elicit relaxation by inhibiting the bronchoconstrictor-induced mobilisation of intracellular Ca^{2+} in HASM cells [130, 135]. The effects of different TAS2R agonists on the histamine-induced mobilisation of intracellular Ca^{2+} were therefore examined in HASM cells. The TAS2R agonists chloroquine, denatonium, dextromethorphan and quercetin were selected for these investigations, because these agonists induced relaxations in human airways that were pre-contracted by histamine.

Following a thirty-second baseline measurement, the addition of histamine caused a rapid increase in intracellular Ca^{2+} in HASM cells. This increase in intracellular Ca^{2+} peaked after fifteen seconds and then slowly returned to baseline. HASM cells were pre-treated with TAS2R agonists or vehicle for 5-minutes before this measurement. Chloroquine prevented this histamine-induced mobilisation of intracellular Ca^{2+} in a concentration-dependent manner (**Figure 11)**. It is noteworthy, that chloroquine relaxed the human bronchi at the same concentrations that inhibited Ca^{2+} fluxes in HASM cells. Similarly, quercetin also inhibited mobilisation of intracellular Ca^{2+} to an extent that was comparable to the relaxations observed in human airways (**Figure 11)**.

In contrast, dextromethorphan and noscapine, agonists that relaxed human bronchi well, only modestly inhibited the mobilisation of intracellular Ca^{2+} in HASM cells (**Figure 11)**. It may be that the time of pre-incubation was not sufficient to reveal an inhibition, and it is therefore a limitation that the effects of longer pre-incubation times with the TAS2R agonists were not assessed in this study. However, our data in human bronchi demonstrated that these TAS2R agonists induce relaxations within 5-minutes of exposure. Therefore, the observed inhibitions of intracellular Ca^{2+} mobilisation in HASM cells do not seem to explain the bronchorelaxation that these agonists elicit in human bronchi. Taken together, these studies demonstrate the distinct inhibitory actions of TAS2R agonist on intracellular Ca^{2+} mobilisation in HASM cells, indicating that TAS2R agonists do not induce bronchorelaxation via one common mechanism.

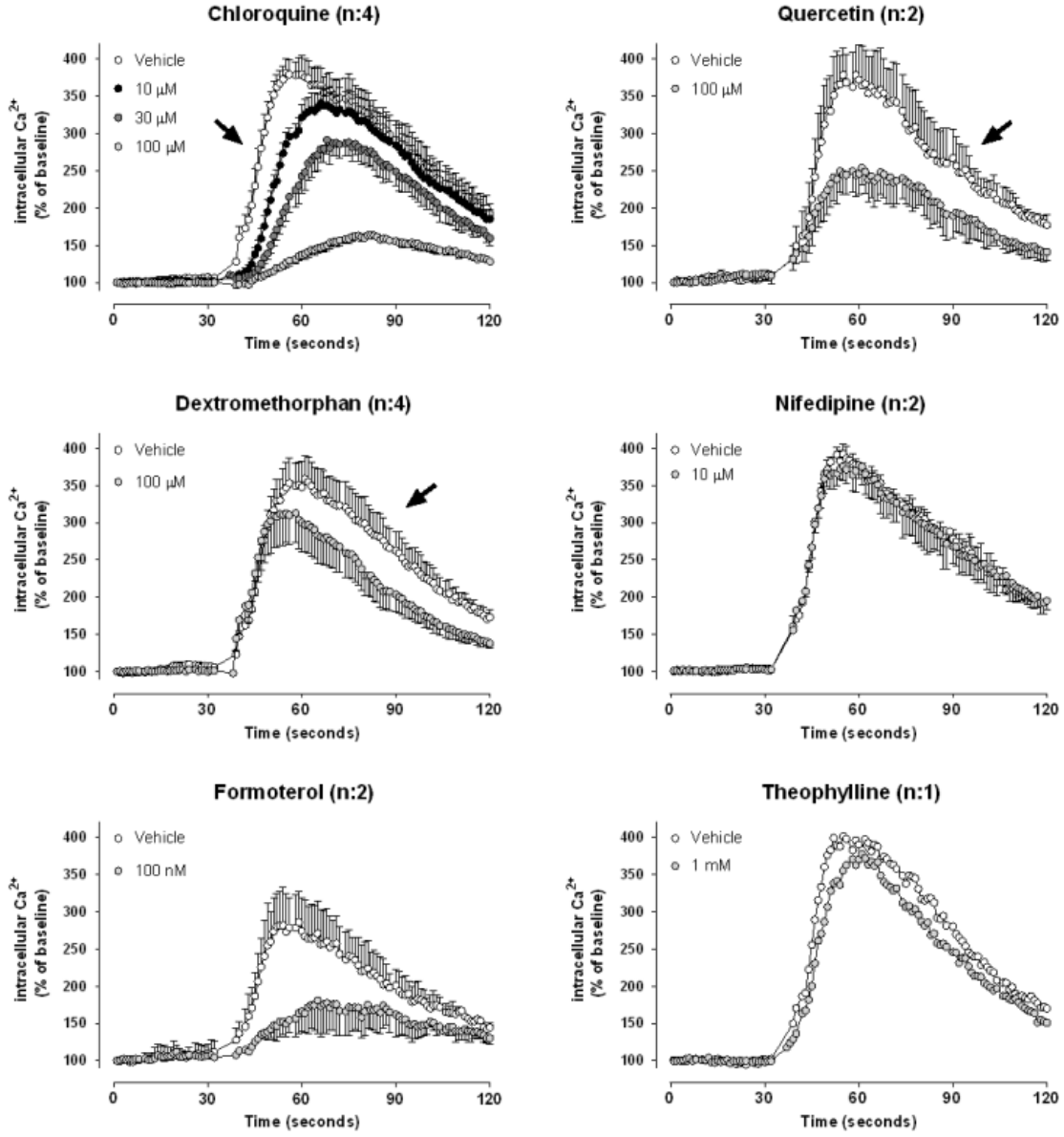

Figure 11: Histamine-induced mobilisation of intracellular Ca^{2+} in HASM cells measured by Fluo-4 in the presence of TAS2R agonists, the L-type Ca^{2+} channel blocker nifedipine, the β_2-adrenoceptor agonist formoterol and the phosphodiesterase inhibitor theophylline. Increases in intracellular Ca^{2+} were presented as percentile increase of baseline. Data for each time-point were obtained from 200-400 cells. **Arrows:** indications for which component of the induction (the initiation phase or the sustained phase) of intracellular Ca^{2+} is inhibited.

Moreover, different components of the histamine-induced mobilisation of intracellular Ca^{2+} were decreased by the different TAS2R agonists tested. Chloroquine inhibited the initial phase of the Ca^{2+} mobilisation, whereas quercetin predominantly decreased the later stage of the intracellular Ca^{2+} mobilisation (**Figure 11, arrows**). This distinction is most clearly exemplified by comparing the inhibitions caused by chloroquine (30 µM) and quercetin (100 µM). At these concentrations the two agonists decreased the maximal induction of intracellular Ca^{2+} to a similar degree, but the time to the peak of the Ca^{2+} induction was only reduced by chloroquine. Similarly to quercetin, dextromethorphan specifically inhibited the later phase of the calcium mobilisation that seemed to be critical for the sustainment of intracellular Ca^{2+} (**Figure 11, arrows**).

Histamine has been shown to induce mobilisation of intracellular Ca^{2+} in HASM cells through IP_3, and to sustain levels of intracellular Ca^{2+} via the influx of extracellular Ca^{2+} [146]. The patterns of the inhibitions of intracellular Ca^{2+} mobilisation observed in our study therefore provide suggestions for the Ca^{2+}-dependent pathways that TAS2R agonists interact with. The sustained phase is considered to be dependent on the influx of extracellular Ca^{2+} via an L- type Ca^{2+} independent pathway[146]. The latter is confirmed in our study as nifedipine had no effects on the histamine-induced mobilisation of intracellular Ca^{2+} (**Figure 11**).

4.2.5 Inhibition of IP_3 signalling

The observation that chloroquine particularly inhibits the initial phase of the intracellular Ca^{2+} mobilisation in HASM cells, suggests that chloroquine interferes with IP_3 signalling. Although, our own studies did not specifically investigate the effects of chloroquine on IP_3 signalling, there is evidence in the literature that supports such a statement, as Tan and colleagues demonstrated that 100 µM of chloroquine inhibited IP_3-induced Ca^{2+} release in HASM cells [130]. The same authors observed that IP_3-induced Ca^{2+} release in HASM cells was inhibited by quinine, and to a lesser extent by denatonium [130]. Moreover, studies by Misra and colleagues demonstrated that chloroquine- and quinine-mediated inhibitions of IP_3 signalling were caused by a blockade of IP_3 receptors and not a decreased synthesis of IP_3 [147].

Our data in the different smooth muscle preparations demonstrated that chloroquine consistently inhibited contractions induced by agonists of G-protein coupled receptors (**Paper I and II**). Because all of these agonists depend on IP_3-induced Ca^{2+} for their contractions, it is plausible that chloroquine exerts its effects in smooth muscle via an inhibition of IP_3 signalling. This mechanism could be also valid for the structurally related TAS2R agonist quinine, as its potency for IP_3 inhibition in HASM cells was comparable to the effect of quinine in airway smooth muscle [130]. On the other hand, the data from Tan and colleagues showing that denatonium inhibited IP_3 signalling [130], are more difficult to link to our observations in airway smooth muscle, as denatonium failed to relax contractions induced by histamine in the guinea pig trachea.

In addition, our experiments in HASM cells showed that both chloroquine and the β_2-adrenoceptor agonist formoterol prolonged the time to peak for the induction of intracellular Ca^{2+} in HASM cells (**Figure 11**). The observed effects of formoterol on IP_3 signalling are in line with previous reports that have shown that β_2-adrenoceptor agonists can reduce IP_3 synthesis or inhibit IP_3 signalling via cAMP and PKA [63, 148]. Overall, it can be concluded that chloroquine and quinine presumably relax airway smooth muscle by inhibiting IP_3 signalling, but this mechanisms does not apply to all TAS2R agonists.

4.2.6 Inhibition of L-type Ca^{2+} channels

An alternative mechanism proposed to be responsible for TAS2R-mediated relaxations is that TAS2R agonists inactivate voltage-dependent L-type Ca^{2+} channels [135].

To test this hypothesis, we assessed the potential L-type blocking activities of the different TAS2R agonists using potassium chloride-mediated contractions in the guinea pig trachea, a functional assay that was shown to be sensitive to the action of the L-type Ca^{2+} channel blocker nifedipine. Contractions induced by potassium chloride were prevented by dextromethorphan and quinine, whereas noscapine induced only minor inhibitions of the potassium-induced contractions, and denatonium and chloroquine were without effects in this assay (**Paper II, figure 3B**). These experiments indicated that chloroquine and denatonium do not exert L-type blocking activities at a concentration that was sufficient to induce relaxations of airway smooth muscle. On the other hand, dextromethorphan and quinine may act as inhibitors of L-type channels.

Our follow-up investigations in the guinea pig aorta demonstrated that all TAS2R agonists elicited complete relaxations of phenylephrine-induced pre-contractions in an experimental set-up where L-type channel activities should be completely inhibited (**Paper II, figure 3C**). Although, for certain TAS2R agonists, these observations could be due to antagonism of noradrenergic receptors (**paragraph 4.2.7.2**), the data clearly indicated that the TAS2R agonists chloroquine and noscapine also induce relaxation of airway and vascular smooth muscle independently of an interaction with L-type channels.

Dextromethorphan-induced inhibitions of contractions in airway smooth muscle (KCl, carbachol, histamine), ileum (histamine, acetylcholine and PGE_2) and bladder (carbachol, α,β-methylene-ATP) (**Figure 10**), can be mimicked by nifedipine, suggesting that dextromethorphan induces relaxation by inhibiting L-type-Ca^{2+} channels. However, this preliminary conclusion can be questioned by the observation that dextromethorphan failed to prevent its own induction of myogenic activity in detrusor smooth muscle, whereas nifedipine could. Moreover, our Ca^{2+} mobilisation studies in HASM cells revealed that dextromethorphan inhibits a specific component in the induction of intracellular Ca^{2+} that is linked to the influx of extracellular Ca^{2+} [146], but does not require the involvement of L-type Ca^{2+} channels (**Figure 11**). This suggests that dextromethorphan exerts parts of its effects in smooth muscle via inhibition of other types of voltage-dependent Ca^{2+} channels [96-98].

4.2.7 Antagonism of G-protein coupled receptors

4.2.7.1 Muscarinic antagonism

As denatonium selectively relaxed pre-contractions induced by carbachol in airway smooth muscle, the question arose as to whether denatonium antagonized muscarinic (M_3) receptors. However, acetylcholine-induced relaxations in guinea pig aorta, which are dependent on muscarinic receptor activation [149], were not affected by denatonium. Moreover, denatonium failed to inhibit carbachol-mediated contractions in guinea pig detrusor smooth that are dependent on M_3 receptor activation [137]. Therefore, the data suggest that relaxations caused by denatonium are not due to antagonism of muscarinic receptors, but rather are the consequence of a specific inhibition of the muscarinic excitation-contraction coupling process in airway smooth muscle.

4.2.7.2 Noradrenergic antagonism

Similarly, TAS2R agonists were shown to selectively relax noradrenergic contractions in vascular smooth muscle. The possible antagonism of α-adrenoceptors was therefore evaluated in the guinea pig taenia coli, a preparation in which α_1-adrenoceptor activation results in relaxation [124]. In this assay, denatonium and quinine completely prevented relaxations induced by phenylephrine, indicating that the effects of these TAS2R agonists in vascular smooth muscle are mediated by antagonism of α-adrenoceptors.

4.2.8 Summary on the mechanisms of TAS2R agonists

Our studies demonstrate that TAS2R agonists do not induce bronchorelaxation via one single common pathway involving cAMP/cGMP, BK channels or L-type Ca^{2+} channels. Instead, the TAS2R agonists were shown to interact individually with different pathways implicated in the mobilisation of Ca^{2+} from intracellular and extracellular sources. Chloroquine, quercetin and quinine seem to relax airway smooth muscle by inhibiting IP_3 signalling, whereas dextromethorphan may exert its effects via the inhibition of voltage-dependent Ca^{2+} channels. The pathways underlying denatonium-, and especially noscapine-, induced responses require further investigations.

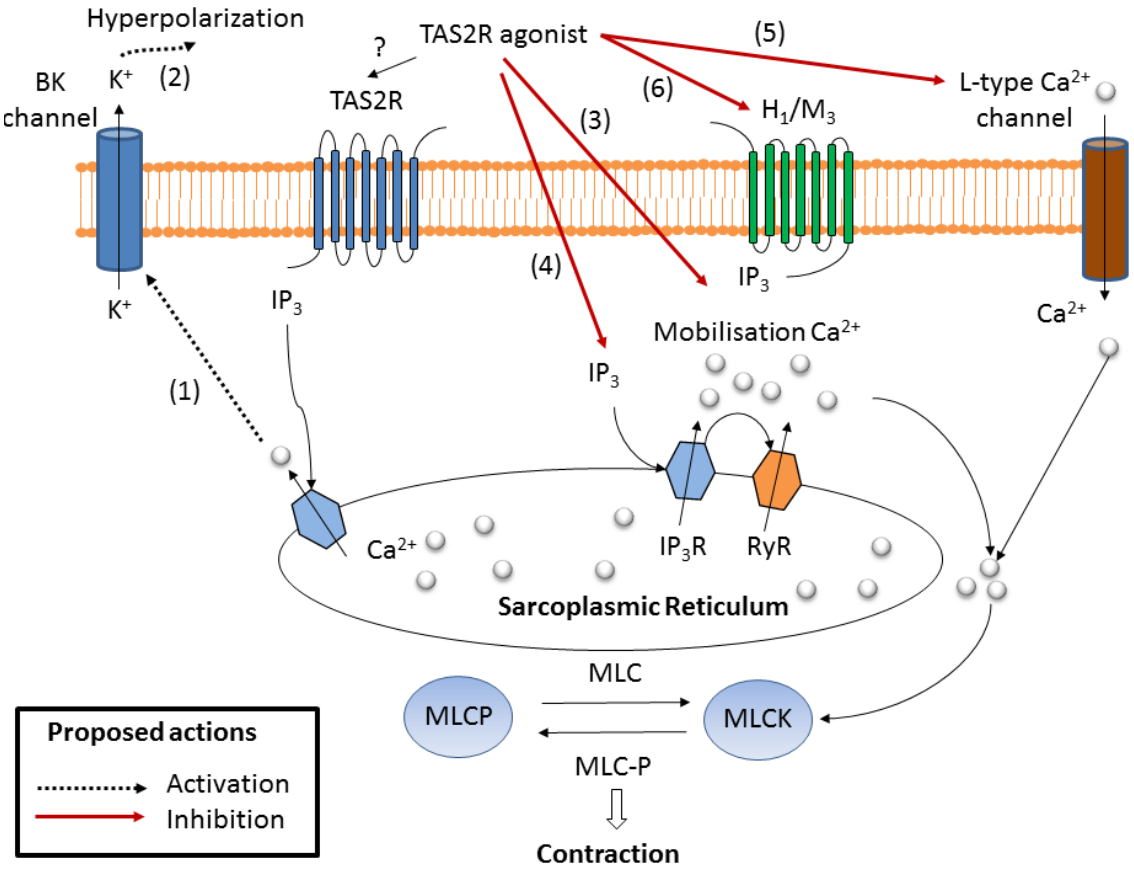

Figure 14: Summary of proposed pathways for TAS2R-agonist mediated relaxation, interpretation of these mechanisms is provided in the text:

1) Localised induction of intracellular Ca^{2+}
2) Hyperpolarization via opening BK channels
3) Inhibition of bronchoconstrictor-induced mobilisation of intracellular Ca^{2+}
4) Inhibition of IP3 signalling
5) Inhibition of L-type Ca2+ channels
6) Antagonism of G-protein coupled receptors

4.3 REQUIREMENT OF TAS2RS FOR BRONCHORELAXATION

The critical question that emerges is whether or not the observed actions of TAS2R agonists in the airway smooth muscle and other cell-types are mediated via the TAS2Rs. This question is particularly relevant as TAS2Rs are low affinity receptors activated by relatively high concentrations of TAS2R agonists [78, 150], which increases the possibility that TAS2R agonists may interact with other pathways, and exert their effects, including bronchorelaxation, via TAS2R-independent pathways.

The optimal way to assess this would be to investigate whether TAS2R agonists cause relaxation of airway smooth muscle from TAS2R-deficient animals, or in the presence of antagonists. To our knowledge, only a TAS2R5-deficient mouse has been developed [72] and although TAS2R antagonists are under development, no antagonists are currently available against the receptors for chloroquine (TAS2R3), denatonium (TAS2R10), dextromethorphan (TAS2R1 and TAS2R10), noscapine (TAS2R14) and quercetin (TAS2R14) [151-155].

Therefore, an alternative two-step strategy was employed to determine TAS2R involvement in bronchorelaxation. First, studies were performed that aimed to define conditions that could increase/decrease TAS2R expression in HASM cells. Thereafter, the functional consequence of modulated TAS2R expression was evaluated by examining TAS2R-agonist mediated inhibitions of intracellular Ca^{2+} mobilisation in HASM cells, as this mechanism seems to be responsible for TAS2R agonist-induced bronchorelaxation (**paragraph 5.2.4**).

4.3.1 Inflammation

The impact of inflammatory mediators and asthma-mediations on TAS2R expression was investigated in HASM cells, as studies have suggested that TAS2R expression is regulated by asthmatic inflammation [82]. The mRNA expression of all TAS2Rs was reduced by TNFα and dexamethasone, whereas the cytokines IL-13 and TGFβ that are implicated in the pathogenesis of asthma did not affect TAS2R expression (**Paper 3, table 1**).

Although the functional consequences of these effects were not further investigated in HASM cells, our finding that the effects of chloroquine and noscapine in IL-13-treated human bronchi are essentially unaltered (**Paper 4, figure 3C-D**), is in line with the lack of effect of IL-13 on TAS2R expression in HASM cells.

4.3.2 Serum-deprivation

As studies in cardiomyocytes have suggested that TAS2R expression increases upon starvation, the effects of glucose and serum-deprivation on TAS2R expression in HASM cells were investigated [85]. In contrast to the results in cardiomyocytes, glucose-deprivation failed to affect TAS2R expression in HASM cells. On the other hand, serum-deprivation increased the expression of mRNA for TAS2R3, TAS2R4, TAS2R10 and TAS2R14 in HASM cells in a time-dependent matter (**Paper 3, figure 2**). These increases were accompanied by an increased expression of the smooth muscle marker α-actin that reflects the serum-deprivation-induced induction of a more contractile phenotype of cultured HASM cells [156].

Interestingly, our findings are in line with the relatively high TAS2R expression that Deshpande and colleagues reported in their HASM cells following 7 days of serum-deprivation [67].

When examining the effect of chloroquine on histamine-induced mobilisation of intracellular Ca^{2+} in HASM cells, chloroquine-mediated inhibitions were greater following 48-hours of serum deprivation (**Paper 3, figure 5**). On the other hand, the inhibitions of histamine-induced mobilisation of intracellular Ca^{2+} by the TAS2R14 agonists noscapine (≈15%) and quercetin (≈50%) remained similar in this set-up, despite an increase in TAS2R14 mRNA expression.

Methodological concerns prevent strong conclusions to be drawn from these observations. For example, the absence of antibodies prevented us from verifying whether increased TAS2R mRNA expression resulted in increased protein expression at the time-points that the functional experiments were conducted. Moreover, it is difficult to conclude whether the increased effects of chloroquine are a direct consequence of increased TAS2R3 expression, or whether they are related to serum-deprivation-induced phenotypic changes in the HASM cells.

4.3.3 siRNA-mediated knock-down of TAS2Rs

A more selective approach to modulate the expression of TAS2Rs in HASM cells was therefore chosen, involving the use of siRNAs. For these investigations, an efficient siRNA transfection protocol for HASM cells was developed that decreased MAPK1 mRNA by 79% two days-after the initial transfection (**Figure 4**).

Attempts to decrease TAS2R3 using pooled and individual siRNAs were ineffective, and prevented us from assessing the requirement for TAS2R3 in responses towards chloroquine. On the other hand, pooled siRNAs against TAS2R14 reduced TAS2R14 mRNA expression in HASM cells by 50% (**Paper 3, Figure 6**). The efficacy of this TAS2R14-mediated knockdown in HASM cells was comparable to recent siRNA attempts at reducing TAS2R1 [157], and higher than the 35% reduction for TAS2R10 reported by Deshpande et al, when the function of TAS2Rs in HASM cells was first described [67].

Functional measurements were conducted one day after the evaluation of mRNA knock-down, in order to allow any effect on TAS2R14 protein to become apparent. In these experiments, quercetin-mediated inhibitions of intracellular Ca^{2+} mobilisation were no different between mock, negative siRNA and TAS2R14 siRNA treated HASM cells (**Paper III, figure 7**). Although it is possible that TAS2R expression is regulated by mechanisms other than transcription, the data suggest that TAS2R14 is not critical for the effects of the TAS2R14 agonist quercetin on Ca^{2+} mobilisation in HASM cells [150].

4.4 AIRWAY HYPERREACTIVITY TO IL-13

4.4.1 Human bronchi - potency

To investigate the effects of IL-13 on airway smooth muscle function in intact human small airways, contractile and relaxant responses were assessed in paired human bronchial rings that were cultured for two days in the presence or absence of IL-13. IL-13 profoundly increased the potencies of the spasmogens histamine (3.1-fold) and carbachol (2.5-fold) in intact human small airways (**Paper IV, figure 1**). These findings confirm and extend previous observations made in precision-cut lung slices (PCLS) of human small airways, where in a limited study, IL-13 was shown to increase the potency of carbachol [121].

4.4.2 Human bronchi - amplitude

To examine whether IL-13 affected the amplitude of contractile responses in the human bronchi, a novel re-culture procedure for human bronchial rings was developed, enabling assessment of the impact of IL-13 on individual bronchial rings. In this re-culture assay, we evaluated contractile responses towards KCl and histamine in untreated human bronchial rings on Day 1. After an extensive wash-out, these same bronchial rings were transferred back into culture and divided into two groups, one treated with a vehicle control and the other with IL-13. Following two-days of culture, contractile responses towards KCl and histamine were re-assessed in these bronchial segments (Day 3). The main advantage of this re-culture assay compared to investigations using paired bronchial segments, is that the outcome cannot be biased by variations in inner diameter, length and the organization and amount of airway smooth muscle in the bronchial segments.

The re-culture procedure itself did not affect the amplitude of contraction, as the contractions towards KCl and histamine in the bronchi of the vehicle group were similar in magnitude before (day 1) and after the re-culture period (day 3) (**Paper IV, figure 2**). Neither were the maximal contractions induced by KCl and histamine changed after the period of re-culture with IL-13 (**Paper IV, figure 2**). Nonetheless, in this assay the administration of IL-13 did result in an increased potency of histamine, indicating that IL-13 specifically increases the potency and not the amplitude of contractile responses in intact human airways (**Figure 13**).

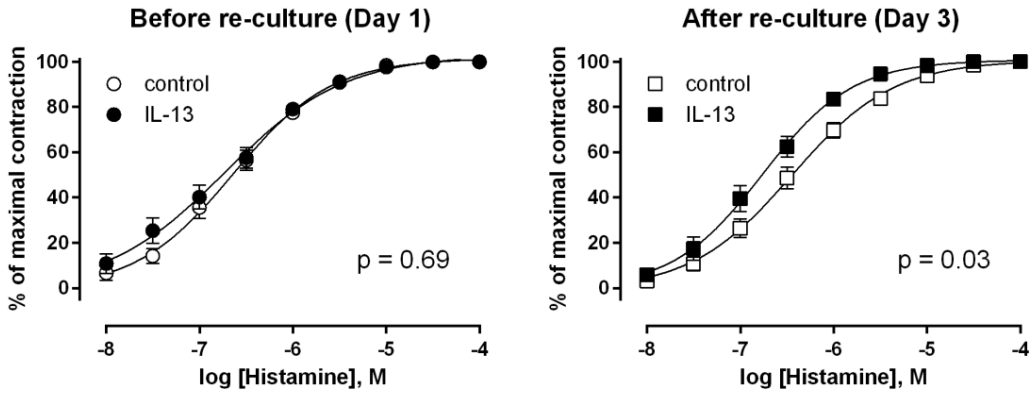

Figure 13: Cumulative concentration-responses towards histamine in human bronchial rings that did not receive any treatment (Day 1) or following a two day re-culture period during which the same bronchial rings were treated with vehicle or IL-13 (100ng/ml). Data are presented as mean ± SEM (n=15).

Our findings regarding the effects of IL-13 in human small airways differ from previous observations made in the airway smooth muscle of mice and rabbits, where IL-13 primarily increased the amplitude and not the potency of contractile responses [113-116]. These studies were however conducted in tracheal smooth muscle, which may explain the different results, because the excitation-contraction coupling is markedly different between bronchial and tracheal smooth muscle [158]. However, in the smaller airways of the mouse, IL-13 also increases only the amplitude, and not the potency of contractile agonists [159], indicating that IL-13 induces similar changes along the airway smooth muscle of the respiratory tree. Therefore, the discrepancies between the observed effects of IL-13 in animal airways (amplitude) and human airways (potency) must be a consequence of species differences and possibly implicate different mechanisms.

4.4.3 Mobilisation of intracellular Ca^{2+}

Airway smooth muscle contraction is initiated by agonist-induced mobilisation of intracellular Ca^{2+} [61], which has encouraged studies to examine the effects of IL-13 on intracellular Ca^{2+} mobilisation in HASM cells. These studies have shown that IL-13 can enhance the maximal induction of intracellular Ca^{2+} by different spasmogens in HASM cells, thereby providing a plausible mechanism for the IL-13-induced increase in the amplitude of contractions in intact airway smooth muscle [114, 129, 159-161].

However, previous studies have not investigated whether IL-13 also changes the potency of agonist-induced intracellular Ca^{2+} mobilisation in HASM cells. Therefore, we decided to study the consequences of IL-13 on the concentration-dependent mobilisation of intracellular Ca^{2+} in HASM cells. These studies clearly showed that IL-13 also increases the potency of histamine-induced intracellular Ca^{2+} mobilisation in HASM cells (**Paper IV, figure 4**), strongly supporting the view that a lower threshold for the mobilisation of intracellular Ca^{2+} in HASM cells is responsible for the IL-13-induced increase in potency of histamine in intact human airways.

4.4.4 Mechanisms underlying IL-13 induced-responses

Different pathways have been implicated in the effects of IL-13 in airway smooth muscle. This work did not permit in-depth studies to uncover the mechanisms underlying IL-13-induced hyperreactivity in the human bronchi. Therefore a summary of possible mechanisms in relation to our findings is discussed below and presented schematically in (**Figure 14**).

As discussed in the previous paragraph, IL-13 increases both the threshold and maximal mobilisation of intracellular Ca^{2+} in HASM cells (1). However, the pathway(s) that IL-13 utilises to increase the mobilisation of Ca^{2+} in HASM cells remain undefined. One possibility is that IL-13 increases the expression of the G-protein coupled receptors for the different spasmogens (2), which has been demonstrated for the $CysLT_1$ [162] and H_1 receptor [160, 163]. An increased receptor expression would result in more IP_3 formation and probably facilitate a greater influx of Ca^{2+} from intracellular pools and thus explain our effects in HASM cells.

Another pathway that has been linked to intracellular Ca^{2+} mobilisation in HASM cells is the CD38 /cyclic adenosine diphosphate ribose (cADPR) pathway (3). In HASM cells, IL-13 was shown to increase CD38 expression which controls the signalling molecule cADPR that mobilises Ca^{2+} via ryanodine channels [129]. This IL-13-induced induction of CD38 was shown to be sensitive to glucocorticoids [164]. However, as our studies demonstrated that dexamethasone failed to prevent the IL-13-induced hyperreactivity of human bronchi and HASM cells (**Paper IV, figure 5**), it is unlikely that CD38 is crucial for the IL-13-induced airway hyperreactivity.

IL-13 has been suggested to augment airway smooth muscle contraction via a Ca^{2+} independent pathway that involves Ca^{2+} sensitisation (4), as IL-13 increases RhoA expression [115]. RhoA can activate Rho kinases, which in their turn maintain contractions by inactivating/inhibiting the myosin light chain phosphatases (MLCP) in airway smooth muscle [165]. Further investigations in human bronchi should assess the involvement of Rho-kinase in IL-13-induced airway hyperreactivity.

One question that evolves from our findings is why IL-13 particularly affected the potency, and not the amplitude of contractions in the human bronchi, as previous studies have mainly observed effects on the amplitude of responses. To answer this question, it is important to emphasize that contractions are the end-result of a consecutive series of signalling events that are required for excitation-contraction coupling [40, 57]. Thus, although receptor number, the degree of IP_3 synthesis and the levels of intracellular Ca^{2+} are important in this series of events, the outcome or amplitude of contraction is still dependent of the rate-limiting steps of the system, which in this case are the down-stream effectors of contraction; calmodulin, actin and myosin. However, an increased receptor expression in a tissue that already contains a high density of receptors could induce a potency shift, rather than a change in amplitude [166]. This phenomenon has been demonstrated for the H_1 receptor in airway smooth muscle [167]. Therefore, the increased potency and maximal mobilisation of intracellular Ca^{2+} in

HASM cells, could explain why we observe an IL-13-induced increase in the potency of contractions in the human bronchus.

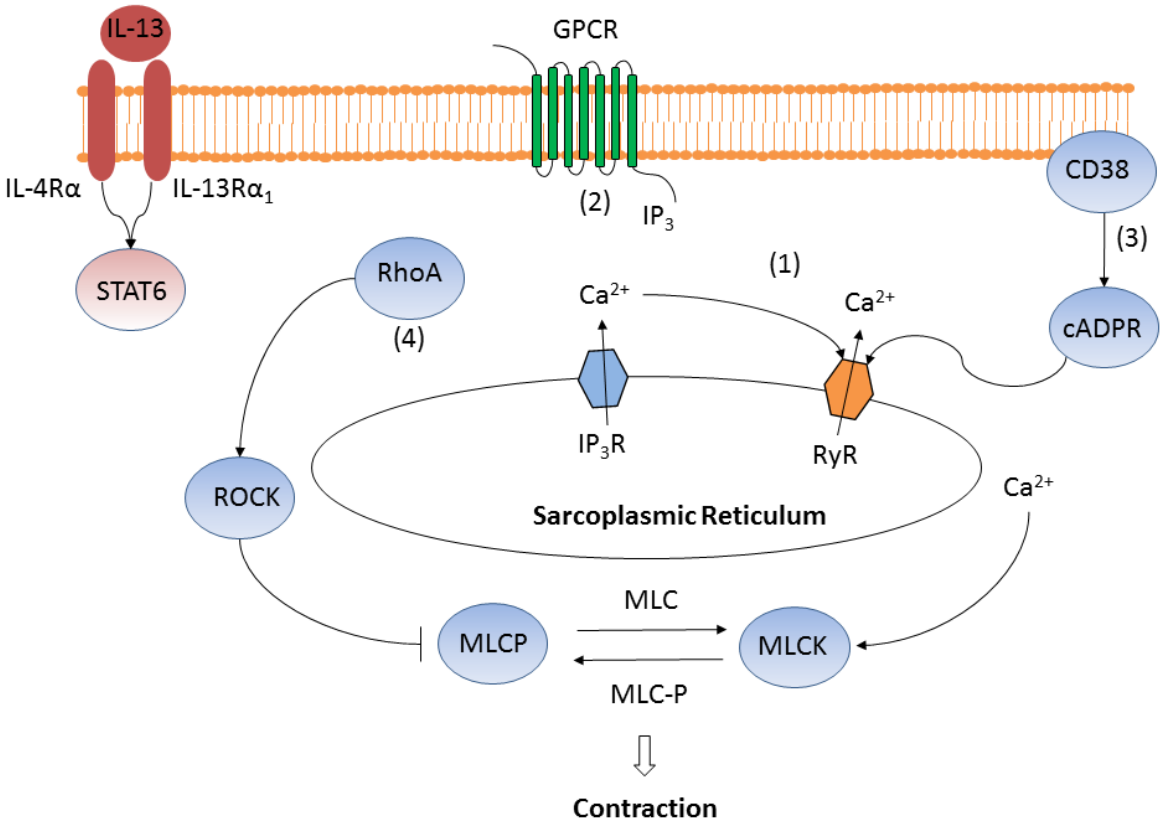

Figure 14: Proposed pathways via which IL-13 induces hyperreactivity of airway smooth muscle.

5 CONCLUSIONS

The main conclusions from this thesis are:

- TAS2R agonists induce strong relaxations in guinea pig, mouse and human airways that vary depending on the agonist used for pre-contraction.

- TAS2R agonists exhibit strong inhibitory actions in vascular, gastrointestinal and detrusor smooth muscle, indicating that the effects of TAS2R agonists are not restricted to airway smooth muscle.

- TAS2R agonists induce bronchorelaxation via distinct pathways, as they each inhibit specific components of the bronchoconstrictor-induced mobilisation of intracellular Ca^{2+} that reflect different pathways of Ca^{2+} entry.

- The involvement of TAS2Rs in the effects of TAS2R agonists on smooth muscle can be questioned because:
    - *TAS2R agonists exhibit different responses when pre-contractions are induced via the TP receptor.*
    - *TAS2R agonists distinctly affect histamine-induced mobilisation of* Ca^{2+}
    - *Agonists for the same receptor (TAS2R14) exert different effects.*
    - *Responses by TAS2R14 agonists remain unchanged despite altered TAS2R expression.*

- IL-13 induces hyperreactivity of intact human airways by increasing the sensitivity of airway smooth muscle cells to bronchoconstrictor-induced Ca^{2+} mobilisation. This study indicates that functional remodelling of the airway smooth muscle can contribute to airway hyperresponsiveness.

6 GENERAL DISCUSSION

The airway smooth muscle is a major contributor to the pathogenesis of asthma due to its involvement in airway obstruction and AHR. Targeting the airway smooth muscle to achieve bronchorelaxation via different pathways has therefore been an attractive therapeutic strategy for relieving asthma symptoms. The activation of relaxant pathways, via β_2-adrenoceptor agonists (and in certain cases theophylline), the antagonism of contractile receptors ($CysLT_1$, M_3) on the airway smooth muscle [168-170], as well reducing the amount of airway smooth muscle via bronchial thermoplasty [171] have all demonstrated clinical benefit for patients with asthma. Nonetheless, there remains an unmet need for new therapeutic bronchorelaxants, as patients with severe asthma may still experience severe airway obstruction, despite the use of high doses of glucocorticoids and a mix of short- and long-acting β_2-adrenoceptor agonists.

"Bitter treats for better breathing?" This question is derived from the initial work by Deshpande et al which suggested that TAS2Rs might be a potential novel target on the airway smooth muscle as TAS2R agonists were shown to induce efficacious relaxation [67, 172]. The aim of this thesis was to define the pharmacological actions of TAS2R agonists in (airway) smooth muscle and determine the potential of TAS2Rs and their agonists as new therapeutic agents for asthma.

To investigate the effects of TAS2R agonists on airway smooth muscle function, the guinea pig trachea was chosen as an experimental model, because the receptor pharmacology of the guinea pig closely resembles that of human airways [128]. By using a selection of defined TAS2R agonists [78], our studies demonstrated that TAS2R agonists are strong, but not potent, relaxants of airway smooth muscle (**Paper I-II**). These observations were subsequently confirmed in intact human airways both by ourselves and others (**Paper III-IV**) [144], thereby supporting the potential of TAS2R agonists as bronchorelaxants.

One requirement for a potential novel bronchorelaxant is that it should induce a strong bronchorelaxation that is not influenced by environmental factors such as increased bronchial tone or inflammation. In this aspect, our data in the guinea pig, as well as human bronchi, demonstrate that under conditions when responses to the short-acting β_2-adrenoceptor agonist salbutamol are impaired, for instance under high tone (**Paper I**) or during IL-13 induced inflammation (**Paper IV**), TAS2R agonists can induce a more efficacious relaxation than the commonly used β_2-adrenoceptor agonists.

The strong responses that TAS2R agonists display in smooth muscle preparations are themselves of great interest, particularly as their relaxations were shown not to be a consequence of muscarinic antagonism, activation of BK channels, or earlier described pathways of relaxation that involve cAMP, PKA or PKG (**Paper I**). Instead, our data in HASM cells clearly demonstrate that TAS2R agonists can decrease the mobilisation of intracellular Ca^{2+} induced by contractile agonists (**Paper III**). These data provide support for

previous data showing that inhibition of IP_3 signalling could be an important pathway that is affected by TAS2R agonists[130]. However, this pathway is not affected by all TAS2R agonists, as noscapine exhibited only minor effects on intracellular Ca^{2+} mobilisation, whereas dextromethorphan seemed to inhibit the entry of extracellular Ca^{2+} (**Paper III**). Irrespective of whether the effects of TAS2R agonists are mediated via the TAS2Rs or not, these undefined pathways of relaxation alone present new potential targets for bronchorelaxation.

The involvement of TAS2Rs in the effects induced by TAS2R agonists is not currently easily evaluated, as gene-deficient animals and antagonists for the receptors of the TAS2R agonists are lacking. However, to circumvent these issues, we used the strategy of selectively decreasing the expression of TAS2R14 with siRNA and then examining possible effects on the inhibition of intracellular Ca^{2+} mobilisation in HASM cells caused by the TAS2R14 agonist quercetin. However, a reduction in TAS2R14 mRNA expression did not affect the inhibitory effects of quercetin in HASM cells (**Paper III).** Although these data suggest that TAS2R14 is not critical for the effects of quercetin, no firm conclusions can yet be drawn from these experiments, as we did not confirm that the siRNA-induced reduction of TAS2R14 mRNA also resulted in decreased protein levels.

Indirect evidence presented in this thesis does suggest that the TAS2R agonists elicit bronchorelaxation independently of TAS2R activation. This preliminary conclusion is based on the observations that the relaxations induced by TAS2R agonists are not mediated via a common pathway, as TAS2R agonists differently relax TP-receptor mediated contractions (**Paper I and II**) and distinctly inhibited the mobilisation of intracellular Ca^{2+} (**Paper III**). In addition, noscapine and quercetin, which both act on TAS2R14, differently inhibited the mobilisation of intracellular Ca^{2+} in HASM cells (**Paper III**). Furthermore, our statement regarding the lack of TAS2R involvement is supported by the recent observation that chloroquine inhibited the contractility of the mouse heart via a G-protein independent pathway [86]. Of relevance is also the finding that TAS2R agonists exert effects in cells that do not express TAS2Rs, for instance in the amoeba *Dictyostelium* [173]. Therefore, it would be of great interest to study responses towards TAS2R agonists in gene-deficient mice lacking α-gustducin, the G-protein assumed to be required for TAS2R signalling [73], as such investigations would allow more definitive conclusions to be drawn regarding the contribution of TAS2Rs to TAS2R agonist-induced bronchorelaxation.

Until TAS2R-deficient animals are developed, the tools available to explore the oral, and proposed extra-oral functions of TAS2Rs will be heavily dependent on the TAS2R agonists. Although the activities of the TAS2R agonists have been carefully characterized in TAS2R-overexpressing HEK-293 cells [78, 150], their use at relatively high concentrations increases the risk of interaction with TAS2R-independent pathways. Thus, in the absence of TAS2R antagonists, caution is warranted when the experimental effects of TAS2R agonists observed in cells/tissues are directly extrapolated to the biological functions of the TAS2Rs. Instead, the pharmacological analysis of the effects of the TAS2R agonists and their possible

interactions with TAS2R-dependent/independent pathways as presented in this thesis (**Paper I-III**), may serve as a guide for future investigations that intend to define the biological functions of TAS2Rs and their related agonists.

The last part of this thesis relates to the role of the airway smooth muscle in the development of AHR. Although the importance of the airway smooth muscle in AHR is undisputed, the mechanisms in airway smooth muscle that promote exaggerated airway narrowing remain unclear [30, 42, 52, 55]. The remodelling of the airways in asthma is thought to involve hyperplasia and hypertrophy of airway smooth muscle cells, but it is also possible that the intrinsic contractile properties of the airway smooth muscle are "remodelled" as a consequence of the inflammatory environment. Most of the data supporting such a role have been obtained from studies in cultured HASM cells and animal airways [30, 55]. However, such a phenomenon has only sparsely been investigated in humans, and if so, the proposed effects of certain mediators (e.g. TLR3) could not be reproduced in human airways [121].

Our study addressed the question of whether IL-13 could alter airway smooth muscle function in intact human airways, as IL-13 has been implicated in the development of hyperreactivity in animal airway smooth muscle [113-115, 117, 159]. Our investigations in human small airways clearly demonstrated that IL-13 can increase the potency of common spasmogens and thus induce direct hyperreactivity of the airway smooth muscle (**Paper IV**). This study is therefore unique as it is one the first studies to prove that inflammation (IL-13) can indeed alter the intrinsic contractile properties of intact human airway smooth muscle.

Our study primarily demonstrated the existence of IL-13-induced hyperreactivity in human airways, yet the mechanisms underlying this phenomenon remain incompletely understood. It would be important to investigate the involvement of different components of the IL-13 signalling pathway and assess whether the observed effects are dependent on activation of the IL-4Rα/IL-13Rα1 receptor complex and STAT-6 phosphorylation. Such investigations would also increase our understanding of why the glucocorticoid dexamethasone failed to prevent IL-13-induced hyperreactivity in both human bronchi and HASM cells (**Paper IV**), as one possible explanation is that glucocorticoids do not affect STAT-6 signalling [174]. Furthermore, it will be essential to determine the mechanisms involved in the IL-13-induced increases in contraction potency in human bronchi, and mobilisation of Ca^{2+} in HASM cells. One strategy to achieve this would be to perform microarrays in IL-13-treated human bronchi and HASM cells in parallel, in order to define mutual effector protein(s) that induce hyperreactivity in both human bronchi and HASM cells.

Finally we ask, what is the clinical importance of our findings? To answer this question we have to consider the source of IL-13, as IL-13 is not solely produced by T_h2 cells but also in significant amounts by mast cells [175]. Mast cells infiltrate the airway smooth muscle of patients with asthma [13] and their numbers within the airway smooth muscle correlate significantly with AHR [176]. This raises the possibility that localized IL-13 release from mast cells in close proximity to airway smooth muscle cells could induce hyperreactivity of the latter, and thus contribute to AHR. Notably, patients with eosinophilic bronchitis

displayed increased airway smooth muscle mass in the absence of AHR, indicating that AHR is not simply a consequence of increased airway smooth muscle mass [176]. Instead, these data suggest that inflammation causes a phenotypic alteration of airway smooth muscle cells which, in contrast to the morphologically apparent increase in cell growth, affects their function. In this thesis we propose the term "functional remodelling" to describe this process.

With respect to the observed IL-13-induced shifts in the potency of common spasmogens, it is important to emphasize that relatively small potency changes can result in profound effects *in vivo*, as airway resistance is inversely related to the 4^{th} power of the airway radius [177]. One query that needs to be addressed is whether the effects of IL-13 on the potencies of individual spasmogens are additive when the mediators are released together, for instance during the antigen-induced response, as this would provide more information on the maximal capacity of this IL-13-induced hyperreactivity.

There are certainly other pathways that contribute to AHR as well, but our data demonstrating that IL-13 induces functional remodelling of intact human airways, suggests that inflammation-induced alterations of the airway smooth muscle can contribute to AHR. Importantly, IL-13 is only one of the mediators released in the inflammatory environment of the airways in asthma. Thus, these data introduce the possibility that other mediators, alone or in combination, could have similar effects to IL-13, causing airway smooth muscle hyperreactivity and the development of AHR.

This thesis aimed to define the potential of the TAS2Rs and IL-13, in relation to their effects on the airway smooth muscle, to be targets for the development of future asthma therapies. Although, the importance of the TAS2Rs may be questioned, the strong actions of bitter tastants on smooth muscle function are undisputed and therefore warrant further investigations into their efficacious and intriguing pathways of relaxation. On the other hand, IL-13 was clearly shown to induce functional remodelling of the ASM in human airways that can contribute to the phenomenon of airway hyperresponsiveness.

7 POPULAR SUMMARY

Asthma is a disease of the airways in the lungs that affects more than 300 million people worldwide. The life-time risk of developing asthma life is around 35%, but most people develop the disease during childhood. Patients who have asthma experience symptoms of cough and shortness of breath, which result in breathing difficulties. These problems are caused by inflammation of the airways, which causes the smooth muscle layer around the airways to constrict (bronchoconstriction). This bronchoconstriction impairs the flow of air through the airways and causes the periods of shortness of breath. The airways of patients with asthma are also hypersensitive, as their airways can constrict in reaction to environmental factors (like cold air or tobacco smoke), which normally have no effect in healthy individuals. This phenomenon is called "airway hyperresponsiveness".

As patients often suffer from asthma for long periods of their lives, it is important that they receive good treatments to control the symptoms of their disease. Asthma therapy is focused on the control and relief of symptoms. Airway inflammation is controlled to prevent the long term effect of lung injury (using drugs called glucocorticoids). In case of an asthma attack, short-acting drugs (relaxants) can be used immediately to relieve the symptoms by reversing constriction of the airway smooth muscle. Although relaxants of the airway smooth muscle have been very successful in the treatment of asthma, patients with the most severe asthma do not always benefit from these treatments. There is therefore a need for new drugs to relax the airway smooth muscle.

In order to develop new treatment strategies for asthma, research aims to find new drug targets. A target of interest is the bitter taste receptors. These receptors (TAS2Rs) that allow us to sense the taste of bitter on our tongues, have also been found on airway smooth muscle cells. It is proposed that bitter substances relax airway smooth muscle via these receptors. The first part of this thesis investigated the effects of bitter substances on the airway smooth muscle, in order to evaluate their potential in the future treatment of asthma.

We found that many bitter substances relax the airway smooth muscle of humans, mice and guinea pigs. This was the case for quinine (the bitter component of tonic water), denatonium (used to prevent thumb sucking), dextromethorphan and noscapine (the latter both found in cough medicine). The contractions of airway smooth muscle depend on increases in intracellular calcium and we found that the bitter tastants reduce intracellular calcium in different ways. The pathways that caused the relaxation of airway smooth muscle were also found to be different from previously known pathways. This indicates that new pathways might be involved in the effects of the bitter tastants, and suggests that the bitter tastants might even relax the airway smooth muscle independently of TAS2Rs. Nonetheless, bitter tastants are strong relaxants in airway smooth muscle, presumably via novel pathways that warrant further investigation.

The last study of this thesis focused on the question of why the airways of patients with asthma are hypersensitive (airway hyperresponsiveness). One possible explanation for this phenomenon is that the inflammatory environment of the asthmatic airway changes the way in which the airway smooth muscle contracts. This hypothesis was tested in the lab by stimulating isolated human airways with interleukin-13 (IL-13), an important pro-inflammatory substance in asthma. Our experiments showed that human airways exposed to IL-13 contracted more easily. This indicates that inflammation can indeed alter the properties of airway smooth muscle and provides a possible explanation for why the airways of patients with asthma are hypersensitive.

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