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PROSTAGLANDIN E_2 AS MEDIATOR AND MODULATOR OF AIRWAY SMOOTH MUSCLE RESPONSES

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To boldly go where a lot of people have gone before

ABSTRACT

Prostaglandin E_2 (PGE_2) is a lipid mediator produced by virtually every cell of the human body. Because common non-steroidal anti-inflammatory drugs (NSAIDs) inhibit its biosynthesis, PGE_2 is usually considered to be a 'pro-inflammatory' mediator. The role of PGE_2 in the lung and airways has however always been unclear. In particular, the airway responses caused by activation of its four different EP receptors have been debated. Research on the mechanisms involved in the actions of PGE_2 has previously been limited by the low potency and selectivity of available pharmacological tools.

Recently, a number of potent receptor antagonists and enzyme inhibitors have become available. The aim of this thesis was therefore to characterise airway responses to PGE_2 in greater detail, focusing on the role of its receptors on baseline smooth muscle function and during antigen-induced contractions. Alongside investigating PGE_2 responses, the newly discovered relaxant effects of bitter tasting substances acting at their respective receptors (TAS2Rs) were examined.

The project mainly involved analysis of isometric contractions and relaxations in isolated airways from guinea pigs and humans in organ baths. In addition, mRNA expression of receptors and enzymes was analysed by PCR and prostanoid release was measured by chemical or immunological methods.

It was found that all four EP receptors, the two cyclooxygenases (COX-1 and COX-2) and three PGE synthases were expressed in the guinea pig trachea and lung. Exogenous PGE_2 induced a bell-shaped concentration-response curve, causing contraction at lower concentrations mediated by the EP_1 receptor and relaxation mediated by the EP_2 receptor at higher concentrations. The spontaneous airway tone was maintained by biosynthesis of endogenous PGE_2, mainly catalysed by COX-2 in the airway epithelium. The level of tone in the preparation was determined by the balance between activation of EP_1 and EP_2 receptors. The EP_1 receptor, but not the EP_2 receptor, displayed homologous desensitisation to endogenous PGE_2.

When the antigen-induced contractions of the guinea pig trachea were studied, it was found that the primary effect of PGE_2 was again to maintain the spontaneous airway tone. When this effect was blocked by EP_1 and EP_2 receptor antagonists, it was revealed that a component of the antigen-response was mediated by prostaglandin D_2 and thromboxane A_2, acting at the thromboxane TP receptor.

In human small airways, PGE_2 induced relaxations mediated by EP_4 receptors at low concentrations and contractions through TP receptors at higher concentrations. In addition, it was discovered that the IgE-dependent contraction of human bronchi could be abolished by the action of exogenous PGE_2 at the EP_2 receptor, an effect presumably involving inhibition of mast cell mediator release.

The bitter tasting substances chloroquine, denatonium, thiamine, and noscapine caused relaxations of human and guinea pig airways with a greater efficacy than beta-adrenergic bronchodilators. TAS2R3, TAS2R4 and TAS2R10 were expressed in the guinea pig trachea. The EP receptor-mediated tone could be relaxed by chloroquine and noscapine, but not by denatonium and thiamine. Although the mechanisms underlying these powerful relaxations remain unknown, the data support the involvement of several different pathways.

In summary, PGE_2 causes contractions and relaxations of guinea pig and human airways, but the receptors involved differ between the two species. Furthermore, the data gathered suggest that EP, TP and TAS2R receptors may be potential targets for the development of drugs to treat asthma and other forms of airway obstruction.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. **Säfholm J**, Dahlén S-E, Delin I, Maxey K, Stark K, Cardell L-O, Adner M.
PGE_2 maintains the tone of the guinea pig trachea through a balance between activation of contractile EP_1 receptors and relaxant EP_2 receptors.
Br J Pharmacol. 2013 Feb;168(4):794-806

II. 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

III. **Säfholm J**, Dahlén S-E and Adner M.
Antagonising EP_1 and EP_2 receptors reveal the antigen associated TP receptor mediated contraction in the guinea pig trachea
Submitted to Eur J Pharmacol 2013

IV. **Säfholm J**, Manson M, Dahlén S-E and Adner M
Prostaglandin E_2 responses in human small airways
Manuscript

During my time at Karolinska Institutet, two additional research articles were published, however they are not included in this thesis:

Adner M, Larsson B, **Säfholm J**, Naya I, Miller-Larsson A.
Budesonide prevents cytokine-induced decrease of the relaxant responses to formoterol and terbutaline, but not to salmeterol, in mouse trachea.
J Pharmacol Exp Ther. 2010 Apr;333(1):273-80

Säfholm J, Lövdahl C, Swedin L, Boels PJ, Dahlén SE, Arner A, Adner M.
Inflammation-induced airway smooth muscle responsiveness is strain dependent in mice.
Pulm Pharmacol Ther. 2011 Aug;24(4):361-6

CONTENTS

LIST OF COMMON ABBREVIATIONS

AA	Arachidonic acid
AIA	Aspirin-intolerant asthmatics
ASM	Airway smooth muscle
BK_{Ca}	Large conductance calcium-activated potassium channels
COX	Cyclooxygenase
cPGES	Cytosolic prostaglandin E synthase
CysLT	Cysteinyl-leukotrienes
EAR	Early allergic reaction
EC_{50}	Effective concentration for half maximal response
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
Fc□RI	High-affinity receptor for IgE
FLAP	5-lipoxygenase activating protein
GPCR	G-protein coupled receptor
GPT	Guinea pig trachea
HB	Human bronchi
IgE	Immunoglobulin E
IL	Interleukin
LAR	Late allergic reaction
LT	Leukotrienes
5-LOX	5-lipoxygenase
MC_T	Mucosal mast cells
MC_{TC}	Connective tissue mast cells
mPGES	Microsomal prostaglandin E synthase
NSAID	Non-steroidal anti-inflammatory drugs
OVA	Ovalbumin
PG	Prostaglandin
PLA_2	Phospholipase A_2
TAS2R	Bitter taste-sensing type 2 receptors
TX	Thromboxane

1 INTRODUCTION

Despite modern therapies using corticosteroids and β_2 adrenoceptor agonists to combat airway constriction, a substantial number of patients suffering from airway ailments still remain insufficiently controlled.

The main focus of this thesis is on the function of the eicosanoid prostaglandin E_2 (PGE_2), a central messenger molecule with diverse biological effects. In the airways, several clinical studies have described PGE_2 as a potent smooth muscle relaxant that also displays anti-inflammatory properties. However, some studies have reported opposing responses after exposure to PGE_2 with powerful bronchoconstriction and cough. Multiple effects may be explained by the activation of several different receptors. Although the first observations of the contractile and relaxant effects of PGE_2 were made over 30 years ago, research on the mechanisms involved in the actions of PGE_2 has been limited by the low selectivity of the previously available pharmacological tools. During the last few years new subtype selective receptor antagonists and potent enzyme inhibitors have become available and thus the role of PGE_2 can be elucidated with greater precision.

The overall aim of this thesis was to characterise the role of PGE_2 in the airways and to describe the receptors responsible for its action, both under normal conditions and during reactions triggered by antigen-challenge. This was primarily achieved by intervention with selective enzyme inhibitors as well as selective receptor antagonists in both guinea pig and human tissue, mainly using a classical pharmacological model; to record smooth muscle contractions isometrically in isolated segments of airways. Alongside the investigations into the mechanisms of action of PGE_2, it was discovered that bitter tasting substances caused powerful relaxations of the airways. As initial experiments revealed marked interactions between bitter tastants and PGE_2, this line of research was included in the thesis.

2 AIMS OF THE THESIS

The overall aim of this thesis was to investigate the role of PGE_2 on airway smooth muscle function under normal resting conditions and during antigen-induced responses.

Specific aims

- To define the response to PGE_2 in isolated human and guinea pig airway smooth muscle studied *in vitro*.

- To examine which receptors are responsible for the action(s) of PGE_2 in human and guinea pig airway smooth muscle.

- To investigate how PGE_2 is produced in the airways.

- To describe the role of endogenous and exogenous PGE_2 during antigen-induced responses in human and guinea pig airway smooth muscle.

- To investigate the relaxant properties of bitter taste agonists and their possible interactions with PGE_2, as suggested by preliminary experiments.

3 BACKGROUND

3.1 ASTHMA

Asthma, a multifactorial and heterogeneous disorder of the airways, is one of the most common chronic diseases in the world. It is estimated that over 300 million people worldwide suffer from asthma with a rising prevalence in societies adopting a western lifestyle or becoming urbanized (Eder *et al.*, 2006; Masoli *et al.*, 2004). The lifetime risk of developing chronic diseases such as cancer (Fay *et al.*, 2003), diabetes (Narayan *et al.*, 2003) and coronary heart disease (Lloyd-Jones *et al.*, 1999) ranges from 30-35% with a low occurrence in early life and a substantial increase during middle age to old age. Asthma, although exhibiting the same lifetime risk as other chronic diseases, displays a completely different profile, initiating during early childhood and increasing throughout life (To *et al.*, 2010). Also, during childhood, asthma is more common among boys than among girls, a ratio that is shifted to the opposite in adult individuals (Almqvist *et al.*, 2008; To *et al.*, 2010).

Although the origin of asthma is still unknown, several factors have been proposed to explain the increasing prevalence. Genetic susceptibility accounts for one part (Moffatt *et al.*, 2010), but it is also recognised that genetic changes in populations would be too slow considering such a rapid increase. Another factor is that of environmental exposures where allergens were considered to be the main driving force (Lemanske *et al.*, 2010), but it is now clear that while most children with asthma are allergic, only a minority of allergic patients actually develop asthma and most adults with asthma are actually non-allergic. A recently identified factor is the microbiome, where loss of certain microorganisms has been shown to negatively influence the immune system exerting deleterious effects in asthma (Heederik *et al.*, 2012). It is most likely that several known and unknown factors co-contribute.

Common triggers that can incite an exacerbation include an antigen-induced response, exercise, temperature change, smoke, strong odours, dust and other airborne irritants (Jackson *et al.*, 2011; Wenzel, 2012). However, respiratory tract viruses have emerged as the most frequent cause of exacerbations in both children and adults (Busse *et al.*, 2010).

The cardinal symptoms of asthma typically feature recurrent episodes of breathlessness, chest tightness, airway hyperresponsiveness, wheezing and cough (Fireman, 2003). The airflow obstruction is caused by bronchial wall oedema, mucus plugging and bronchoconstriction of the airway smooth muscle (ASM) in the bronchi and bronchioles, causing a partially or complete closure of the small airways (Tashkin, 2002; Tulic *et al.*, 2006). This impairs O_2/CO_2 exchange resulting in reduction of blood oxygenation with a potential to cause hypoxia and death by respiratory failure (Fuller, 1996; Wasserfallen *et al.*, 1990).

The structural changes observed in an asthmatic airway, often referred to as remodelling, include denudation or disruption of airway epithelium, collagen deposition within the sub-basement membrane, hyperplasia of goblet cells and both hyperplasia and hypertrophy of bronchial smooth muscle fibres (Bara *et al.*, 2010; Folli *et al.*, 2008). The fact that a significant proportion of asthma is associated with atopy has led the disease to be regarded largely as an inflammatory disorder of the airways associated with T helper type 2 cell-dependent promotion of IgE production and recruitment of mast cells and eosinophils in concert with increased cytokine production (Galli *et al.*, 2012; Holgate, 2012; Wenzel, 2012). However, although asthma was once considered to be a single disease entity, different phenotypes are now recognized with distinct pathophysiology, clinical expression and response to treatment (Wenzel, 2012). It is now clear that the presence of eosinophils in the airways is neither sufficient nor necessary to cause symptoms of asthma.

Guidelines for asthma diagnosis and management are recommended by an international consortium titled the Global Initiative for Asthma. The goals are not only to achieve day-to-day asthma control, but also to minimize future risk of exacerbations and prevent decline in lung function. The current paradigm is based on a stepwise increase in medication over five treatment steps with a preferred option for each step. Initially (step 1) only short-acting β_2 adrenoceptor agonists is advised, however if the asthma is uncontrolled, treatment needs to be increased to the next step. For treatment of mild asthma (step 2) low-doses of inhaled corticosteroids or leukotriene modifiers (either receptor antagonists or synthesis inhibitors) is recommended. For moderate asthma (step 3 and 4) increasing concentrations of inhaled corticosteroids, long-acting β_2 adrenoceptor agonists and leukotriene modifiers are advised. Severe asthma (step 5) includes all the previous treatments plus oral glucocorticosteroids and in some cases

anti-IgE treatment (GINA, 2011). Although current therapies for asthma are relatively safe and effective at controlling symptoms, it is debatable whether they change the chronic course of the disease. One major therapeutic approach for a long-term disease-modifying effect involves allergen-specific immunotherapy to induce specific immune tolerance (Akdis, 2012).

3.2 AIRWAY PHYSIOLOGY

The respiratory system includes the upper airways consisting of the nose, nasal cavity, pharynx and larynx connected to the lower airways consisting of the trachea, bronchi and alveoli. The lower airways can be further divided into a proximal portion referring to the trachea and the main bronchi and a distal portion referring to the bronchioles and alveoli. The conducting airways form a complex series of branching tubes that culminate in the gas exchange area. At each generation, the branching is essentially dichotomous, each airway being divided into two smaller daughter airways. Overall there are about 23-27 divisions of the bronchial tree from the trachea to the terminal bronchioles and the border between proximal and distal airway is positioned at the eighth division where the airway diameter is < 2 mm, also commonly recognized as the border to the "small airways" (Hogg *et al.*, 2004; Marieb, 2004).

3.2.1 Trachea

The trachea descends from the larynx into the mediastinum dividing into two primary bronchi at mid-thorax. It is a flexible and mobile tubular structure consisting of several cell-layers; the mucosa, submucosa and adventia. The mucosa is built up by an innervated pseudostratified ciliated epithelium with inclusions of goblet cells resting on the lamina propria, whereas the submuscosa is a connective tissue layer containing seromucous glands. The adventia is also a connective tissue layer structurally reinforced by integrated hyaline cartilage, preventing the trachea from collapsing and maintaining a normal structure during breathing (Marieb, 2004).

3.2.2 Small airways

The distal airways, often referred to as "small airways", comprise the 8th–19th generation of airways with a diameter ranging between 2– 0.5 mm (Holz *et al.*, 2000; Tashkin, 2002). Anatomically, the small airways differ from the larger airways because they contain fewer ciliated epithelial cells and rely on pulmonary surfactant to protect against excessive volume-related changes in diameter as compared to a structural

protection in the larger airways (Kraft, 1999). As the airways become smaller and more distal, cartilage support becomes scarce, and in the terminal bronchioles (< 0.5 mm in diameter) no cartilage is found. Distal to the terminal bronchioles are the respiratory bronchioles and alveoli where gas exchange takes place. (Macklem, 1998; Marieb, 2004).

3.2.3 Airway smooth muscle

Smooth muscles are found in several places in the body, often located within the walls of organs and structures such as the stomach, intestines, airways, uterus, bladder, blood vessels, and erector pili in the skin. Smooth muscle is different from skeletal muscle in several respects, *e.g.* it is non-striated, the control is involuntary and each muscle fibre contains a single nucleus (Stephens, 2001).

The ASM plays an important role both in healthy and asthmatic individuals (An *et al.*, 2007). Traditionally, the ASM was considered to primarily respond with a contraction or relaxation to mediators or drugs, however, that view is now antiquated. It is now clear that ASM is a very active tissue responsible for a profound synthesis of several mediators, cytokines, chemokines and growth factors (Black *et al.*, 2002; Knox *et al.*, 2000).

Increased ASM mass, one hallmark of asthma, may be caused by hypertrophy or hyperplasia. A recent study has found that hypertrophy occurs in the large airways in both nonfatal and fatal cases of asthma whereas hyperplasia was only present in the large and small airways in fatal asthma cases (James *et al.*, 2012b).

Humans and several other species exert an inherent ASM tone with a suggested purpose to maintain homeostasis within the airway as it is exposed to both constrictive and relaxant stimuli. In the guinea pig trachea (GPT), prostanoids and especially prostaglandin E_2 have been associated with maintenance whereas in the human airways, cysteinyl-leukotrienes are proposed as the responsible mediator (Orehek *et al.*, 1973; Schmidt *et al.*, 2000).

In the present thesis, the ASM was mainly investigated in the context of its contractile and relaxant properties.

3.2.4 Airway epithelium

The airway epithelium has many important functions. Firstly, it is a physiological barrier that prevents allergens, irritants, agonists and gases from stimulating the underlying ASM and intra-epithelial nerves (Sparrow *et al.*, 1995). Secondly, the epithelial cells have a metabolic function for several relevant endogenous mediators, such as acetylcholine by acetylcholinesterase and histamine by diamine oxidase (Koga *et al.*, 1992; Lindstrom *et al.*, 1991). Thirdly, the epithelial layer liberates a variety of bronchoactive mediators, *e.g.* lipoxygenase, cyclooxygenase products and 'epithelium-derived relaxing factors' such as nitric oxide and prostaglandin E_2 (Munakata *et al.*, 1990; Ruan *et al.*, 2011). Furthermore, the epithelium is a major source of inflammatory cytokines and chemokines, such as IL-25, IL-33, TSLP and eotaxin involved in local inflammatory responses and tissue repair mechanisms (Holgate, 2011).

3.3 THE MAST CELL AND ITS MEDIATORS

Mast cells are tissue resident immune cells often located close to blood vessels and in proximity to surfaces that border the external environment, such as the skin and respiratory tract. The mast cell displays a multitude of functions, including important roles in tissue homeostasis, wound healing and host defence against pathogens and parasites (Metcalfe *et al.*, 1997). Generally, the mast cell is classified according to the different serine proteases contained in its secretory granules, either tryptase (MC_T) alone or both tryptase and chymase (MC_{TC}). Mast cells residing in the bronchi, bronchioles and the alveolar parenchyma primarily belong to the MC_T classification and are often referred to as "mucosal" mast cells, in contrast to MC_{TC} which are largely found in the pulmonary vessels and the pleura, as well as in the skin, and are commonly described as "connective tissue" types (Andersson *et al.*, 2009; Irani *et al.*, 1986).

In the pathophysiology of asthma, mast cells have been implicated as one of the key cells responsible for the early phase reaction, both for allergic and non-allergic exacerbations, as well as the late phase reaction and chronic induction of hyperresponsiveness (Bradding *et al.*, 2006; Brightling *et al.*, 2002; Wenzel, 2012). Mast cells also display a different pattern of localisation within the airway of subjects with asthma compared to healthy controls; with increased numbers in airway smooth muscle and epithelial layers and decreased numbers in the submucosa (Balzar *et al.*, 2011; Brightling *et al.*, 2002; James *et al.*, 2012a).

3.3.1 Mast cell activation

Mast cell activation is primarily initiated via cross-linking of immunoglobulin E (IgE) antibodies attached to the high affinity receptor (Fc□RI) on the cell surface. When crosslinking of adjacent Fc□RI bound IgE antibodies by allergen or anti-IgE antibodies occurs, aggregation of FcεRI triggers a complex intracellular signalling process that results in degranulation and secretion of biologically active products. Three separate classes of mediators are released, including pre-formed mediators stored in cytoplasmic granules (*e.g.* histamine, proteases and heparin), *de novo* synthesised lipid mediators (*e.g.* prostanoids, leukotrienes and other oxylipins) and both preformed and newly synthesised cytokines and chemokines (*e.g.* TNF-α, IL-4 and IL-13) (Galli *et al.*, 2008). Importantly however, mast cell activation also occurs through non-IgE mediated pathways, such as osmotic stimulation, cytokines, neuropeptides or adenosine (Forsythe *et al.*, 2000; Gulliksson *et al.*, 2006; Metcalfe *et al.*, 1997). Such IgE-independent pathways may explain *e.g.* exercise-induced bronchoconstriction in asthma.

3.3.2 Early phase reactions

Early-phase reactions, also known as "type I immediate hypersensitivity reactions" occur within minutes of mast cell activation and mainly reflect the secretion of mediators at the affected location. They begin with the fusing of cytoplasmic granules to the mast cell membrane causing an exocytosis of preformed substances to the external environment. The mediators released include biogenic amines (mainly histamine in humans and guinea pig, but serotonin in rats and mice) together with serglycin proteoglycans and serine proteases acting in an autocoid manner (Galli *et al.*, 2012).

Histamine has the possibility to activate at least four different receptors ($H_{1\text{-}4}$). In the airways, the H_1 receptor has been implicated in bronchoconstriction whereas the H_2 receptor is more associated with mucus secretion (Barnes, 1991; White, 1990). H_3 receptors are often found on sensory nerves where they are shown to modulate cholinergic neurotransmission and H_4 receptors are considered to play an immunomodulatory role (Barnes, 1991; Dunford *et al.*, 2010). Today, the use of H_1 receptor antagonists, so called "antihistamines" has proven beneficial for the treatment

of allergic symptoms, especially in patients with rhinitis and allergic conjunctivitis (Simons, 2004).

Upon mast cell activation, the production of lipid mediators is promptly initiated and these mediators also contribute to the immediate reaction. These diverse lipid mediators are generated primarily from arachidonic acid and have the capability to either amplify or dampen the tissue response. Several different pathways are activated, such as the cyclooxygenase (COX) pathway generating prostanoids or the 5-lipoxygenase (5-LOX) producing leukotrienes (Boyce, 2007). These mediators and pathways will be covered in more depth in the eicosanoid-section of the thesis.

Taken together, the acute effects of the mast cell mediators include contraction of the airway smooth muscle, increased vascular permeability and enhanced secretion of mucus resulting in acutely reduced airflow. This response is usually resolved within a few hours. However, in some individuals a second phase of obstruction, often referred to as “the late phase reaction”, develops a few hours after antigen exposure (Galli *et al.*, 2012).

3.3.3 Late phase reactions

The late phase reaction has a prevalence of up to 50% in adults who previously developed an early phase response. Usually it commences within 2-6 hours after antigen exposure and is fully developed after 12 hours. It presents as a slowly progressing smooth muscle constriction causing a decrease in lung capacity of a similar extent to that caused during the early phase response (O'Byrne *et al.*, 1987). The degree of the reaction has been linked with increased numbers and activation of inflammatory cells, such as eosinophils and neutrophils (Aalbers *et al.*, 1993; Smith *et al.*, 1992) as well as an increase in airway hyperresponsiveness which may occasionally last for months (Cartier *et al.*, 1982). Although the late response can be partially reversed by different bronchodilators, it does not appear to be truly preventable. It is, however, blocked by prophylactic treatment with inhaled sodium cromoglycate, nedocromil, anti-IgE antibodies, anti-leukotrienes and corticosteroids (Cockcroft, 1988; Fahy *et al.*, 1997; Krawiec *et al.*, 1999; Roquet *et al.*, 1997), supporting mast cell involvement.

3.4 EICOSANOIDS

One of the most important classes of lipid derived mediators in mammalian cells are the eicosanoids. The word itself is derived from the Greek word for twenty; *eicosa (εἴκοσι)*, referring to the 20 carbon polyunsaturated fatty acid derivatives synthesized *de novo* from liberated phospholipids. Its existence and significance was hinted at in a few, seemingly unrelated, observations published around 1930 (Burr *et al.*, 1929; Kurzrok *et al.*, 1930; von Euler, 1934). More than 30 years later, Bergström and Samuelsson revealed the structures of the primary prostaglandins and described a link to its major precursor, arachidonic acid (Bergstroem *et al.*, 1964). In 1971, Vane discovered that aspirin-like drugs, used to treat fever and inflammation, inhibited prostaglandin biosynthesis (Vane, 1971). Bergström, Samuelsson and Vane jointly received the Nobel Prize in Physiology or Medicine in 1982 "for their discoveries concerning prostaglandins and related biologically active substances".

Arachidonic acid (20:4ω6; four double bonds with the first located at the 6th carbon position) is the main precursor for synthesis of eicosanoids in mammalian cells. It is stored as an ester in the phospholipid cell membrane until liberated by Ca^{2+}-dependent phospholipase A_2 (PLA_2). PLA_2 hydrolyses the polyunsaturated fatty acid from the *sn*-2 position (the 2nd carbon of the glycerol molecule), and when released, these can be metabolized to form various bioactive lipid mediators. Although there are several different types and forms of PLA_2, the cytosolic version of type IV is considered responsible for eicosanoid production since cells lacking this particular enzyme generally have no synthesis (Six *et al.*, 2000). Hormones, autocoids, antigens, bacterial peptides, immune complexes and mechanical stress are among the many stimuli that can induce PLA_2 activation, causing a translocation of the enzyme to the nuclear envelope and endoplasmic reticulum and a release of arachidonic acid (Smith, 1989).

3.4.1 Essential fatty acids

Essential fatty acids (EFAs) are important elements of all cell membranes with the capacity to alter membrane fluidity and thus, determine and influence the behaviour of membrane-bound enzymes and receptors. They are essential for human survival and cannot be synthesised by the body, so therefore have to be obtained through our diet. In the human body two types occur; the omega (ω) -6 series derived from cis-linoleic acid (LA, 18:2) and the ω -3 series derived from α -linolenic acid (ALA, 18:3). There are

also fatty acids derived from oleic acid (OA, 18:1ω9), however they are not an EFA (Das, 2006a).

LA is converted to γ-linoleic acid (GLA, 18:3ω6) by the enzyme Δ^6-desaturase and GLA is elongated to form dihomo-GLA (DGLA, 20:3ω6), the precursor of the 1 series of prostaglandins. DGLA can also be converted to arachidonic acid by Δ^5-desaturase forming the precursor of 2 series of prostanoids and the 4 series of leukotrienes. Also, ALA is converted to eicosapentaenoic acid (EPA, 20:5ω3), via $\Delta^{5\text{-}6}$-desaturase, forming the precursor for the 3 series of prostanoids and the 5 series of leukotrienes (Das, 2006b).

As there are many enzymes, the possibilities for lipid metabolite formation from a single pre-cursor are numerous **(Figure 1)**.

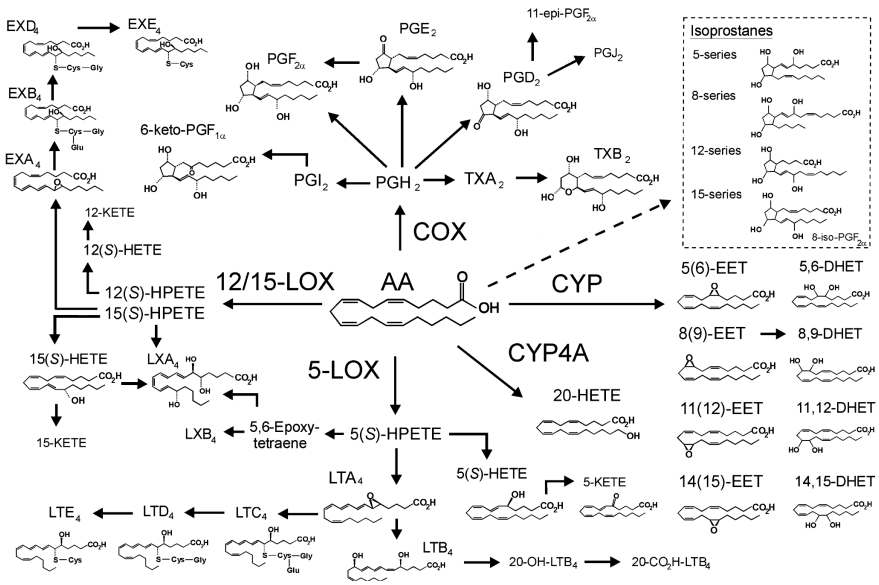

Figure 1. Schematic overview of eicosanoid metabolism. Enzyme catalysed pathways are indicated by filled arrows: COX=Cyclooxygenase; 5-LOX=5-lipoxygenase; 12/15-LOX=12/15-lipoxygenases; CYP=Cytochrome P450 monooxygenases. Non-enzymatic formation of isoprostanes indicated by dashed arrow.

3.4.2 Biosynthesis and catabolism in the cyclooxygenase pathway

Prostanoids are generated from free arachidonic acid in a two-step reaction catalysed by prostaglandin endoperoxide H synthases, commonly known as cyclooxygenases

(COX). Firstly, arachidonic acid is liberated from the phospholipid cell membrane by $cPLA_2$ and translocated to the endoplasmic reticulum (ER) or nuclear envelope where it is converted by membrane-bound COX enzymes to the unstable intermediate PGG_2 via a cyclooxygenase reaction. Secondly, PGG_2 is converted to PGH_2, the precursor for all prostanoids, via a lipid peroxidation resulting in reduction of a hydroperoxyl to form a hydroxyl at the 15th carbon. PGH_2 is then further metabolised to form bioactive prostaglandins (PG) D_2, E_2, $F_{2\alpha}$, I_2 and thromboxane A_2 (TXA_2) through tissue specific isomerases or synthases. The prostaglandins produced by a given cell largely depend on the expression profile of the individual prostaglandin synthase enzymes (Smith *et al.*, 2000) **(Figure 2)**.

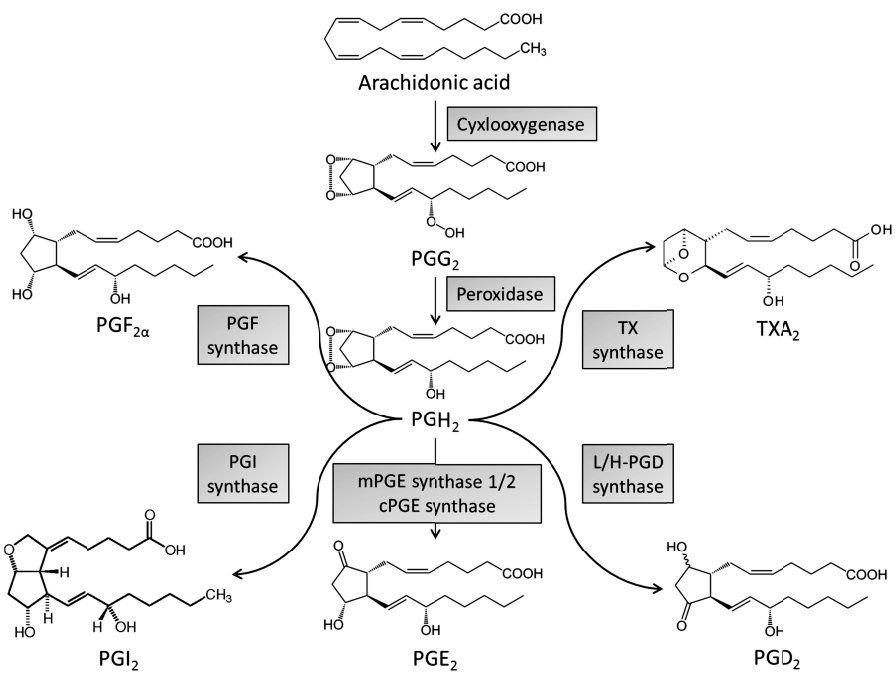

Figure 2. Biosynthesis of prostanoids from arachidonic acid via the cyclooxygenase pathway. PG=Prostaglandin; TX=Thromboxane; mPGE synthase=microsomal PGE synthase; cPGES synthase=cytosolic PGE synthase.

There are currently two accepted isoforms of COX commonly referred to as COX-1 (Picot *et al.*, 1994) and COX-2 (Kurumbail *et al.*, 1996) which have the capacity to perform the cyclooxidation and peroxidation action. Both isoenzymes are found in the membrane of the ER whereas only COX-2 is found expressed on the nuclear envelope (Morita *et al.*, 1995). They share 60-65% sequence homology within the same species

and 85%–90% identity among individual isoforms from different species (Smith *et al.*, 2000). In most tissues, COX-1 is considered to be constitutively expressed and believed to play a role in tissue homeostasis and tissue regulation whereas COX-2 is considered to be inducible, especially during inflammation (FitzGerald, 2003). There are however exceptions, such as constitutive COX-2 in renal tubular cells (Schaefers *et al.*, 1996).

Prostaglandins have a half-life of less than 1 minute and are extensively degraded through first-pass metabolism in the lung, thus the concentration is greatly reduced before it reaches the systemic circulation (Hamberg *et al.*, 1971). The majority of prostaglandins follow the same path which is initiated by removal of the hydrogen at the 15th carbon-OH group by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) forming an unstable 15-keto-prostagladin **(Figure 3)**. The second enzyme to be involved is the 13-reductase (Δ^{13}-reductase) which reduces the double bond located at the 13th carbon position generating 15-keto-13,14-dihydroprostaglandins (Piper *et al.*, 1970). Further shortening of the carbon chain to achieve complete inactivation is then often carried out by β- or ω-oxidaton, in the mitochondria and ER respectively before being excreted through the kidneys (Tai *et al.*, 2002).

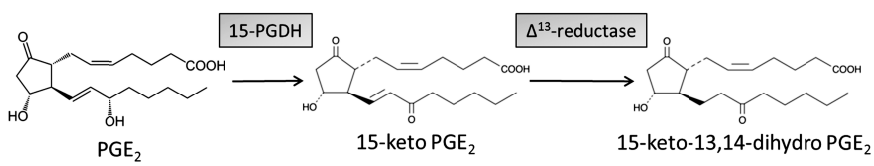

Figure 3. Metabolism of PGE_2, as an example of general prostaglandin degradation. 15-PGDH=15-hydroxyprostaglandin dehydrogenase; Δ^{13}-reductase= 13-reductase.

3.4.3 Prostanoid receptors, signalling pathways and biological effects

Prostaglandins act locally, through specific receptors, in an autocrine or paracrine manner to elicit diverse effects modulating many physiological systems. They are G-protein-coupled receptors, a family of rhodopsin-like seven transmembrane spanning receptors (GPCRs). Currently there are eight members (DP_1, $EP_{1–4}$, FP, IP and TP), all named after the agonist for which they have the highest affinity **(Table 1)**. Furthermore, there is a ninth prostanoid receptor; DP_2 (also named $CRTH_2$) with the capacity to bind PGD_2, however, this receptor is more closely related to the chemoattractant leukotriene B receptors (BLTR) superfamily (Hirai *et al.*, 2001).

The precise roles of prostaglandin receptors are determined by an intricate set of ligand-receptor interactions such as ligand affinity, receptor expression profile and different coupling to signal transduction pathways. It is not surprising, given their structural similarities, that prostaglandins activate more than one subtype of prostaglandin receptor (Abramovitz *et al.*, 2000; Coleman *et al.*, 1994).

Table 1. Pharmacological properties of the prostanoids.

Agonist	Primary receptor	Primary signalling pathway	
PGD_2	DP_1	G_s	↑ cAMP
	DP_2	G_i	↓ cAMP, ↑ Ca^{2+}
PGE_2	EP_1	G_q	↑ Ca^{2+}
	EP_2	G_s	↑ cAMP
	EP_3	G_i	↓ cAMP, ↑ Ca^{2+}
	EP_4	G_s	↑ cAMP
$PGF_{2\alpha}$	FP	G_q	↑ Ca^{2+}
PGI_2	IP	G_s	↑ cAMP
TXA_2	TP	G_q	↑ Ca^{2+}

cAMP=Cyclic adenosine monophosphate

G_q signalling pathway

Signalling through G_q-coupled receptors is of particular interest in the airways since it promotes ASM contraction (Billington *et al.*, 2003). Upon agonist binding, the receptor undergoes a conformational change exposing a high-affinity binding site for a G-protein complex ($G\alpha_q\beta\gamma$) in its GDP state (inactive). Once the G-protein is bound to the receptor, the GDP is released into the cytosol and replaced by a GTP which induces a conformational change dissociating the $G\alpha_q$ from the $G\beta\gamma$-dimer. The $G\beta\gamma$ unit remains in the lipid bilayer whereas the $G\alpha_q$ activates the phospholipid-cleaving enzyme phospholipase C (PLC) promoting hydrolysis of phosphoinositol 4,5-bisphospate (PIP2) to form 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG remains in the membrane where it induces a translocation and activation of protein kinase C (PKC). IP_3 translocate and binds to IP_3 receptors located on the sarcoplasmic reticulum (SR) which in turn opens Ca^{2+} channels resulting in further influx of extracellular Ca^{2+}. Elevated Ca^{2+} promotes binding to calmodulin forming a Ca^{2+}-calmodulin complex that, in turn, activates the myosin light chain kinase which phosphorylates myosin light chains (MLC) and enables actin to activate myosin

ATPase activity; resulting in cross-bridge cycling and contraction (Billington *et al.*, 2003).

G_s signalling pathway

The Gs pathway is also of great importance in the airways since it induces smooth muscle relaxation. Agonist binding to the receptor induces a reaction similar to $G\alpha_q$ with the exception that the $G\alpha_s$ subunit does not activate PLC, but membrane bound adenylyl cyclase (AC). AC activation catalyses the formation of cyclic AMP (cAMP) from free cytoplasmic ATP. Binding of cAMP to the regulatory subunits of protein kinase A (PKA) will induce a conformational change releasing the PKAs catalytic subunits. These catalytic subunits have tremendous effects in the cell with the capacity to inactivate PLC, IP_3 receptors and cross-bridge cycling through MLC phosphorylation. The net outcome in airway smooth muscle cells is a decrease in intracellular calcium with a subsequent inhibition of contraction (Billington *et al.*, 2003).

G_i signalling pathway

Activation of $G\alpha_i$ associated receptors has the possibility to initiate and modulate intracellular signalling through both the α- and βγ-subunit. Primarily, $G\alpha_i$ binds to AC making it difficult for $G\alpha_s$ to activate it. At the same time, Gβγ has the possibility to activate PLC generating IP_3 and DAG, resulting in calcium mobilization (Billington *et al.*, 2003). Furthermore, $G\alpha_i$-coupled receptors have been linked to ASM growth (Ediger *et al.*, 2002) and Rho-dependent changes in actin polymerisation (Hirshman *et al.*, 1999). The Gi pathway has less known effects in the airway smooth muscle.

A summary with interaction of all three signalling pathways is described in **Figure 4**.

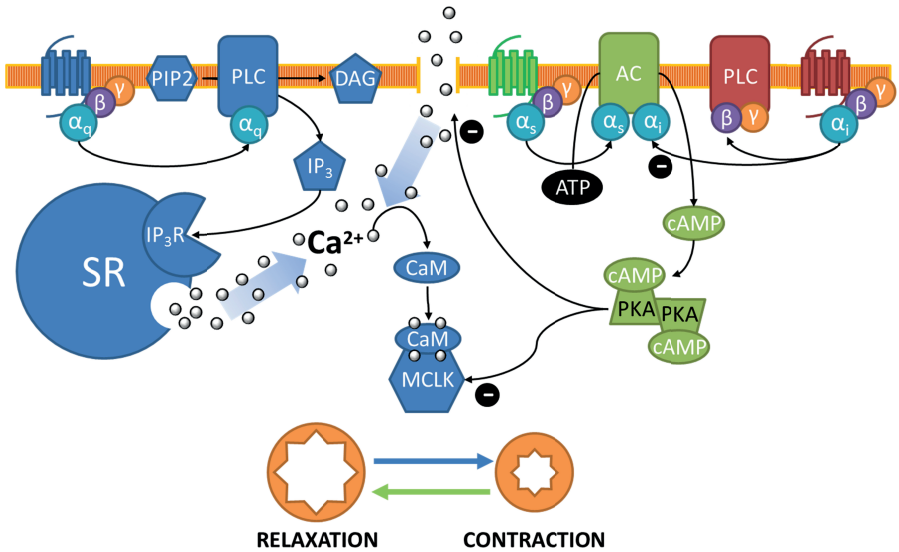

Figure 4. GPCR signalling in ASM involves sequential activation of receptors, G proteins (α, β and γ), second messengers, and effectors. Upon agonist binding, the receptor undergoes a conformational change which causes an activation of the heterotrimeric G protein through an exchange of GDP for GTP. This induces a dissociation of the Gα from the Gβγ dimer. In the **blue pathway**, $G\alpha_q$ activates phospholipase C (PLC) promoting hydrolysis of phosphoinositol 4,5-bisphosphate (PIP2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG remains membrane bound whereas IP_3 translocate and bind to IP_3 receptors (IP_3R) located on sarcoplasmic reticulum (SR) resulting in the opening of Ca^{2+} channels and calcium efflux into the cytosol. The rise in intracellular Ca^{2+} cause an influx of extracellular Ca^{2+} which promotes Ca^{2+} binding to calmodulin forming calcium-calmodulin (CaM) complexes that activate myosin light chain kinase (MLCK). Subsequently MLCK phosphorylates myosin light chains and enables actin to activate the myosin ATPase activity required for cross-bridge cycling and contraction. In the **green pathway**, $G\alpha_s$ activates adenylyl cyclase (AC) to catalyse formation of cyclic AMP (cAMP) from cytoplasmic ATP. cAMP activate protein kinase A (PKA) enabling PKA to phosphorylate various intracellular proteins, such as PLC, IP_3R, MLCK and ion channels to inhibit their action which consequently cause relaxation. In the **red pathway**, both $G\alpha_i$ and Gβγ have the capacity to initiate or modulate signalling. Activated $G\alpha_i$ can act as a negative modulator of $G\alpha_s$-induced signalling by binding to AC whereas Gβγ can activate PLC promoting IP_3 formation and a subsequent Ca^{2+} influx.

DP receptors

DP_1 receptors are connected to AC via a $G\alpha_s$ protein, thus promoting cAMP generation (Boie *et al.*, 1995). Activation of DP_1 receptors leads to vasodilatation as well as inhibition of platelet activation (Giles *et al.*, 1989). The primary agonist is PGD_2, however in the airways, PGD_2 signals mainly through the TP receptor to induce contraction via $G\alpha_q$ (Hamid-Bloomfield *et al.*, 1990). The DP_2 receptor formerly known as $CRHT_2$ (chemoattractant receptor homologous molecule expressed on T-Helper type 2 cells) is not yet well characterized, but has been shown to modulate eosinophil morphology and degranulation as well as increase eosinophil and T-Helper type 2 cell motility (Bos *et al.*, 2004).

EP receptors

As a broad generalization, EP_1 and EP_3 receptors mediate excitatory effects, while EP_2 and EP_4 receptors mediate inhibitory effects. Although EP receptor distribution among cells within the lung is not well established, it is clear that various airway cells can express multiple EP receptors increasing the complexity of subsequent activation (Tilley *et al.*, 2003). The EP_1 receptor has been shown to increase intracellular Ca^{2+} which is associated with bronchoconstriction in experimental models (Coleman *et al.*, 1985), a response that has not yet been translated into humans. Recently, the EP_1 receptor has been described to modulate the β_2-adrenergic receptor (β_2AR) response, causing an EP_1:β_2AR heterodimeric complex to form which reduces cAMP formation and thus decreases bronchorelaxation (McGraw *et al.*, 2006). The EP_2 receptor is coupled to a $G\alpha_s$ protein stimulating AC and cAMP formation. It has been shown to mediate bronchorelaxation in several models (Birrell *et al.*, 2013; Fortner *et al.*, 2001) and also mediate ASM migration inhibition (Aso *et al.*, 2013). Furthermore, due to a short intracellular domain it is resistant to arrestin-induced internalisation (Penn *et al.*, 2001) as compared to other EP receptors. In order to turn off an activated GPCR response, phosphorylation by G protein coupled receptor kinases (GRKs) occurs on the cytoplasmic part when the G proteins are disassociated from the receptor. GRK acts as a binding site for arrestin, which in turn blocks further G protein-mediated signalling and targets receptors for internalization (Magalhaes *et al.*, 2012) The EP_3 receptor, generally inhibits AC via $G\alpha_i$, and is one of the most complex EP receptors with at least ten isoforms, defined by unique C-terminal cytoplasmic tails, identified across species, six of these being expressed in man (An *et al.*, 1994). The isoforms differ in their G-protein coupling resulting in a wide spectrum of EP_3 actions, such as smooth muscle

contraction, enhancement of platelet aggregation and inhibition of autonomic neurotransmitter release (Bos *et al.*, 2004). Recently, the EP_3 receptor has been implicated as the possible culprit promoting PGE_2-induced cough (Maher *et al.*, 2009). The EP_4 receptor couples, like the EP_2 receptor, to AC *via* a $G\alpha_s$ protein. The receptors structural design is however very dissimilar with a substantially longer cytoplasmic tail including multiple phosphorylation sites which enables a rapid desensitization (Nishigaki *et al.*, 1996). Recently, the EP_4 receptor has been implicated in PGE_2-induced HB relaxation, showing similarities with the rat trachea (Buckley *et al.*, 2011; Lydford *et al.*, 1994).

FP receptors

Not much is known regarding FP receptors in the airways. They are indicated to be coupled via the $G\alpha_q$ pathway and induce strong contractions in blood vessels. In airways, the agonist with the highest affinity, $PGF_{2\alpha}$, induces contraction (Mathe *et al.*, 1975) mainly through the TP receptor (Devillier *et al.*, 1997).

IP receptors

IP receptors are coupled via a $G\alpha_s$ protein, stimulating AC induced cAMP production. Its role in the airways is still at large, but it is known to relax vascular smooth muscle and inhibit platelet aggregation (Mitchell *et al.*, 2006). In experimental knock-out mice models, loss of IP receptors has been linked to increased goblet cell hyperplasia and fibrosis (Nagao *et al.*, 2003). Unfortunately, many of the agonists used to investigate the IP receptor have a high affinity for the EP_1 and/or EP_3 receptors, making it difficult to draw conclusions (Abramovitz *et al.*, 2000).

TP receptors

TP receptors have been found in nearly all mammalian blood vessels, in the airways and on blood platelets, where their primary task is to mediate smooth muscle contraction and platelet aggregation (Nakahata, 2008). There are claims for two splice variants, TPα and TPβ, with a small variance in the carboxyl-terminal resulting in a more rapid internalization of the β–version (Raychowdhury *et al.*, 1994). The TP receptor is functionally coupled to the $G\alpha_q$ protein increasing Ca^{2+}.

The TP receptor has the highest affinity for TXA_2, but other prostanoids such as PGD_2, PGE_2 or $PGF_2\alpha$ can activate it (Abramovitz *et al.*, 2000; Coleman *et al.*, 1989;

Featherstone *et al.*, 1990), making it an attractive target for prevention of allergy-associated mast cell induced bronchoconstriction (Beasley *et al.*, 1989). With a half-life of 30 seconds in the presence of water, TXA_2 is rapidly metabolised to the inactive metabolite TXB_2 (Hamberg *et al.*, 1975). Therefore, U-46619, a stable synthetic PGH_2 analogue acting as a TXA_2 mimetic is often used during pharmacological experiments.

3.4.4 Prostaglandin E_2

PGE_2 was first isolated from the vesicular gland of sheep in 1962 (Bergström *et al.*, 1962). Since then, numerous physiological effects have been described to be caused by this particular prostaglandin, which is considered the most abundant in the body (Legler *et al.*, 2010). It is formed by PGE synthases (PGES) and to date three enzymes have been cloned; cytosolic PGES (cPGES), microsomal PGES-1 (mPGES-1) and microsomal PGES-2 (mPGES-2) (Murakami *et al.*, 2002). mPGES-1 is a membrane bound member of the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily (Jakobsson *et al.*, 1999) and co-localized with COX-2 at the perinuclear membrane. Both mPGES-1 and COX-2 are often found to be upregulated in concerted action during pro-inflammatory conditions (Samuelsson *et al.*, 2007). mPGES-2 and cPGES are constitutively expressed in various tissues and cell types, and both are believed to serve a homeostatic function. mPGES-2 is often localized either in the cytosol or on the Golgi whereas cPGES is located only in the cytosol. Both enzymes favour COX-1, but mPGES-2 is known to also couple with COX-2 (Kudo *et al.*, 2005).

Both COX-1 and COX-2 are localized to the ER where the prostaglandins primarily are synthesized (Morita *et al.*, 1995). In order for the prostanoids to exert their effects they need to exit the cell in which they were produced. This was originally thought to be a simple passive diffusion, due to the fact that PGs are anions in contrast with the cells electronegative interior (Bito *et al.*, 1975). However, the kinetics of PG synthesis could not be explained by slow diffusion (Baroody *et al.*, 1981) and in 1995, Kanai *et al.* identified a prostaglandin transporter (PGT) that was associated with the rapid transport of PGE_1, PGE_2, and $PGF_{2\alpha}$ mainly into the cell (Kanai *et al.*, 1995). This was followed by Reid *et al.* who found that MRP (multidrug resistance protein)-4 was used specifically for the transport of PGE_1 and PGE_2 out of cells in the presence of ATP and glutathione. Interestingly, MRP4-dependent PGE efflux is reduced by both non-E prostaglandins and NSAIDs (non-steroidal anti-inflammatory drugs), suggesting that

other PGs could be substrates for the same protein and that NSAIDs could exert their pharmacologically beneficial actions by both inhibiting PG synthesis and by inhibiting the secretion of PGE_2 (Reid *et al.*, 2003).

The biological effects of PGE_2 are diverse and of a high complexity since they can be produced by virtually any cell of the human body and signal through different receptors. PGE_2 also regulates numerous physiological functions *e.g.* reproduction, neuronal, metabolic and immune functions. In the central nervous system, PGE_2 controls body temperature, sleep–wake activity and an increased sensitivity to pain. It has been described as a regulating factor for bone formation and bone healing (Fortier *et al.*, 2008). In addition to the respiratory system, its powerful effects on smooth muscle cells are also evident in processes such as childbirth, blood pressure control and in gastrointestinal motility (Dey *et al.*, 2006). PGE_2 is involved in all the classic signs of inflammation: redness, swelling and pain. Because of this, it is often referred to as a 'pro-inflammatory' mediator, however it can exert 'anti-inflammatory' actions on innate immune cells like macrophages, neutrophils, monocytes and NK cells (Funk, 2001; Harris *et al.*, 2002).

Besides the clinical use of PGE_2 in inducing childbirth or abortion (Brunnberg, 1978), and in severe ischemia (McCullough *et al.*, 2004), the current pharmaceutical focus lies in the inhibition of responses trough synthase inhibition and receptor antagonism.

3.4.5 Inhibitors of the cyclooxygenase pathway

Blocking the formation of prostanoids has been shown to reduce pain sensation, fever and inflammatory effects. Aspirin (acetylsalicylic acid) was the first NSAID introduced in 1889, which was followed, 70 years later, by other NSAIDs such as indomethacin and ibuprofen (Adams *et al.*, 1969; Winter *et al.*, 1963). Although they exert the same main effect, their COX-1/COX-2 inhibitory ratio and pharmacological properties are very different, with both aspirin and indomethacin causing an irreversible inhibition (aspirin covalently and indomethacin non-covalently) whereas ibuprofen is competitive (Rome *et al.*, 1975). Today, many different NSAIDs exist, all of them exerting their main effect through COX inhibition, however, some of them have other mechanisms of action as well, such as MRP-4 inhibition (Reid *et al.*, 2003), TP receptor antagonism (Selg *et al.*, 2007) or DP_2 receptor agonism (Hirai *et al.*, 2002).

In the beginning of the 1990s a second COX-isoform was discovered that was induced by inflammatory responses (Xie *et al.*, 1991). One structural difference between the isoenzymes, which also happens to be a major drug target, is the slightly larger active cyclooxygenase site for COX-2 (Smith *et al.*, 2000). With this knowledge, selective COX-2 enzyme inhibitors (coxibs) were developed with the assumption that they would dampen known secondary effects caused by the unselective inhibitors, such as removal of the protective function for the mucosa of the gastrointestinal tract (Drews, 2006). Unfortunately, COX-2 is not only an induced pro-inflammatory enzyme, but also involved in important vascular homeostasis. Consequently, long-term use of COX-2 inhibitors was found to result in an increased rate of serious cardiovascular events possibly due to a change in the PGI_2/TXA_2 ratio. Normally PGI_2 is formed by both COX-1 and COX-2 in vascular endothelial cells and dilates blood vessels reducing aggregation and adhesion of platelets whereas TXA_2, formed primarily by COX-1 in platelets promotes aggregation. A change in this ratio to a more pro-thrombotic environment makes the formation of pathological blood clots more likely (FitzGerald, 2003). In contrast, the cardio-protective effect of aspirin (Theroux *et al.*, 1988) is due to the irreversible long-term inhibition of platelet COX-1 and hence of TXA_2 biosynthesis by platelets in the circulation (aspirin has little effect on PGI synthesis).

In this thesis several COX-enzyme inhibitors with different COX-1/COX-2 preferences have been used **(Table 2).**

Table 2. IC_{50} values of COX-inhibitors towards COX-1 and COX-2

	COX-1 (µM)	COX-2 (µM)	COX-1/COX-2
Indomethacin	0.03 [A]	1.7 [A]	0.02
Diclofenac	1.6 [A]	1.1 [A]	1.5
Ibuprofen	12 [B]	80 [B]	0.15
FR-122047	0.028 [C]	65 [C]	0.0004
SC-560	0.0048 [B]	1.4 [B]	0.003
Lumiracoxib	67 [D]	0.13 [D]	515
Etoricoxib	116 [E]	1.1 [E]	105

A (Laneuville *et al.*, 1994), **B** (Kato *et al.*, 2001), **C** (Ochi *et al.*, 2000), **D** (Esser *et al.*, 2005) and **E** (Riendeau *et al.*, 2001). IC_{50} was measured in murine macrophages, in human recombinant blood assay or human whole blood assay.

3.4.6 Biosynthesis and catabolism in the 5-lipoxygenase pathway

The word "leukotriene" is derived from the Greek word for white, leuko (λευκό), in combination with triene referring the presence of three conjugated double bonds in the compounds. As the name implies leukotrienes were originally identified in leukocytes in 1979 (Borgeat *et al.*, 1979; Murphy *et al.*, 1979; Samuelsson *et al.*, 1979), but have since been found to be produced by many other cell types. They are important mediators in various inflammatory disease states (Peters-Golden, 1999), especially in the pathogenesis of asthma (Drazen *et al.*, 1999).

Leukotrienes (LTs) are, like PGs, produced from AA in response to cellular activation **(Figure 5).** Synthesis is initiated by a reversible translocation of the 5-lipoxygenase (5-LOX) enzyme from either the nuclear envelope or cytoplasm, depending on cell type, to the nuclear envelope. 5-LOX in corporation with another enzyme, 5-LOX activating protein (FLAP), convert AA to the unstable 5-hydroperoxyeicosatetraenoic acid (5-HPETE). 5-HPETE is then non-enzymatically reduced to 5-hydroxyeicosatetraenoic acid (5-HETE) or via 5-LOX converted into the very reactive epoxide LTA_4 (Smith, 1989).

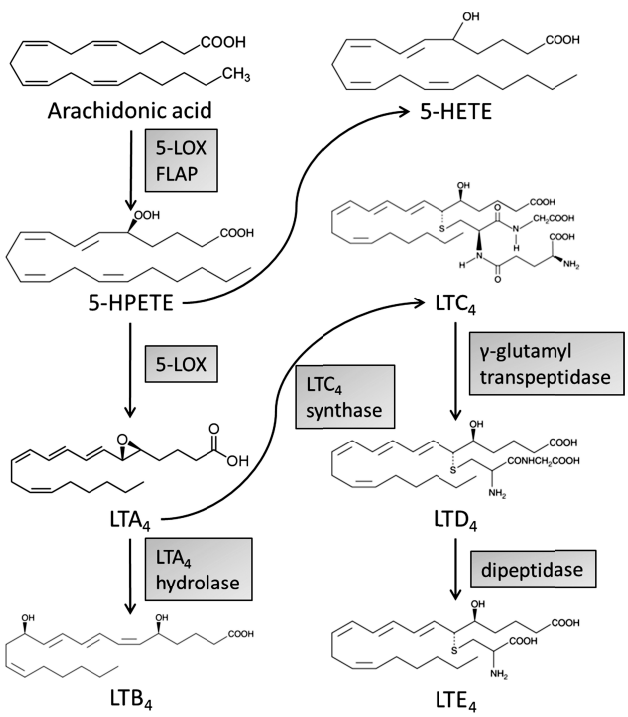

Figure 5. Biosynthesis of leukotrienes generated from arachidonic acid. 5-LOX: 5-lipoxygenase; FLAP=5-LOX activating protein; LT=leukotriene.

In cells containing LTA_4 hydrolase, LTA_4 can be converted to LTB_4 by a net addition of water (Haeggstrom *et al.*, 2007). LTA_4 can also be conjugated with glutathione (GSH) in cells which contains LTC_4 synthase (an enzyme belonging the MAPEG family) to form the first of the cysteinyl-leukotrienes (CysLTs); LTC_4 (Welsch *et al.*, 1994). In human mast cells a secondary pathway exists where microsomal GSH-s-transferase type 2 (MGST2) can conjugate LTA_4 with GSH to produce LTC_4 (Sjostrom *et al.*, 2002).

LTB_4 is transported out of the cell by the LTB_4-transporter (Yokomizo *et al.*, 2001) whereas LTC_4 uses a conjugation with MRP-1 (Pulaski *et al.*, 1996). Outside the cell, the glutamic acid on LTC_4 is cleaved off by γ-glutamyl transpeptidase to form LTD_4 which is further metabolised via cleavage of a glycine by dipeptidase to form LTE_4 (Peters-Golden *et al.*, 2007). Before the structure of the CysLTs was known they were collectively known as Slow Reacting Substance of Anaphylaxis (SRS-A). LTE_4 is the final metabolic product of the CysLTs and can be measured in urine as a marker for CysLT produktion (Kumlin *et al.*, 1992).

3.4.7 Leukotriene receptors, signalling pathways and biological effects

BLT receptors

The major activities of LTB_4 include the recruitment and activation of leukocytes (Dahlen *et al.*, 1981). LTB_4 is known to be a particularly potent neutrophil chemoattractant (Ford-Hutchinson *et al.*, 1980). Two GPCRs for LTB_4 have been identified, BLT_1 and BLT_2 (Peters-Golden *et al.*, 2007). BLT_1 is the high-affinity receptor and mediates most of the chemoattractant and pro-inflammatory actions of LTB_4. The BLT_2 receptor is a low-affinity receptor for LTB_4 that can also be activated by other lipoxygenase products. Little is known about its physiological function, however, it is highly conserved across species suggesting a possible, yet undetermined, important biological role (Tager *et al.*, 2003).

CysLT receptors

CysLTs are a group of compounds with a diverse range of effects in the airways. They are known to induce bronchoconstriction, mucus secretion, vascular permeability, oedema, ASM hyperplasia and hypertrophy and airway remodelling (Bäck *et al.*, 2011). Their effects are mediated through activation of two different receptors, $CysLT_1$

and $CysLT_2$, both GPCRs inducing calcium mobilization through the $G\alpha_q$ pathway (Heise *et al.*, 2000; Lynch *et al.*, 1999; Sarau *et al.*, 1999). Certain reported effects of CysLTs are not fully explained by either $CysLT_1$ or $CysLT_2$ receptor responses, raising the possibility that other, as yet unknown, CysLT receptors may exist (Yoshisue *et al.*, 2007). One candidate receptor is GPR17 (Ciana *et al.*, 2006) whereas another is GPR99 (Kanaoka *et al.*, 2013), both with the capacity to bind CysLTs. In HB, all three CysLTs are equipotent at inducing contraction through $CysLT_1$, whereas in the guinea pig airway LTC_4 primarily mediates its effects through the $CysLT_2$ receptor and LTD_4 and LTE_4 *via* $CysLT_1$ receptor (Bäck, 2002).

3.4.8 Inhibitors of the 5-lipoxygenase pathway

Anti-inflammatory glucocorticosteroids do not reduce LT responses (Gyllfors *et al.*, 2006) and classical NSAIDs have actually been shown to increase the production of LTs (Laekeman *et al.*, 1979; Walker, 1980). There are several approaches that may be taken to inhibit 5-LOX metabolite responses, such as inhibition of the 5-LOX enzyme, or FLAP, and thus removal of LT production. One synthase inhibitor that has been approved for the treatment of asthma is the 5-LOX inhibitor zileuton (Dube *et al.*, 1999). There are currently no FLAP inhibitors on the market, however there are several undergoing clinical trials (Sampson, 2009). One potent and selective FLAP inhibitor used frequently within this thesis is MK-886 (Gillard *et al.*, 1989). Another approach is to block the receptors mediating the response. $CysLT_1$ receptor antagonists, such as montelukast (Singulair) or zafirlukast (Accolate), have been evaluated in several clinical studies where they improve pulmonary function, reduce inflammation and exacerbations, increase quality of life and are now considered standard treatment in asthma (Bäck *et al.*, 2011; Dahlen, 2006).

3.5 BITTER TASTE RESPONSES IN THE AIRWAYS

The gustatory taste perceptions that can be sensed on the tongue upon exposure to bitter compounds are mediated by bitter taste-sensing type 2 receptors (TAS2Rs) (Meyerhof *et al.*, 2010). TAS2Rs have recently been suggested to have important extra oral functions in the respiratory and gastrointestinal tracts (Behrens *et al.*, 2011; Deshpande *et al.*, 2010) proposing a possible defence mechanism against noxious compounds. Furthermore, correlations between the expression of the TAS2Rs and clinical markers of asthma severity have been found in both adults and children (Pietras *et al.*, 2012). As

initial experiments performed also found an interaction between PGE_2 and several, but not all TAS2R agonists, this connection was investigated further.

3.5.1 Bitter taste receptors, signalling pathways and biological effects

TAS2Rs are different from many other GPCRs (currently 25 known expressed in human and 10 in guinea pig), as the receptors are capable of binding a wide range of compounds with a relatively low specificity and affinity (Meyerhof *et al.*, 2010). Initially it was discovered that activation of these receptors increased intracellular Ca^{2+}, suggesting that they could play a role in bronchospasm. However, several *in vitro* and *in vivo* studies in mice, guinea pigs and humans, now confirm that despite increasing internal Ca^{2+}, TAS2R agonists have a potent bronchorelaxant effect, not mediated *via* cAMP (Deshpande *et al.*, 2010). The mechanism for this relaxation is still unknown.

4 MATERIAL AND METHODS

A more detailed description regarding the material and methods used in the separate studies can be found in the different publications and manuscripts within this thesis. The section below provides a simplified overview regarding some of the key methods used.

4.1 GENERAL

To study the action of PGE_2 and other agonists on smooth muscle tissue experiments were performed on airway preparations (trachea and bronchi) and vessels (aorta) using the classical organ bath setup (papers I-IV). To further investigate the response subsequent activation of mast cells two different methods was employed; either an active sensitization towards chicken egg albumin (OVA; paper III) or exposure to monoclonal anti-IgE antibodies (paper IV). The release of PGE_2 was analysed using enzyme immune assay (EIA; paper I). The experiments (guinea pig, rat and human) were approved by the local ethics committee in Stockholm, Sweden. All animals were housed in cages (5 animals together) under 12-hour light/dark cycle with food and water *ad lib.*

4.2 TISSUE COLLECTION

Animals, rat and guinea pig, used in the functional studies were sacrificed either by inhalation of CO_2 or overdose of sodium pentobarbital followed by rapid removal of the heart-lung-package which was placed in ice-cold Tyrode's or Krebs-Ringer PSS buffer. For functional studies, tracheal segments were cut along the cartilage into eight intact rings of equal length whereas the thoracic aorta or rat trachea was cut into four intact ring-segments. The tissue was placed in a 5 mL tissue organ bath containing Krebs-Ringer PSS and kept at 37 °C, constantly bubbled with carbogen (5% CO_2 in O_2) to maintain pH at 7.4.

Macroscopically healthy human lung tissue was obtained from patients undergoing lobectomy (after confirmed structural changes within the lobe) and placed immediately in ice-cold Krebs-Ringer PSS buffer. Within 3 hours, bronchial tissue was, using light microscopy, gently dissected clear from lung parenchyma under ice-cold Krebs-Ringer PSS buffer condition, cut into intact rings (approximately 0.5-1 mm inner airway diameter and 3-5 mm in length) and placed in separate wells containing Dulbecco's

modified Eagle medium (DMEM) supplemented with 1% penicillin (100 IU·mL^{-1}) and streptomycin (100 µg·mL^{-1}) under sterile conditions. The culture plates were placed in a humidified incubator (37 °C at 95% O_2 and 5% CO_2) for 17-24 hours.

4.3 ORGAN BATH EXPERIMENTS

Changes in smooth muscle force upon administration of substances were detected using an isometric force-displacement transducer linked to a Grass polygraph or a myograph. During an equilibration period with washes every 15 min, the resting force was mechanically adjusted (30 mN for guinea pig trachea, 10 mN for rat trachea, 8 mN for guinea pig aorta and 1.5 mN for HB). As a control of reactivity and viability, either histamine or KCl were added which was followed by a second wash period and a new equilibration period. All experiments were finished by adding histamine (1 mM), acetylcholine (1 mM) and KCl (60 mM) as a reference for the maximal contraction followed by theophylline (1 mM) or a combination of papaverin (0.1 mM) and sodium nitroprusside (0.1 mM) as a reference for the maximal relaxation. In aorta, the presence of an intact endothelium was assessed at the end of the experiment by relaxation to acetylcholine after pre-contraction with phenylephrine prior to obtaining the maximal contractibility.

Pharmacological tools, such as receptor antagonists or enzyme inhibitors, were given a minimum of 45 min to equilibrate.

To study the response after mast cell activation, either OVA (0.1 ng/mL to 0.1 mg/mL in sensitized animals) or monoclonal anti-IgE (1/1000 dilution in HB), generating a contraction was elicited in the absence and presence of pharmacological tools which were given 45 min prior. Only one antigen response was investigated in each preparation.

4.4 ORGAN CULTURE EXPERIMENTS

During organ culture, intact individual guinea pig tracheal rings were placed in separate wells containing DMEM-F12 supplemented with 1% penicillin (100 IU·mL^{-1}) and streptomycin (100 µg·mL^{-1}) under sterile conditions. The culture plates were placed in a humidified incubator at 38 °C at 95% O_2 and 5% CO_2. Segments were incubated for 96 hours in total and transferred into new wells containing fresh media every 24 hours (Adner *et al.*, 2002).

4.5 ANTIGEN SENSITISATION

Male albino guinea pigs (Dunkin-Hartley; 400-450 g) were sensitized by a single peritoneal injection containing 100 μg OVA grade V and 0.1 g aluminium hydroxide dissolved in 0.8 mL sterile PBS. This induced an active sensitisation producing both IgE and IgG_1 antibodies towards OVA (Andersson, 1980). Animals were sacrificed 14 to 28 days later by an overdose of sodium pentobarbital.

4.6 ENZYME IMMUNOASSAY

Two separate investigations were carried out using EIA; PGE_2 and cAMP measurement.

The determination is based on competition of the investigated free substrate (PGE_2 or cAMP) and a substrate-acetylcholinesterase (AChE) conjugate, also known as a tracer. These substances compete for binding to a specific antibody that is mounted on the bottom of a 96-well plate. A fixed amount of tracer and substrate is added and while the concentration of the investigated substrate may differ between samples, the concentration of the tracer is known. Since this is a competition, the more tracer that will bind to the antibodies, the less substrate and *vice versa*. To detect the amount bound to the coated antibodies, a method to convert the concentration to a spectophotometrically visible signal is carried out using Ellman's reagent (acetyltiocholine) which absorbs at 412 nm. The more tracers bound to antibodies the brighter the signal, thus, there is an inverse relationship with the investigated substrate.

PGE_2 levels were investigated in paper I and II. The detection limit was 15 $pg \cdot mL^{-1}$and the PGE_2 monoclonal antibody displayed cross-reactivity towards PGE_3 (43%), 8-iso PGE_2 (37.4 %), PGE_1 (18.7%), 6-keto $PGF_{1\alpha}$ (1%), 8-iso $PGF_{2\alpha}$ (0.25%), 13,14-dihydro-15-keto prostaglandin E_2 (0.02%) and <0.01% to PGD_2, $PGF_{2\alpha}$ and TXB_2

cAMP levels were investigated in paper II. The detection limit was 3 $pmol \cdot ml^{-1}$ and the antibody displayed cross-reactivity towards cGMP (1.5%) and other compounds (<0.01%).

4.7 MASS SPECTROMETRY

Oxylipins were isolated by solid phase extraction in HLB Oasis cartridges. The quantification was done by tandem liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS). Liquid chromatography was performed in an Acquity instrument with a UPLC BEH C_{18} column. The mass spectrometer was a Xevo TQ system.

4.8 RNA PREPARATION AND REAL TIME-PCR

Animals from which mRNA was extracted were all sacrificed by an overdose of sodium pentobarbital and tissue preparations were removed and stored in RNAlater® at room temperature until use (0-4 days later). Trachea, lung parenchyma and thoracic aorta were gently dissected from the surrounding connective tissue and tracheal epithelium was denuded using a scalpel after which the airway smooth muscle layer was cut out

Guinea pig RNA was isolated using the RNeasy Mini Kit followed by concentration and purity measurement using a Nanodrop 3300. Aliquots of RNA were reverse-transcribed into cDNA using a QuantiTect® Reverse Transcription Kit.
All PCR primers were designed towards available guinea pig sequences from the Ensembl database (www.ensembl.org) and the NCBI Genebank Sequences (www.ncbi.nih.gov) from the 2nd scaffold of the guinea pig (Cavia porcellus) genome comprised by the Feb. 2008 Cavia porcellus draft assembly (Broad Institute cavPor3). Primers for the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were adapted from previous studies (Bourne *et al.*, 2005; West *et al.*, 2005). All primers were titrated using increasing concentrations of cDNA to get a shift of the CT values, expressed as a regression slope of 3.32 ($2^{3.32}$=10) with less than 10% deviation. Furthermore, a melt curve analysis was performed to ensure the quality of the amplification product.

Real-time PCR was performed using Power SYBR® Green PCR Master Mix. Each sample was analysed in duplicate in MicroAmp® Fast optical 96-well Reaction Plates using an Applied Biosystems 7500 Real-Time PCR system. The program was set to 50 °C 2 min followed by a hot start at 95 °C for 15 min, followed by 6 cycles; 94 °C 30 sec, 66 °C 1 min, followed by 40 cycles; 94 °C 30 sec, 55 °C 1 min and followed by a melt curve analysis. mRNA levels were calculated using the comparative CT method

and normalized to the housekeeping gene β-actin and expressed as 10^5 mol·mol^{-1} β-actin.

4.9 CALCULATIONS AND STATISTICS

All data are presented as mean ± SEM. Data accumulated from the organ bath were expressed as a relation to the maximum contractions relative to maximal relaxation. For agonists, a non-linear regression with a variable slope fit was used to calculate E_{max}, pEC_{50} and Hill slope **(Table 3)**. For the antagonist assay, agonist concentration-response curves were globally fitted to the modified Gaddum/Schild model using GraphPad Prism®.

Table 3. Basic pharmacological terms used within this thesis

Term	Definition
E_{max}	Maximal response elicited of an agonist
pEC_{50}	The $-\log^{10}$ concentration of an agonist that produces 50% of the maximal possible effect of that agonist
pK_B	The dissociation constant expressed as $-\log^{10}$ concentration where occupancy is 0.5, reflecting the affinity of the antagonist to its receptor
Hill slope	Cooperativity of the agonist mediated reactions, reflected by the steepness of the concentration-response curve
pA_2	The value that is generated by a Schild plot analysis at Y = 0; (X-intercept)
Schild Plot	A pharmacological method of antagonist characterisation. A plot of log (concentration ratio − 1) versus log concentration of antagonist. If the slope of the regression is not different from unity then by definition the antagonist is a competitive antagonist (provided parallel shifts with maintained maximal effect)
Affinity	How the agonist binds to the receptor
Efficacy	How effective the drug is at activating the receptor

Normally distributed unpaired data comparison between two groups was assessed by unpaired *t*-test with Mann Whitney U-test as the post hoc test. For more than two groups, the parametric test One-Way Analysis of Variance followed by the post hoc

Bonferroni's Multiple Comparisons Test was used. A *p*-value of less than 0.05 was considered significant. All statistical analysis was performed using GraphPad Prism®.

5 RESULTS AND DISCUSSION

5.1 CHARACTERISATION OF EP RECEPTORS IN AIRWAYS (PAPERS I AND IV)

5.1.1 Guinea pig trachea

To characterize the receptors involved in the PGE_2 response, experiments were initiated by obtaining the profile and activity within our model. PGE_2 produced a bell-shaped concentration-response curve with a pEC_{50}-value for the contraction of 8.2±0.2 and 6.7±0.1 for the relaxation **(Figure 6A)**, closely similar to the one described by Coleman over 30 years ago (Coleman *et al.*, 1980). This bell-shaped pattern, with an initial contraction displayed at low concentration, and relaxation at high concentrations indicated activation of multiple signalling pathways. Although experiments using the previous generation of drugs support a general concept where bronchoconstriction is mediated by EP_1 and EP_3 receptors, and airway relaxation by EP_2 and EP_4 receptors, (Buckley *et al.*, 2011; Jones *et al.*, 2009), it had not previously been possible to simultaneously assess the role of each EP receptor in any airway preparation.

The role of PGE_2 was investigated by identification of mRNA expression for both COX enzymes, all three PGE synthases and all four receptors in the airway of the guinea pig in our study in combination with a new class of available subtype selective pharmacological tools **(Table 2 and 4).**

Table 4. Subtype selective pharmacological tools used within this thesis

Name of receptor antagonist	Primary target
ONO-8130	EP_1
PF-04418948	EP_2
ONO-AE5-599	EP_3
ONO-AE3-208	EP_4
SQ-29,548	TP

Using guinea pig specific primers, we found mRNA expression of all four EP receptors in GPT. To further characterize the EP receptors involved in the PGE_2 response, initial experiments were performed using the novel and selective EP_1 antagonist ONO-8130. ONO-8130 shifted the contractile part of the bell-shaped concentration-response curve

for exogenous PGE_2 to the right and also, simultaneously depressed the peak contractions at higher concentrations **(Figure 6B)**. This suggests that EP_1 receptors are involved in the contraction. The responses to ONO-8130 did not demonstrate a classical competitive antagonism, possible due to interference by other receptors activated by PGE_2. Therefore, the selective EP_1/EP_3 receptor agonist 17-phenyl trinor PGE_2 was used in the characterisation of the contractile component. In these experiments no bell-shaped profile was observed and ONO-8130 caused a classic, competitive, parallel shift to the right of concentration-response curve with maintained maximal response, clearly depicting the EP_1 receptor as a major contractile receptor in the GPT **(Figure 6C)**. From these results, a pA_2 value of 8.93 for ONO-8130 acting on the EP_1 receptor could be calculated **(Figure 6D)**. Thus, ONO-8130 is a far more potent EP_1 receptor antagonist than the commonly used AH6809 with previously estimated pA_2 values of 6.4 - 7.0 for the EP_1 receptor and also with similar affinities for EP_2, EP_3 , DP_1 and TP receptors (Abramovitz *et al.*, 2000).

With EP_1 receptors identified as a contractile receptor in the GPT, the presence of mRNA expression for the EP_3 receptor suggested the possibility of another contractile EP receptor within the ASM. The EP_3 receptor have previously been implicated in the modulation of neural responses (Maher *et al.*, 2009) and activation has been linked to inhibitory responses on parasympathetic nerves innervating the GPT (Clarke *et al.*, 2004). However, the selective EP_3 receptor antagonist ONO-AE5-599 displayed no effect on the concentration-response curves to either PGE_2 or 17-phenyl trinor PGE_2. Furthermore, the EP_1/EP_3 receptor agonist sulprostone did not induce a contraction of the guinea pig trachea in the presence of ONO-8130. This suggests that EP_3 is not directly involved in induction of ASM contraction and that EP_1 is the sole contractile EP receptor in the GPT.

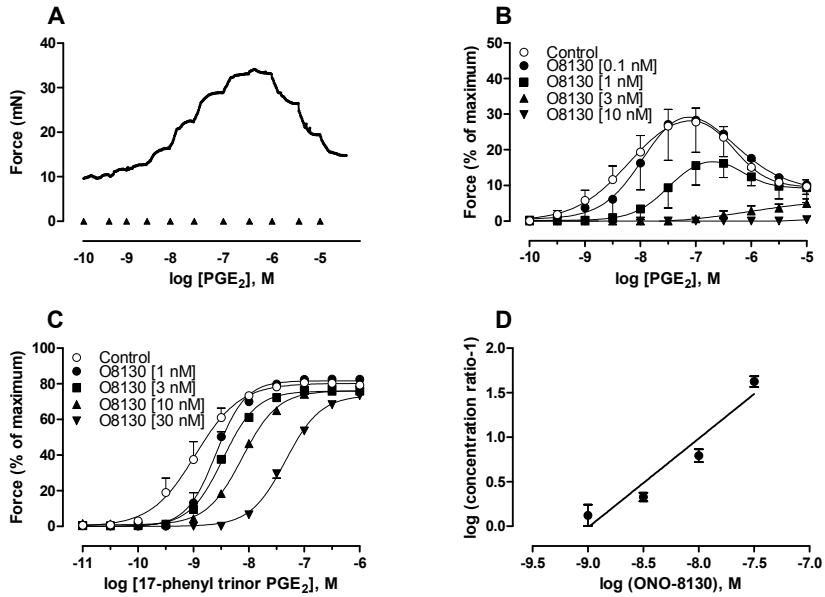

Figure 6. (A) Representative trace of PGE_2 in a segment treated with indomethacin. (B) Concentration-response curves of PGE_2 in guinea pig trachea in the presence of the selective EP_1 receptor antagonist ONO-8130 at different concentrations. (C) Contraction induced by cumulative concentrations of 17-phenyl trinor PGE_2 in guinea pig tracheal segments in the presence of ONO-8130 at different concentrations (1 nM to 30 nM). (D) Schild plot calculated from the concentration-response curves in figure 6C. Constraining the Schild slope to unity resulted in a pK_B value of 8.9.

Since the PGE_2 profile in GPT was bell-shaped, it suggested activation of receptors mediating a relaxation that counteracts the induced contraction. To characterise the role of the EP_2 receptor, the new and selective EP_2 receptor antagonist PF-04418948 (Forselles *et al.*, 2011) was used. PF-04418948 caused a concentration-dependent increase of peak contraction by PGE_2, in contrast to ONO-8130 that caused a decrease. PF-04418948 also induced a rightward shift of only the relaxation part of the concentration-response curve **(Figure 7A)**. Thus, the relaxation could not be analysed properly at baseline due to opposite actions of PGE_2. To isolate the relaxation, the ASM tone was pre-contracted with the cholinergic agonist carbachol in the presence of ONO-8130. Increasing concentrations of PF-04418948 caused a parallel rightward shift of the concentration-response curve with maintained maximal response for PGE_2-induced relaxation **(Figure 7B)** that was used to calculate a pK_B value of 7.48 **(Figure 7C)**. This value is almost 50 to 100-fold lower compared to the pK_B value of 8.3 and 8.9

found in human myometrium and mouse trachea, respectively (Forselles et al., 2011), suggesting marked species differences. The relaxations also occurred at lower PGE_2 concentrations than observed for the relaxant part of the bell-shaped concentration-response curve, suggesting an overlap in concentrations for the contractile and relaxant effects mediated by activation of EP_1 and EP_2 receptors.

The EP_4 receptor, known to induce relaxation in rat trachea (Lydford *et al.*, 1994), was highly expressed at mRNA-level in GPT and was thus investigated using the selective EP_4 receptor antagonist ONO-AE3-208. However, neither the potency nor the maximal relaxation to PGE_2 was changed suggesting that EP_4 receptors are not involved in PGE_2 mediated relaxation in GPT.

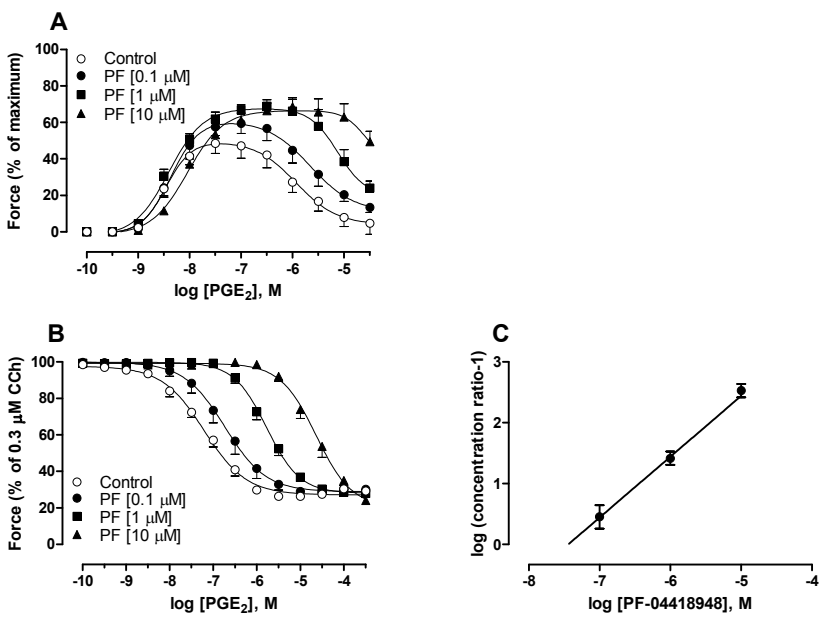

Figure 7. Concentration-response curves of PGE_2 in guinea pig trachea. (A) In the presence of the selective EP_2 receptor antagonist PF-04418948 at different concentrations. (B) In segments pre-contracted with 0.3 μM carbachol after treatment with PF-04418948 at different concentrations. (C) Schild plot calculated from the concentration-response curves in figure 7B. Constraining the Schild slope to unity resulted in a pK_B value of 7.48.

5.1.2 Human bronchi

To characterise the contractile EP receptors involved in the PGE_2 response in HB experiments were performed in presence of antagonists for the presumed relaxant

receptors. Both PGE_2 and 17-phenyl trinor PGE_2 induced a powerful contraction at high agonist concentrations. The contractions were not reduced when the EP_1 receptor was antagonised with ONO-8130, but a substantial reduction was observed by the TP receptor antagonist SQ-29,548. This indicates that human small bronchi do not contract to PGE_2 *via* EP_1 or EP_3 receptors, but instead via the TP receptor.

Since PGE_2 is known to induce bronchorelaxation in humans (Kawakami *et al.*, 1973; Melillo *et al.*, 1994; Pavord *et al.*, 1993; Walters *et al.*, 1982), the two cAMP –inducing EP_2 and EP_4 receptors were investigated. To observe a relaxation, the contraction of the TP receptor was blocked and the HB segments were pre-contracted with histamine. In these experiments it was shown that PGE_2 caused a concentration-dependent relaxation which was inhibited by ONO-AE3-208, but not by PF-04418948.

Initially it was proposed that EP_2 was the relaxant PGE_2 receptor, based on a study using AH6809 and the receptor agonist misoprostol (Norel *et al.*, 1999) whereas recent studies, using more selective receptor antagonists, implicates the EP_4 receptor (Benyahia *et al.*, 2012; Buckley *et al.*, 2011) as the responsible relaxant receptor. Thus, our study can confirm that PGE_2 relax human small airways through the EP_4 receptor and that the EP_2 receptor is not involved in bronchorelaxation directly.

5.1.3 Summary

Expression of all four EP receptors was found at mRNA-level in GPT. However, using new and selective pharmacological antagonists and agonists, we could functionally identify the EP_1 receptor mediating contraction and EP_2 receptors mediating relaxation. Thus, the mRNA levels do not completely reflect the functional outcome, possible due to regulation at post-transcriptional levels. In human small airways no EP receptor is involved in contraction whereas the EP_4 receptor is responsible for relaxation. Thus, in both GPT and human small airways, PGE_2 have the possibility to contract and relax airway smooth muscle, however the receptors involved differ completely.

5.2 SPONTANEOUS AIRWAY TONE (PAPERS I, III AND IV)

5.2.1 Guinea pig trachea

The observation that the spontaneous airway tone could be completely reduced by NSAIDs and ONO-8130 as well as increased by PF-04418948 was made during the

characterisation of the EP receptors. This suggested that PGE_2 acting on the EP_1 and EP_2 receptors are involved and thus warranted closer investigations.

After the wash and resting period, following the initial standard assessment of histamine responsiveness, GPT segments develop a spontaneous contractile tone that was reduced by administration of NSAIDs, such as indomethacin, ibuprofen and diclofenac, reaching the same level acquired by high concentration of papaverine or theophylline, *i.e.* maximally obtainable relaxation of the smooth muscle. This reduction was also observed upon administration of ONO-8130 whereas the PF-04418948 caused a substantial increase of the tone instead **(Figure 8)**. It was also shown that the tone was more rapidly decreased by ONO-8130 than that caused by the enzyme inhibitors, suggesting a mechanism involving the immediate blockade of receptors rather than a gradually diminished biosynthesis. Since ONO-8130 and PF-04418948 were the only receptor antagonists that modulated the tone became apparent when other prostanoid receptor antagonists against DP_1, EP_3, EP_4, FP, IP or TP failed to elicit a change, suggesting that PGE_2 is the likely compound to mediate the effect. This conclusion obtained strong support when it was found that a high affinity neutralizing monoclonal antibody against PGE_2 relaxed the preparations concentration-dependently.

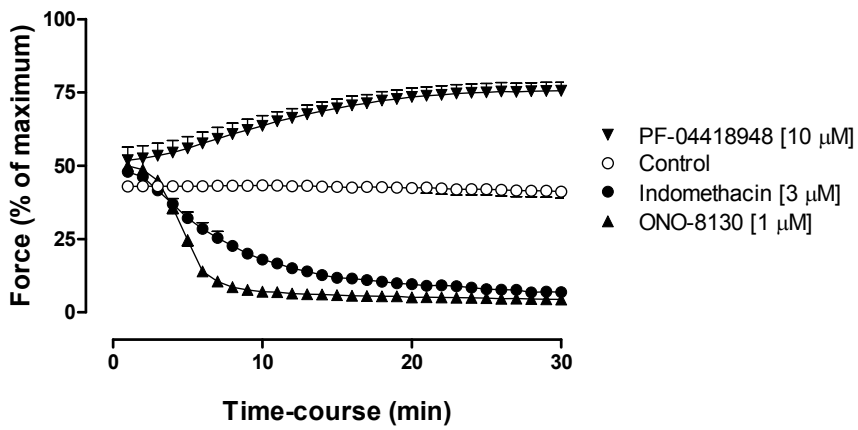

Figure 8. Development of spontaneous tone after addition of EP_1 receptor antagonist (ONO-8130), EP_2 receptor antagonist (PF-04418948) and COX inhibitor (indomethacin).

To investigate which of the two COX isoenzymes that mediated and maintained the spontaneous tone, four selective COX inhibitors with different COX-1/COX-2 ratio were used. The more COX-1 selective inhibitors FR-122047 and SC-560 had a smaller

effect on spontaneous tone compared to the more COX-2 selective inhibitors etoricoxib and lumiracoxib, confirming that COX-2 is the major enzyme catalysing formation of PGE_2.

Evidence was obtained to support that a significant part of the biosynthesis of PGE_2 originate from the tracheal epithelium since the active tension was lower in epithelium denuded segments than in epithelium intact. The epithelium has previously been suggested to be an important source of PGE_2 (Hay *et al.*, 1988). The tone was however not completely reduced with epithelium denudation suggesting that the ASM also contributes in the synthesis of PGE_2. This is in line with the high expression of COX-2 in both smooth muscle and epithelial cells.

The involvement of LTs in mediating the tone was investigated when the 5-LOX inhibitor zileuton reduced the spontaneous tone. However, neither the FLAP inhibitor MK-886 nor $CysLT_{1\text{-}2}$ receptor antagonist Bay-u9773 had any effect. The reason why zileuton reduced the spontaneous tone is due to that it, together with its potent 5-LOX inhibitory action, also inhibits prostanoid production at higher concentration (Rossi *et al.*, 2010).

Since the spontaneous tone play a central and always present role for GPT ASM function, there is an importance of incorporating it when interpreting the response of other agonists. As is shown in absolute force, histamine causes a contraction and indomethacin reduces the spontaneous tone **(Figure 9A)**. If the reduction of tone is not incorporated in the calculation, the starting point will be the same. If one also neglects to integrate the full contractile span, the increase in regard of maximum will not be related to the maximal relaxation, but to a false zero **(Figure 9B)**. If the full contractile span is included, but not the level of spontaneous tone, there is an even greater discrepancy that could be misinterpreted as indomethacin treated segments contract more than untreated **(Figure 9C)**. To be able to more accurately define the agonist response, it is more correct to define the level of the spontaneous tone and combine it with the full contractile span of the segment, thus Specific Force = (Measurement – Minimum) / (Maximum – Minimum) **(Figure 9D)**.

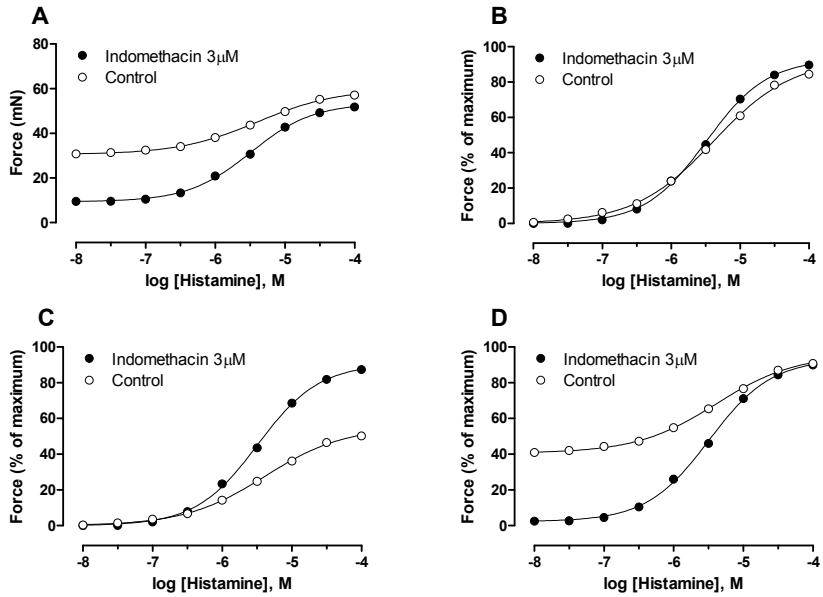

Figure 9. Concentration-response curve to histamine in the presence or absence of indomethacin. The same data are displayed in four different ways: (A) Absolute force, (B) related to maximum, (C) related to the full contractile span (D) incorporating both spontaneous tone and full contractile span.

5.2.2 Human bronchi

Airway preparations of different species possess varying degrees of spontaneous tone mediated by different agonists (Linden *et al.*, 1991; Okazawa *et al.*, 1990; Schmidt *et al.*, 2000). In this study, NSAID treatment of HB did not cause the same complete abolishment of the tone as observed in the GPT. Instead an over-time gradual increase of tone was obtained reaching around 20%. This increased contraction could possibly be as a result of removal of relaxant responses by prostanoids, but antagonising possible relaxant EP_2, EP_4 and IP receptors as well as possible contractile EP_1, EP_3 or TP receptors did not induce a change. Another possibility for the observed increase is removal of inhibition of leukotriene synthesis (Kuehl *et al.*, 1984), resulting in higher concentration of endogenously produced leukotrienes. The decreased tone by FLAP inhibitor MK-886 supports this theory also suggesting that in HB, leukotrienes are maintaining the tone. The phenomenon with change of tone after NSAID administration have previously been described, displaying both increased (Bjorck *et al.*, 1993; Coleman *et al.*, 1996; Hutas *et al.*, 1981), decreased (Ito *et al.*, 1989) or no effect on the tone (Brink *et al.*, 1980). This may be due to limited amount of investigated

tissue, different treatments of patients prior to surgery or possibly varying responses in different compartments of the lung.

5.2.3 Organ culture

Having identified the EP_1 and EP_2 receptors to mediate the response to exogenous PGE_2 in GPT another aim of the current study was to examine how the endogenous production of PGE_2 affects the responses of these receptors. A homologous desensitisation of the EP_1 receptor and not EP_2 receptor has previously been described in cell models (Illes *et al.*, 1975; Penn *et al.*, 2001; Vermue *et al.*, 1987). In our *in vitro* study, we found that during four days culture, tracheal segments released a substantial amount of PGE_2. This resulted in a completely abolished contraction, yet a maintained relaxation towards exogenously added PGE_2 was shown. When different NSAIDs were introduced to the culture, the released PGE_2 was abolished and a complete PGE_2 concentration-response curve could be obtained.

HB was incubated using the same culture method over 8 days, however during that time no EP desensitisation was observed (not shown) suggesting that the release of PGE_2 is not as extensive as in GPT. It could be argued that our lack of function for the EP_1 receptor was due to a desensitisation, but there was no EP_1 response in tissue used immediately after surgery (not shown). Furthermore, the culture of human segments showed to be an optimal method because the spontaneous fluctuations of tone observed in the tissue studied acutely was removed. Also, re-culture of used segments with maintained contractile capacity was possible up to at least three times over eight days, which opens up for a methodology where *ex vivo* "cross-over"-studies are possible.

5.2.4 Summary

In the GPT, the spontaneous tone is maintained by PGE_2 primarily synthesised *via* COX-2 within the airway epithelium, mediating a simultaneous activation of both contractile EP_1 and relaxant EP_2 receptors. In human small airways, by use of selective receptor antagonists, we conclude that PGE_2 does not play a role maintaining the tone, an action mediated by leukotrienes since a FLAP inhibitor have the capacity to reduce it. However, HB tone is modulated by NSAIDs through an unknown mechanism. Both HB and GPT utilize arachidonic acid metabolites as mediators for spontaneous tone, however, by completely different agonists as a result by different enzymatic pathways. Moreover, the organ culture experiments indicated that when culturing GPT, specific

caution should be taken since the EP receptors are constantly activated. Addition of NSAIDs will prevent this phenomenon.

5.3 RELAXATION OF AIRWAYS MEDIATED BY BITTER TASTE RECEPTORS (PAPER II)

5.3.1 Guinea pig trachea

As initial experiments performed also showed an interaction between PGE_2 and several, but not all TAS2R agonists, this connection was investigated further.

Using in-house developed primers based on relevant TAS2Rs from agonists by Deshpande *et al*., expression of mRNA for TAS2R3, TAS2R4 and TAS2R10 were detected in both epithelial and smooth muscle cells. Denatonium, chloroquine, thiamine and noscapine induced strong relaxation of GPT pre-contracted with carbachol **(Figure 10A-D)**. For denatonium and chloroquine the magnitude of relaxation was comparable to that of salbutamol **(Figure 10E)** and formoterol **(Paper II, Figure 7D)**, however with markedly lower potencies. Pre-treatment with either denatonium or chloroquine prior to contraction with contractile agonists showed that denatonium could only reduce the effect of carbachol, whereas chloroquine decreased the effects to histamine, U-46619, LTD_4 and OVA. The selective effects of denatonium to only relax cholinergic responses raised the possibility that it is a muscarinic antagonist. However, this hypothesis was omitted since acetylcholine-mediated relaxation in aorta was unaffected by denatonium.

The inability for denatonium to relax endogenous PGE_2 was demonstrated as it could only cause relaxation to the same contractile level as the spontaneous tone maintained by PGE_2. Upon indomethacin or ONO-8130 treatment, thus abolishing the spontaneous tone by removing endogenously produced PGE_2 or antagonising EP_1 receptor respectively, an enhanced relaxation of GPT was achieved by denatonium. The same enhanced phenomenon was displayed with thiamine, suggesting similar relaxant pathway as for denatonium which clearly differ from both chloroquine and noscapine.

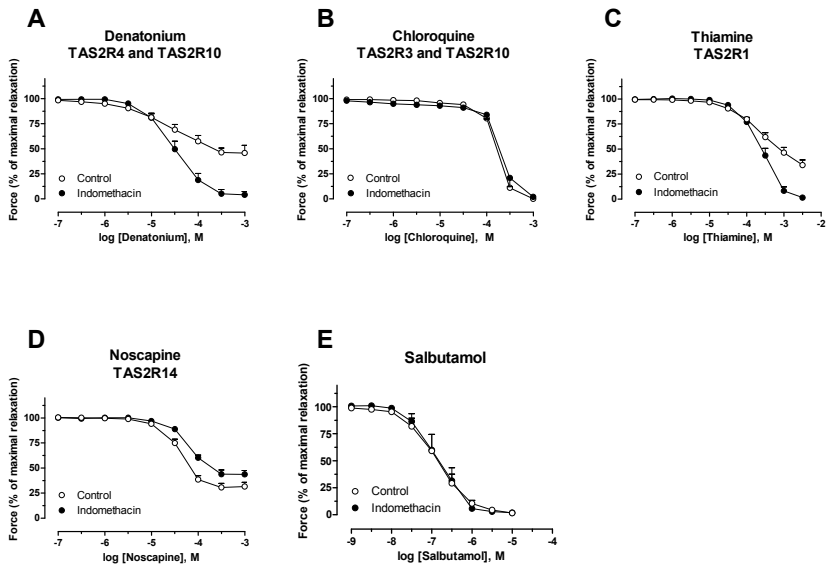

Figure 10. Concentration-response curves to the TAS2R agonists (A) denatonium, (B) chloroquine, (C) thiamine and (D) noscapine as well as (E) the β_2-adrenoceptor agonist salbutamol, in presence or absence of indomethacin.

The previously proposed general pathways for TAS2Rs relaxation are hyperpolarization through large conductance potassium channels (BK_{Ca}) channel opening (Deshpande *et al.*, 2010) and cAMP production (Dasso *et al.*, 2011). In our study, production of cAMP after TAS2R agonist revealed no change in produced levels compared to salbutamol which caused a marked increase. BK_{Ca} channels are also shown to be involved in the relaxation mediated by β_2-adrenoreceptors (Jones *et al.*, 1993; Miura *et al.*, 1992). Blockers of the BK_{Ca} channel (iberiotoxin, charybdotoxin and paxilline) did not affected chloroquine relaxation, whereas the potency, but not efficacy was slightly shifted for denatonium. Furthermore, the relaxation by denatonium was not mediated through PKA, PKC and PKG-dependent pathway as selective kinase inhibitors for these kinases involved in cAMP, contractile machinery and NO-mediated pathways, respectively, had no effect

The level of pre-contraction can influence both the magnitude and the potency of the agonist-induced relaxation (Belvisi *et al.*, 2011). When the preload was increased to supramaximal levels both denatonium and chloroquine induced maximal relaxations whereas neither of the β_2-adrenoceptor agonists did. There was an inverse relationship

regarding the potency and preload for denatonium, whereas the potency for chloroquine was unaffected. This confirmed the observation that bitter taste agonists induced a stronger effect than β_2-adrenoceptor agonists (Deshpande *et al.*, 2010).

Taken together, the difference detected during characterisation suggests that denatonium and chloroquine mediates relaxations through different signalling pathways and that activation of BK_{Ca} channels is only one part of the denatonium mediated smooth muscle relaxation.

5.3.2 Human bronchi

The smooth muscle relaxing properties of bitter tasting agonists denatonium, noscapine quinine and chloroquine could be replicated in isolated human small bronchi pre-contracted with carbachol (preliminary results; **Figure 11**). Similar as in the guinea pig trachea the potency was low whereas the efficacy was high.

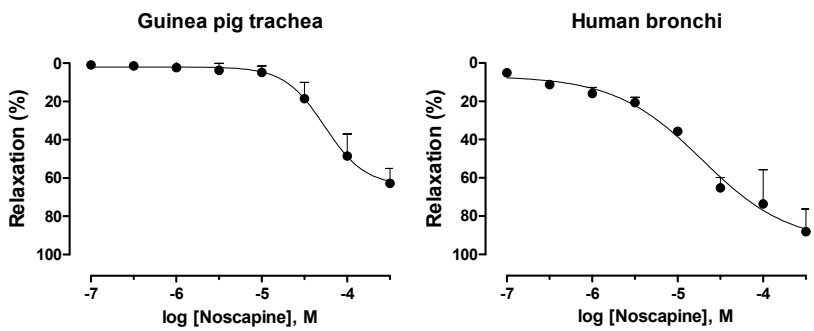

Figure 11. Comparison between concentration-response to the TAS2R agonist noscapine in guinea pig trachea and isolated human bronchi.

5.3.3 Summary

It was documented that bitter taste receptors are expressed in the guinea pig trachea and that agonists for these receptors can induce strong relaxations. In carbachol contracted preparations, denatonium and chloroquine relaxed maximally contracted preparations whereas the β_2-adrenoceptor agonist salbutamol under these conditions had a lower efficacy. Where denatonium only reduced the effects of cholinergic contractions, chloroquine uniformly inhibited contractions evoked also by histamine, contractile prostanoids and cysteinyl-leukotrienes. The different effects on different contractile

agonists suggest that they act through different pathways. In addition, although we could not identify the primary signalling mechanisms for the bitter taste agonist response, relaxations induced by denatonium were sensitive to inhibitors of BK_{Ca} activation. Furthermore, the relaxation could be replicated in human small bronchi suggesting that the mechanism exists in both species and thus could provide a target for bronchorelaxant drugs. In regards of the relaxant mechanism, initially it was described as the activation of BK_{Ca} resulting in hyperpolarization of the cell membrane (Deshpande *et al.*, 2010), however, a recent study oppose that proposal instead suggesting inhibition of L-type voltage-dependent Ca^{2+} channels (VDCCs) (Zhang *et al.*, 2013). As of today, none of these hypothesis presented have been confirmed as the sole mechanism of ASM relaxation by bitter tasting compounds.

5.4 PROSTANOIDS INVOLVED IN THE ANTIGEN-INDUCED CONTRACTION (PAPERS III AND IV)

5.4.1 Guinea pig trachea

In the organ bath, cumulative concentrations of OVA (0.1 ng/mL to 0.1 mg/mL) induced a concentration-dependent antigen-induced contraction irrespectively of initial smooth muscle tone in guinea pigs actively sensitized against OVA **(Figure 12)**. By removing the spontaneous tone with NSAIDs the maximum contractile response to the antigen was increased. When the EP_2 receptor was antagonised, the maximum contraction was increased both with and without EP_1 receptor antagonism, confirming and further specifying that the increased response observed by NSAIDs was due to inhibition of the relaxant effect by PGE_2 on EP_2 receptors **(Paper III, Figure 1)**. This phenomenon was also seen when only the EP_1 receptor was antagonised, where a reduced maximal effect was obtained.

Blocking $H_{1\text{-}2}$ receptors or inhibiting the response to CysLTs using the selective FLAP inhibitor MK-886 and nonselective $CysLT_{1\text{-}2}$ receptor antagonist BAY-u9773 alone or in combination did not affect the maximum antigen response, suggesting that the receptor responses to each separate agonist are close to ASM maximum making additive effects indistinguishable. Combining antihistamines with inhibition of CysLT and prostanoid production caused a marked inhibition resulting in a 70% reduction of the antigen response. This was irrespectively of whether the antigen-induced contraction was reduced using antihistamines in combination with complete inhibition

of COX and 5-LOX enzymes, or by the use of antagonists targeting EP_{1-2}, TP or $CysLT_{1-2}$ receptors. The remaining 30% contraction was completely inhibited by the relaxing effect of the EP_2 receptor (when not antagonised), but was resistant to atropine, ketanserin and HOE-140 indicating that muscarinic M_{1-5} receptors, serotonin 5-HT_{2A} and bradykinin BK-B_2 receptors respectively are not involved antigen-induced contraction in the guinea pig trachea and that there is a, yet undetermined, residual component(s) released.

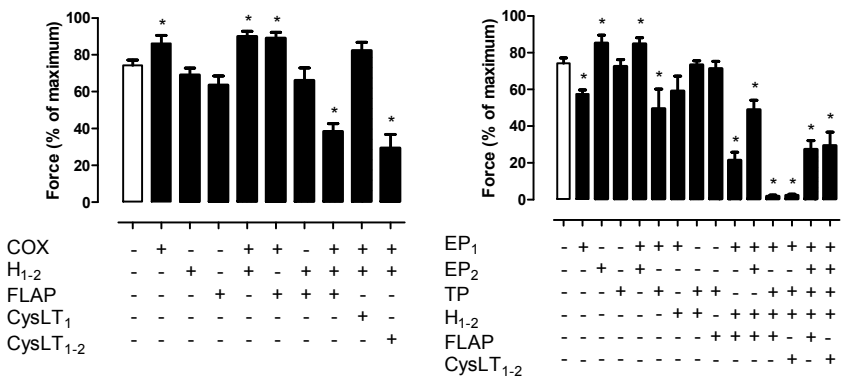

Figure 12. Maximal effect of the antigen-induced responses after treatment with different receptor antagonists and enzyme inhibitors. COX=cyclooxygenase inhibitor indomethaicn; H_{1-2}=histamine 1 and 2 receptor antagonist mepyramine and metiamide; FLAP=5-lipoxygenase activating protein inhibitor MK-886; $CysLT_1$= Cysteinyl-leukotriene 1 receptor antagonist montelukast; $CysLT_{1-2}$=Cysteinyl-leukotriene 1 and 2 receptor antagonist BAY-u9773; EP_1= EP_1 receptor antagonist ONO-8130; EP_2=EP_2 receptor antagonist PF-04418948; TP=TP receptor antagonist SQ-29,548.

To study the release pattern regarding prostanoids in the GPT subsequent antigen exposure, bath fluid was collected during specific time points and analysed by a triple quadrupole mass spectrometer. In control segments, only PGE_2 and PGI_2 (measured as 6-ketoPGF$_{1\alpha}$) were detected in quantifiable amount in the organ bath fluid **(Figure 13)**. Both increased with a similar rate over time (preliminary data). After antigen-exposure there was two distinct patterns of prostanoid release; immediate and slow. Both PGD_2 and TXA_2 (measured as TXB_2) belong to the immediate profile with a substantial release after 15 minutes that continued to increase over time (preliminary data). PGE_2, 6-keto PGF$_{1\alpha}$ and PGF$_{2\alpha}$ were also detected, with a minor immediate release and

displaying a slower gradual increase over time (preliminary data). The levels of all prostanoids were completely abolished upon stimulation with COX inhibitor, whereas the FLAP inhibitor MK-886 did not affect the release (not shown).

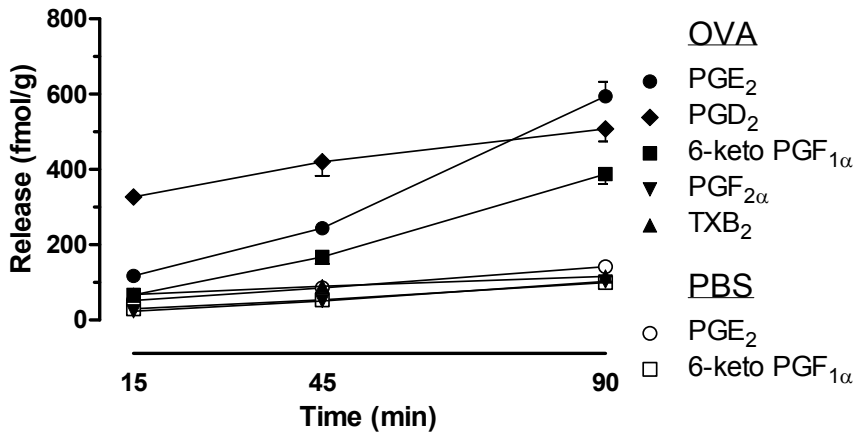

Figure 13. Prostanoid release in relation to wet weight of the tracheal segment during PBS treatment (open symbols) and antigen exposure (closed symbols). Only PGE_2 and PGI_2 (measured as 6-keto$PGF_{1\alpha}$) were detected in quantifiable amount in the organ bath fluid after PBS treatment.

There was a major release of PGD_2, which is a known mediator in the early phase reaction recognized to mediate their contractile effect through the TP receptor (Larsson *et al.*, 2011; Turner *et al.*, 1988). Solely antagonising the TP receptor did not affect the contraction due to the other contractile mediators in the background, but when histamine and CysLT responses were inhibited, a component exerting a 30% contraction could be unmasked that was susceptible to SQ-29,548. Thus, we could for the first time show the magnitude of the antigen-induced contraction mediated through the activation of TP receptors.

5.4.2 Human bronchi

Antigen -induced contractions of human bronchi were studied by challenge with a human monoclonal anti-IgE antibody (anti-IgE). This causes degranulation of the mast cells present in the isolated airway by cross-linking of IgE molecules on their surface. The response to the highest concentration of anti-IgE was close to the maximal contraction of the bronchi. Maximal contraction was reached within 15 min. The IgE-

dependent contraction was abolished by the combination of H_1 receptor antagonist, FLAP enzyme inhibitor and TP receptor antagonist, supporting that the three classes of mast cell mediators histamine, CysLTs and PGD_2 mediated the response. The findings show that the IgE-dependent contraction of small human airways has the same mediators as previously shown in larger bronchi (Bjorck *et al.*, 1993; Dahlen *et al.*, 1983; Schmidt *et al.*, 2000).

In line with the above reported relaxation of HB by PGE_2 acting on EP_4 receptors, previous data have suggested that activation of EP_4 receptors by exogenous PGE_2 may relax bronchial segments pre-contracted with anti-IgE (Benyahia *et al.*, 2012). However, pre-stimulation with an EP_4 receptor antagonist prior to anti-IgE challenge did not affect the anti-IgE induced contraction **(Figure 14A)**. This indicates that the endogenous levels of PGE_2 are insufficient to reach a concentration that effectively activates EP_4 receptors in small airways. Furthermore, as expected, incubation with the EP_2 receptor antagonist prior to anti-IgE treatment had no effect on the contraction **(Figure 14A)**.

As it is known that inhaled PGE_2 protects against allergen-induced bronchoconstriction in subjects with asthma (Pavord *et al.*, 1995), it was investigated if such an effect could be mimicked in the isolated small airways. It was hypothesised that the bronchoprotective action of PGE_2 might be due to inhibition of mast cell mediator release, as this mode of action is well established in animal models (Raud et al 1987) and in human purified mast cells (Kay *et al.*, 2006). In order to remove the contractile effect of PGE_2, the experiments were performed in the presence of a TP receptor antagonist. It was indeed found that pre-treatment with exogenous PGE_2 completely abolished the anti-IgE contractile response **(Figure 14B)**. Moreover, antagonism of the relaxant EP_4 receptors prior to PGE_2 administration did not block the effect of PGE_2 on the anti-IgE induced contraction. In contrast, pre-treatment with the EP_2 receptor antagonist completely blocked the inhibitory effect of PGE_2 on the anti-IgE response **(Figure 14B)**. Although studies of mediator release are required to prove the mechanism, the findings support the hypothesis that the bronchoprotective effect of PGE_2 is exerted at the level of the mast cell rather than involving bronchodilation. Taken together, the findings suggest that the EP_2 receptors are more important in regulating airway responsiveness to exogenous PGE_2 than are the EP_4 receptors.

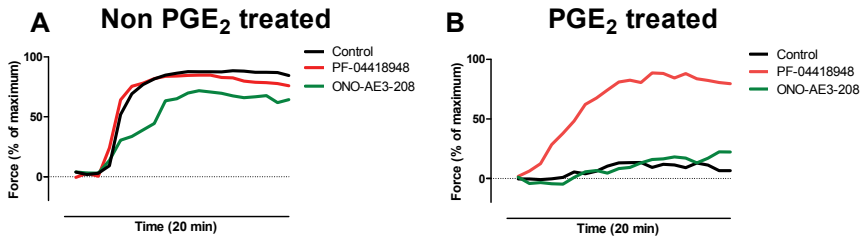

Figure 14. Contractile response in human bronchi after anti-IgE exposure (1/1000 dilution) in presence or absence of EP_2 (PF-04418948) and EP_4 (ONO-AE3-208) receptor antagonists with or without PGE_2 treatment. (A) Only treated with EP receptor antagonists prior to anti-IgE exposure. (B) Treated with EP receptor antagonists followed by PGE_2 treatment prior to anti-IgE exposure.

5.4.3 Summary

In the central airway of the guinea pig, the antigen-induced contractile responses to OVA was due to the release of histamine (acting on H_1), leukotrienes (acting on $CysLT_{1\text{-}2}$) and a COX product acting on TP receptors. The latter component has not been identified previously as it is masked by the powerful effects of PGE_2 on basal tone of the preparation. Using the EP_1 and EP_2 receptor antagonists it was however possible to uncover this component. It is likely that TXA_2 and PGD_2 mediated this part of the response because both are agonists at the TP receptor and both prostanoids were released by challenge. Theoretically, the contractile effect of PGE_2 at the EP_1 receptor might also contribute to the COX component of the antigen response, but the data suggest that PGE_2 predominantly controls the spontaneous tone of the guinea pig. The slow increase of the bath concentration of PGE_2 after the antigen challenge is also consistent with the interpretation that PGE_2 is a modulator rather than a mediator of the antigen-induced contraction.

The mediators of the IgE dependent contractions of small human bronchi were the same as in the guinea pig trachea, namely histamine, CysLTs and prostanoid acting at the TP receptor. There were differences between the preparations however inasmuch that there was no residual response in the human bronchi when all three mediator classes had been blocked and there was no need to use a $CysLT_2$ antagonist in this tissue. The same complete abolishment of the antigen response by combined antagonist of these three receptors (H_1, $CysLT_1$ and TP) has previously been found in the perfused and ventilated guinea pig lung (Sundström et al 2003) and the precision

cut guinea pig lung parenchyma (Ressmeyer *et al.*, 2006). Furthermore, in the latter two guinea pig models, it was possible to identify an inhibitory component by single intervention with any of the three receptors whereas it was remarkable in the GPT that combined antagonism of all three mediator classes was required to have significant effects on the antigen response. Presumably differences with respect to local tissue concentrations of the mediators in relation to the operative ranges for receptor activation explain these differences in sensitivity to receptor antagonism. Nevertheless, by and large, the guinea pig trachea provides a model to study antigen-induced responses which includes the main components of the mast cell dependent contractions of human airways. The model is relevant as mast cell activation is a prominent feature of asthma exacerbations in humans.

The study confirmed that the protective *in vivo* effect of PGE_2 on allergic bronchoconstriction could be replicated in the isolated small human airways. Moreover, it was shown that the effect was mediated by activation of the EP_2 receptor and therefore seemed unrelated to bronchodilation. This is in fact consistent with the effect of inhaled PGE_2 in subjects with asthma where the inhibition of the allergen response occurred at time point when the initial bronchodilation had ceased (Pavord et al 1995).

Focusing on the role of prostanoids, the studies indicate that in humans the TP receptor is a suitable target to block the bronchoconstrictive effects of in particular the major mast cell product PGD_2, but also TXA_2 and possibly PGE_2 if its levels in the tissue are high. Furthermore, the experiment where PGE_2 was administered prior to antigen challenge suggests that EP_2 agonists have potential as inhibitors of mast cell dependent bronchoconstriction. However, combined EP_2 and EP_4 antagonism may be an additional approach as that would add bronchodilation.

6 CONCLUSIONS

The main conclusions obtained from this thesis are:

- In guinea pig trachea, PGE_2 induces a concentration-dependent ASM contraction followed by a relaxation, whereas in HB the actions are reversed.

- Contractile EP_1 receptors and relaxant EP_2 receptors are responsible for the bell-shaped response in the guinea pig trachea whereas relaxant EP_4 receptors and contractile TP mediates the U-shaped action of PGE_2 in HB.

- The spontaneous ASM tone in guinea pig trachea is solely mediated and maintained by PGE_2 simultaneously acting on EP_1 and EP_2 receptors whereas in HB PGE_2 does not regulate.

- In the guinea pig trachea, PGE_2 is produced primarily via COX-2 in the airway epithelium, but also to some extent, by the ASM.

- During antigen-induced response PGE_2 is not one of the major components released from the mast cell in either guinea pig or HB. However, in the guinea pig trachea, PGE_2 plays an important modulatory role on the antigen response by controlling the ASM tone via EP_1 and EP_2. In HB, PGE_2 acts on EP_2 receptors on the mast cell to prevent degranulation.

- Bitter tasting substances have the capability to mediate ASM relaxation in both guinea pig trachea and HB, however, the mechanism is still unknown.

7 GENERAL DISCUSSION

Is PGE_2 really important enough to justify a whole thesis being written on the subject, despite the fact that its structure and biological activities were first described over 50 years ago?

The guinea pig trachea was chosen as the main model to address this question because the anatomy, pharmacological responses and functions resemble those of human airways (Canning *et al.*, 2008; McKenniff *et al.*, 1988; Ressmeyer *et al.*, 2006). Indeed, in the guinea pig trachea and human bronchi, PGE_2 induced both contractions and relaxations. These effects are achieved by PGE_2 activating different subsets of receptors. In the guinea pig trachea, the EP_1 and EP_2 receptors mediate contraction and relaxation, respectively, whereas in human bronchi the same effects are mediated by TP and EP_4 receptors. Thus, both similarities and differences exist regarding the functional properties of the receptors in the two different species.

It was furthermore shown that both in the guinea pig trachea and in human bronchi, lipid mediators maintain the spontaneous tone of isolated airways. However whereas the guinea pig airway mainly employed the COX pathway for this function, the tone of the human airways was mainly underpinned by products of the 5-LOX pathway. This role of eicosanoids for the basal tone of the airways was especially prominent in the guinea pig trachea due to extensive constitutive release of PGE_2 with dual effects on the preparation. The interaction between this effect of endogenous PGE_2 and the direct effects of tested substances needs to be considered when investigating the specific effect of contractile agonists, or relaxant compounds. Indeed, the awareness of this effect led to the discovery that different bitter taste agonists have distinct properties to relax contractions evoked by stimuli that activate different pathways.

The powerful effect of constitutive release of PGE_2 was also present during culture of the guinea pig trachea where it was implicated in the down regulation of the EP_1 receptor. This effect may limit the use of guinea pig trachea for culture procedures as successfully performed with mouse trachea (Adner *et al.*, 2002). Culturing of the human small airways was however established. Perhaps the less prominent constitutive release of PGE_2 and leukotrienes in the human airways explains why culturing of human bronchi was successful. This model is suggested to be useful for investigations

of how human airway smooth muscle cells in their natural surroundings respond to different inflammatory agents.

The present study shows many similarities between the antigen-induced responses in guinea pig trachea and human bronchi. Both are due to the combined action of histamine and CysLTs (Bjorck *et al.*, 1993). Previous data in larger human bronchi have shown that histamine and CysLTs are the major mediators involved. This clearly differs from the isolated airways of mice where serotonin released by mast cells stimulates neural and epithelial release of acetylcholine, that in turn induces contractions (Lei *et al.*, 2013). In the guinea pig experiments, it was furthermore shown that activation of the TP receptor, at which both TXA_2 and PGD_2 act, mediates a significant component of the antigen response. In the human bronchi, the combination of a H_1-antihistamine, a leukotriene biosynthesis inhibitor and a TP receptor antagonist completely abolished the IgE dependent contraction whereas there remained a small yet unidentified component of the antigen response in the guinea pig trachea. The study also confirms and extends the triple antagonism concept, which is that several pathways need to be blocked in order to achieve maximal prevention of bronchoconstriction. Due to the strong action of each agonist, inhibition of only one pathway is not sufficient since the other pathways can still maintain a powerful contraction. Further experiments need to be performed to determine the relative importance of the different mediators in the antigen-induced contraction of small human airways.

In human bronchi, which do not relax in response to EP_2 receptor activation, it was demonstrated that PGE_2 activation of the EP_2 receptor caused a marked inhibition of the antigen-induced response, most likely through inhibition of mast cell activation (Kay *et al.*, 2006; Raud *et al.*, 1987). When the effect of the EP_2 receptor was unopposed during the antigen-induced responses in the guinea pig, a decreased contraction is observed. However, it cannot be concluded from these experiments whether this is due to inhibition of the mast cell response or due to the strong relaxant effect of the EP_2 receptor in the guinea pig.

Clearly, chloroquine, but not denatonium could relax antigen constricted guinea pig trachea, these effects need to be investigated further. In analogy with the suggested capacity for PGE_2 to inhibit mast cell responses, the possibility that bitter taste agonists may inhibit mast cell degranulation also needs to be studied. This is especially important because some bitter taste agonists were found to have anti-inflammatory properties in leukocytes (Pietras *et al.*, 2012).

Despite the introduction of modern therapies, a substantial number of patients suffering from airway ailments still remain insufficiently controlled. Novel bronchorelaxant therapies, utilising both known and unknown pathways for relaxation should therefore be explored further. Inhaled β_2 adrenoceptor agonists are currently the gold standard of bronchodilators, however, since the introduction of long-acting β agonists (LABA) and ultra-LABAs, there has been increasing concern regarding their safety, particularly when used as a monotherapy in the treatment of asthma (Rodrigo *et al.*, 2012). A selective EP_4 receptor agonist would be beneficial and has recently been shown to relax human airways with a higher potency than the β_2 adrenoceptor agonists salbutamol and salmeterol (Benyahia *et al.*, 2012; Gorenne *et al.*, 1995). However, potential cardiovascular side effects needs to be monitored carefully since the EP_4 receptor is involved in the control of vascular tone in *e.g.* cerebral and uterine arteries (Baxter *et al.*, 1995; Davis *et al.*, 2004). Also, activation of the EP_4 receptor has recently been shown to facilitate T_h1 differentiation and T_h17 expansion, two Th-subsets involved in inflammation (Sakata *et al.*, 2010). For these reasons, alternative bronchodilator therapies with an increasing safety profile should be premiered possibly coupled with an anti-inflammatory activity, such as a dual EP_2 and EP_4 receptor agonists. Such a configuration would also not trigger the EP_3 receptor induced cough (Maher et al., 2009), an adverse effect often observed following inhalation of PGE_2 (Cuthbert, 1969). Finally, since current treatment is unable to provide bronchial relief for some patients, the bitter taste agonists might represent a novel therapeutic approach for bronchorelaxation through unknown relaxant pathways. As no previously described pathways have been proven to meditate the relaxation induced by bitter taste agonists, these new findings suggest the presence of new powerful relaxant pathways.

In relation to the question raised in the beginning of this section, it is indeed clear that it has been meaningful to investigate the actions of PGE_2 in airways. With the aid of a new generation of subtype selective receptor antagonists and enzyme inhibitors, it has been possible to perform an extended characterisation of the role of PGE_2 as an important mediator and modulator of airway function, both directly at the level of the smooth muscle and indirectly be control of mast cell mediator release. It is possible that these PGE_2 receptors may become new targets for treatment of asthma and other airway diseases.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Astma är en sjukdom som drabbar andningsorganen. Förekomsten av astma är 5-10% av befolkningen och i nuläget sker en ökning i de flesta länder. Astma kännetecknas av svårigheter att andas och ger symptom i form av hosta och andnöd, i svåra fall kan man avlida genom kvävning. Sjukdomen beror på inflammation och ökad retbarhet i luftrören. En central komponent i astmaattacken är bronkkontraktion, dvs. sammandragning av luftrörens glatta muskulatur. Behandlingen är främst antiinflammatoriska steroider (kortison) och luftrörsvidgande läkemedel som β_2 stimulerare. Detta till trots är det många individer som inte blir symptomfria och behovet av nya terapier är stort. Astma är starkt kopplad till allergen-specifika antikroppar (IgE) mot t.ex. katt, pollen eller kvalster. Genom att IgE binder till mastceller, vilka är vävnadsspecifika vita blodkroppar, har de möjlighet att, vid allergenkontakt, aktivera dessa celler. Vid aktivering utsöndrar mastcellerna en mängd olika substanser, däribland histamin, leukotriener och prostanoider vilka åstadkommer sammandragning av luftrören. Effekterna av dessa substanser förmedlas av specifika mottagarmolekyler, receptorer, som i sin tur kan orsaka kontraktion, relaxation eller ytterligare utsöndring av substanser. Kontraktion av glatt muslukatur, muskelvävnaden i luftvägarna, är den dominerande effekten vid mastcell-aktivering även om substanser som relaxerar också utsöndras. En substans som har förmågan att både kontrahera och relaxera glatt muslukaturen samt påverka mastcellseffekten är prostanoiden prostaglandin E_2. Huvudsyftet med studierna i denna avhandling är att karaktärisera verkan av prostaglandin E_2 i luftvägarna och undersöka vilken roll den har i normala luftvägar samt under allergiska betingelser.

Marsvin valdes som djurmodell då luftvägspreparat från djurslaget uppvisar både fysiologiska och farmakologiska likheter med människa. Ändock viktigare är att marsvin uppvisar ett reaktionsmönster vid allergiska reaktioner som är väldigt likt det man ser hos människan. Bekräftande observationer utfördes även i lungvävnad från människa.

Prostaglandin E_2 har tidigare visat sig primärt binda till fyra olika receptorer, så kallade EP receptorer, men den kan även binda ytterligare receptorer, så som TP receptorn. I luftväg hos både marsvin och människa har den förmåga att kontrahera och relaxera

beroende på vilken receptor som aktiveras. Det visade sig att hos marsvin är det primärt EP_1 receptorn som förmedlar kontraktion samtidigt som EP_2 receptorn orsaker relaxation. Dessa två receptorer medierar även en spontan luftvägstonus, vars syfte är att upprätthålla en dynamisk muskelspänning. I isolerade luftvägar från människa är det TP receptorn som medierar kontraktion och EP_4 receptorn som medierar relaxation vid tillförsel av prostaglandin E_2 med effekten på spontan luftvägstonus mindre framträdande.

Under allergiska förhållanden visades att läkemedel mot histamin, leukotriener och prostanoider (främst via TP receptorn) motverkade majoriteten av luftvägskontraktionen i både marsvin och människa samt att alla tre klasser behövder hämmas för full effekt. Detta ger rationellt underlag att föreslå att liknande kombinationsbehandling borde vara värdefullt vid behandling av astmaattacker och kanske även för att förebygga anfall. Prostaglandin E_2 roll under allergiska förhållanden var olika för marsvin och människa. Hos marsvin påverkade prostaglandin E_2 främst kontraktionen indirekt genom att ändra den spontana luftvägstonusen medan prostaglandin E_2 hos människa kunde hämma den allergiska kontraktionen totalt via en verkan på EP_2 receptorer sannolikt belägna på mast-celler.

Under tiden då arbetet med prostaglandin E_2 fortgick publicerades nya rön som pekade på att bittra substanser, ex. kinin som återfinns i ”*gin&tonic*”, kunde vara luftrörsvidgande samt att en eventuell koppling till prostaglandin E_2 kunde finnas. Det visade sig att relaxationen kunde replikeras hos både marsvin och människa samt att det handlade om aktivering av nya, ännu inte karaktäriserade, signalvägar. Vidare kunde vissa, men inte alla, av de bittra substanserna relaxera luftvägar som kontraherats i en allergisk modell.

Sammanfattningsvis har avhandlingsarbetet identifierat flera nya möjliga angreppspunkter som kan bli mål för utveckling av framtida astmamediciner.

9 ACKNOWLEDGEMENTS

Since this is the page most people will read, here is the take-home message: *PGE_2 is a molecule, highly integrated in vital mammalian homeostatic functions, with the influence to cause both excitatory and inhibitory responses within the same tissue, some of them considered pro- while others anti-inflammatory. The data gathered support that PGE_2 activated EP receptors are potential targets for development of drugs to treat asthma and other forms of airway obstruction.*

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RESEARCH PAPER

PGE_2 maintains the tone of the guinea pig trachea through a balance between activation of contractile EP_1 receptors and relaxant EP_2 receptors

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BACKGROUND AND PURPOSE
The guinea pig trachea (GPT) is commonly used in airway pharmacology. The aim of this study was to define the expression and function of EP receptors for PGE_2 in GPT as there has been ambiguity concerning their role.

EXPERIMENTAL APPROACH
Expression of mRNA for EP receptors and key enzymes in the PGE_2 pathway were assessed by real-time PCR using species-specific primers. Functional studies of GPT were performed in tissue organ baths.

KEY RESULTS
Expression of mRNA for the four EP receptors was found in airway smooth muscle. PGE_2 displayed a bell-shaped concentration–response curve, where the initial contraction was inhibited by the EP_1 receptor antagonist ONO-8130 and the subsequent relaxation by the EP_2 receptor antagonist PF-04418948. Neither EP_3 (ONO-AE5-599) nor EP_4 (ONO-AE3-208) selective receptor antagonists affected the response to PGE_2. Expression of COX-2 was greater than COX-1 in GPT, and the spontaneous tone was most effectively abolished by selective COX-2 inhibitors. Furthermore, ONO-8130 and a specific PGE_2 antibody eliminated the spontaneous tone, whereas the EP_2 antagonist PF-04418948 increased it. Antagonists of other prostanoid receptors had no effect on basal tension. The relaxant EP_2 response to PGE_2 was maintained after long-term culture, whereas the contractile EP_1 response showed homologous desensitization to PGE_2, which was prevented by COX-inhibitors.

CONCLUSIONS AND IMPLICATIONS
Endogenous PGE_2, synthesized predominantly by COX-2, maintains the spontaneous tone of GPT by a balance between contractile EP_1 receptors and relaxant EP_2 receptors. The model may be used to study interactions between EP receptors.

Abbreviations
cPGES, cytosolic PGE synthase; GPT, guinea pig trachea; mPGES, microsomal PGE synthase; NSAID, non-steroidal anti-inflammatory drug; PGI2, prostacyclin; TX, thromboxane

Introduction

PGE_2 is a central messenger molecule with diverse biological effects. The action of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the COX reaction that catalyses its biosynthesis, have implicated PGE_2 as a mediator of pain and inflammation (Flower, 2006). Nevertheless, PGE_2 is continuously released in the airways (Brink *et al.*, 1981; Selg *et al.*, 2009) where it is involved in protective and anti-inflammatory responses. For example, refractoriness to repeated bouts of exercise-induced bronchoconstriction appears to depend upon local formation of PGE_2 (Manning *et al.*, 1993), and accordingly, inhalation of PGE_2 will inhibit bronchoconstriction evoked by exercise, as well as several other triggers of asthma attacks (Pavord and Tattersfield, 1995). Furthermore, the inhalation of PGE_2 is also associated with cough (Pavord and Tattersfield, 1995). The multiple effects of PGE_2 are obviously explained by the presence of several EP receptors that may mediate different and sometimes opposing responses (Coleman *et al.*, 1984).

The first observations of contractile and relaxant effects of PGE_2 in the airways were made in the isolated guinea pig trachea (GPT) (Anggard and Samuelsson, 1965; Coleman and Kennedy, 1980; Gardiner and Collier, 1980) where the cumulative concentration–response curve for PGE_2 was found to be biphasic (Coleman and Kennedy, 1980). In fact, experiments using this standard preparation for airway pharmacology provided early data for the general classification of prostanoid receptors (Coleman and Kennedy, 1980; 1985; Gardiner and Collier, 1980; Kennedy *et al.*, 1982). Along with the observations that aspirin and other NSAIDs (Orehek *et al.*, 1973), as well as the PG antagonist SC-19220 (Farmer *et al.*, 1974), caused relaxation of GPT basal tone, it was assumed that endogenous PGs in this preparation predominantly acted on receptors that mediated contractions. Initially, it was thought that $PGF_{2\alpha}$ or thromboxane (TX) A_2 were the contractile compounds responsible for maintaining the smooth muscle tone (Farmer *et al.*, 1974; Raeburn *et al.*, 1987), but more recent evidence favours PGE_2 as responsible for this role in GPT by activation of contractile EP_1 receptors (Ndukwu *et al.*, 1997).

Research on the mechanisms involved in the actions of PGE_2 in the airways has however been limited by the low selectivity and potency of the pharmacological tools available. Although experiments using the previous generation of drugs support a general concept where bronchoconstriction is mediated by EP_1 and EP_3 receptors, and airway relaxation by EP_2 and EP_4 receptors, (Jones *et al.*, 2009; Buckley *et al.*, 2011), it has not so far been possible to simultaneously assess the role of each EP receptor in any airway preparation. For example, a role for the EP_1 receptor in maintaining the basal tone of the GPT was implied by experiments using the compound AH6809 (Ndukwu *et al.*, 1997), an unselective antagonist with similar affinities for EP_1, EP_2, EP_3, DP_1 and TP receptors (Abramovitz *et al.*, 2000).

More selective agonists and antagonists for the EP receptors have recently become available (Aihara *et al.*, 2007; Forselles *et al.*, 2011). In this study, we have used a new generation of subtype-selective EP receptor antagonists to establish which receptors mediate the contractions and relaxations of GPT to exogenous PGE_2. Intervention with antagonists for each of the EP receptors has to the best of our knowledge not been investigated simultaneously in any airway preparation, although some of the new antagonists have been tested individually (Buckley *et al.*, 2011). The present study in particular examined the role of the different EP receptors in controlling the basal tone of the preparation. As there are considerable species differences regarding responsiveness to prostanoids (Martin *et al.*, 1988; Held *et al.*, 1999; Ressmeyer *et al.*, 2006), one intended outcome of this study was to demonstrate the usefulness of the much employed GPT model for future research on PGE_2 in the airways and in general.

On the basis of experiments with one selective COX-2 inhibitor, it has been proposed that PGE_2 in GPT is generated by COX-2 (Charette *et al.*, 1995). In order to clarify the pathways for PGE_2 formation, we used several isotype selective COX inhibitors in this study. Another objective of the present investigation was to establish whether PGE_2 alone is the critical COX product that maintains basal tone of the preparation, or if other COX products are involved. We therefore assessed the effects of all known prostanoid receptor antagonists, as well a highly specific PGE_2 antibody (Mnich *et al.*, 1995), on GPT basal tone.

In an attempt to provide a stronger molecular framework behind the observed functional responses, the present study for the first time uses real time-PCR with guinea pig-specific primers to document the expression of mRNA for the four EP receptors in different parts of the guinea pig lung and aorta. Although the study is focused on the characterization of EP receptors, we took advantage of the methodology for construction of species specific primers and also explored the mRNA expression of COX and PGE synthase (PGES) enzymes in the pathway for biosynthesis of PGE_2 in the same tissues used for mapping of the receptors.

Methods

Tissue preparation and organ culture

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). The study was approved by the regional animal experimentation ethical review board (N63/07 and N257/09). Trachea and aorta from male albino guinea pigs (Dunkin–Hartley; 350–600 g) and trachea from male albino rats (Sprague–Dawley; 300–350 g) were prepared as previously described (Adner *et al.*, 2002; Morin *et al.*, 2005; Larsson *et al.*, 2007) with some minor changes (Supplementary methods).

Tissue organ bath

Intact guinea pig tracheal and aorta or rat tracheal segments cut as rings were set up in 5 mL organ baths with Tyrode's buffer (Supplementary methods). Changes in smooth muscle force were detected using an isometric force displacement transducer linked to a Grass polygraph. The response was displayed using the IOX data acquisition system (EMKA, Paris, France). During an equilibration period of 60 min with washes every 15 min, the resting force was adjusted to either 30 mN for the guinea pig trachea (GPT), 8 mN for guinea pig aorta and 10 mN for the rat trachea. As a control of guinea pig

tracheal reactivity, histamine (0.1 nM to 0.3 mM) was cumulatively added, whereas for the guinea pig aorta and rat trachea, 60 mM KCl was applied. Before the pharmacological studies, a second wash period and a further 30–45 min equilibration period was completed. In aorta, the presence of an intact endothelium was assessed at the end of the experiment by relaxation to acetylcholine (0.1–10 μM) after pre-contraction with phenylephrine (10 μM).

To investigate the involvement of COX-activity on the spontaneous tone of the trachea, unselective COX inhibitors indomethacin, diclofenac and ibuprofen, selective COX-1 inhibitors FR-122047 and SC-560, selective COX-2 inhibitors lumiracoxib and etoricoxib, monoclonal PGE_2 antibody (2B5), selective DP_1 antagonist BWA868c, EP_1 receptor antagonist ONO-8130, EP_2 antagonist PF-04418948, FP antagonist AL-8810, IP antagonist CAY10441 or TP antagonist SQ-29548 were given either as a single concentration or by cumulative dosing subsequent to the second equilibration period. At the end of these experiments, a single concentration of theophylline (1 mM) or a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM) was given as a reference for the maximal relaxation. To study the action of PGE_2 and other agonists, these were given in a cumulative manner either during spontaneous tone or after 30 min incubation with indomethacin (3 μM). Antagonists were added a minimum of 45 min prior to the agonists. When investigating contractile properties, the experiments were finished by adding histamine (1 mM), acetylcholine (1 mM) and KCl (60 mM) as a reference for the maximal contraction and contractile responses presented as a percentage of this maximum. In cases where maximal contractibility was not obtainable, the contraction was measured as absolute force in Newtons. Histamine was excluded from experiments using rat tissue. Relaxation was studied in segments exposed to carbachol (0.3 μM), to give a stable pre-contraction, prior to addition of the agonists. These experiments were ended with a single concentration of theophylline (1 mM) or a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM) as a reference for the maximal relaxation.

Measurements of mediator release

PGE_2 was measured by enzyme immunoassay (EIA, Cayman Chemical, Ann Arbor, MI, USA; Supplementary methods).

RNA preparation and real-time PCR

Guinea pig RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and real-time PCR, employing primers designed towards available guinea pig sequences, was performed using Power SYBR® Green PCR Master Mix (ABI, Foster City, CA) according to the manufacturers' instructions (Supplementary methods).

Calculations and statistics

All data are presented as mean ± SEM. For agonists, a non-linear regression with a variable slope fit was used to calculate E_{max}, pEC_{50} and Hill slope. Statistical analysis was performed using the one-way ANOVA test, followed by Bonferroni's multiple comparisons test and the Mann–Whitney *U*-test for comparisons between two groups using GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA).

For the antagonist assay, agonist concentration–response curves were globally fitted to the modified Gaddum/Schild model using GraphPad Prism 5.01 (Supplementary methods).

Drugs and suppliers

Stock solutions and dilutions were performed according to manufacturers and suppliers instructions (Supplementary methods).

Results

Expression of mRNA for COX-1, COX-2, mPGES-1, mPGES-2, cPGES and EP_{1-4} in guinea pig ASM, airway epithelium, aorta and lung parenchyma

There was expression of mRNA for all the studied proteins (Figure 1: Supplementary results, Figure S1 and Table S1). For the initial step in the biosynthesis of PGE_2, semi-quantitative analysis revealed a significantly higher expression of COX-2 compared with COX-1 in ASM and airway epithelium ($P < 0.05$; Figure 1A–B). In contrast, the expression of COX-1 was significantly higher than COX-2 in lung parenchyma and aorta ($P < 0.05$; Figure 1C–D).

For the enzymes catalysing the isomerization of PGH_2 to PGE_2, there was a similar expression pattern in all investigated tissues; *viz*. the expression of cPGES was significantly greater than mPGES-1 and mPGES-2 ($P < 0.05$; Figure 1E–H). The expression of mRNA for mPGES-1 and mPGES-2 was similar in epithelium and aorta, whereas mPGES-2 was numerically higher than mPGES-1 in ASM and significantly higher ($P < 0.05$; Figure 1G) in the lung parenchyma.

Tissue-specific patterns of expression were observed for PGE_2 receptors. Thus, the expression of mRNA for EP_4 was significantly higher in ASM compared with EP_1, EP_2, and EP_3 ($P < 0.05$; Figure 1I). The expression of mRNA for EP_1, EP_2 and EP_4 receptors was similar in the airway epithelium, whereas the expression of the EP_3 receptor was lower ($P < 0.05$; Figure 1J). In the lung parenchyma, the expression of mRNA for EP_1, EP_3 and EP_4 receptors was similar, whereas the expression of EP_2 receptors was lower ($P < 0.05$; Figure 1K). The pattern of mRNA expression for the EP receptors was however different in the aorta with the expression of EP_3 being significantly greater than that of EP_1, EP_2 and EP_4 ($P < 0.05$; Figure 1L).

Influence of indomethacin on the concentration–response curve to PGE_2 in GPT

After the wash and resting period, following the initial standard assessment of histamine responsiveness, tracheal segments develop a spontaneous contractile tone that stabilizes within 30 min. This spontaneous tone could be relaxed by administration of indomethacin (3 μM). In line with previous observations (Coleman and Kennedy, 1980), exogenous PGE_2 (0.1–10 000 nM) produced a bell-shaped concentration–response curve, and this response was observed both in absence and presence of indomethacin. Moreover, the peak contraction reached the same amplitude (29.4 ± 3.9 mN and 30.3 ± 2.2 mN) at the same concentration of PGE_2 (100 nM),

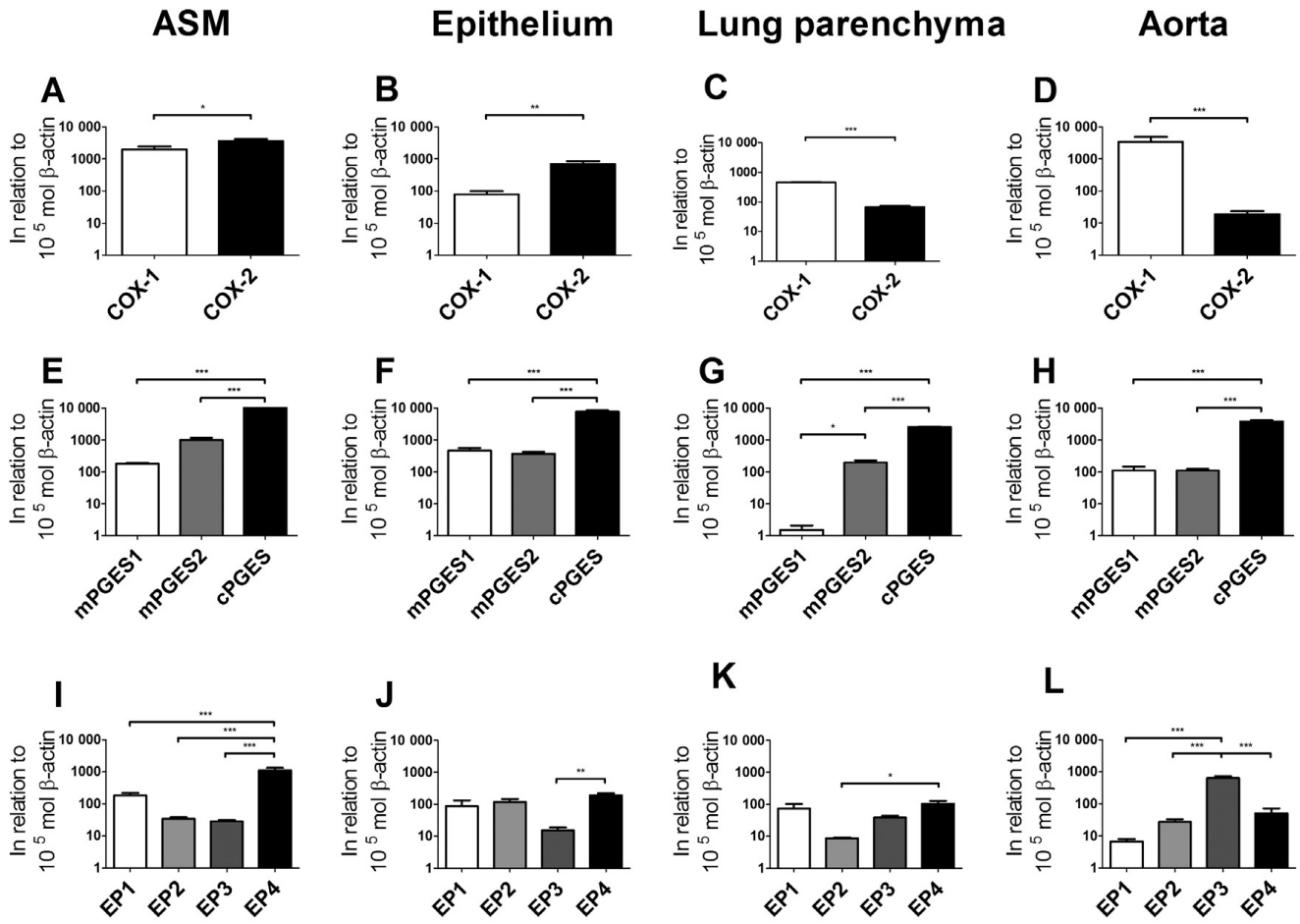

Figure 1

Real-time PCR expression of guinea pig mRNA for COX-1, COX-2, mPGES-1, mPGES-2, cPGES and EP_{1-4} in airway smooth muscle (A, E and I), airway epithelium (B, F and J), lung parenchyma (C, G and K) and aorta (D, H and L). All values are represented as mean ± SEM ($n \geq 5$) mol in relation to 10^5 mol β-actin *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ denotes significance between selected groups.

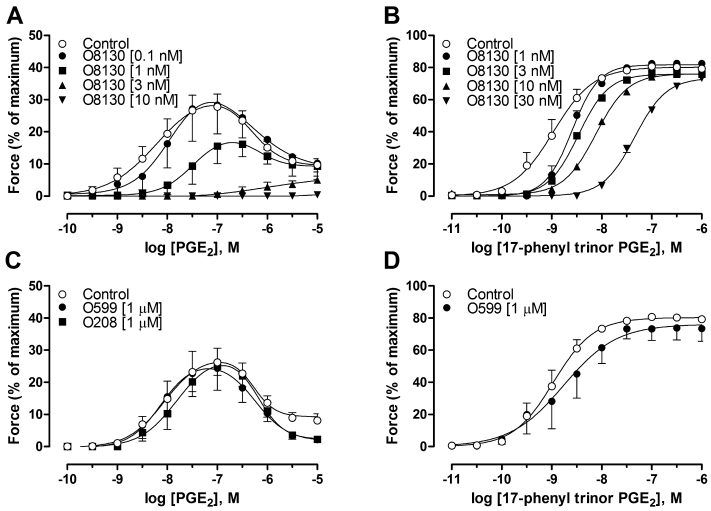

Figure 2

(A) Concentration–response curves to PGE_2 in GPT in the presence of the selective EP_1 receptor antagonist ONO-8130 (O8130) at different concentrations. (B) Contraction induced by cumulative concentrations to 17-phenyl trinor PGE_2 in guinea pig tracheal segments in the presence of ONO-8130 (O8130) at different concentrations (1–30 nM). Schild plot analysis yielding a pA_2 value of 8.9 with a slope of 1.0. (C) Concentration–response curves to PGE_2 in GPT in the presence or absence of the selective EP_3 receptor antagonist ONO-AE5-599 (O599), or selective EP_4 receptor antagonist ONO-AE3-208 (O208). (D) Contraction induced by cumulative concentrations to 17-phenyl trinor PGE_2 in the presence or absence of ONO-AE5-599 (O599). The contraction of each segment in all experiments was calculated as percentage of maximal contraction (histamine (1 mM), acetylcholine (1 mM) and KCl (60 mM)) in relation to maximal relaxation (theophylline (1 mM) or a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM)). All experiments were performed in the presence of 3 μM indomethacin (3 μM). Data represent mean ± SEM (n = 4–11).

irrespective of whether or not the concentration–response curve was raised in the presence of indomethacin. The pEC_{50}-values of PGE_2 for the contractile part (8.2 ± 0.2 and 8.0 ± 0.1, respectively) and the relaxant part (6.7 ± 0.1 and 6.3 ± 0.3, respectively) were similar for both untreated segments and those relaxed by indomethacin.

PGE_2 mediates contraction through the EP_1 receptor in GPT

To characterize the receptors involved in the PGE_2 response, initial experiments were performed using the selective EP_1 antagonist ONO-8130. For PGE_2, ONO-8130 caused a concentration-dependent reduction of the peak contraction response concomitant with a rightwards shift. Concentrations of ONO-8130 above 10 nM abolished the contractile response to exogenous PGE_2 (Figure 2A). To further investigate the action of PGE_2 on the EP_1 receptor and to be able to estimate the potency of ONO-8130, experiments were performed using the EP_1/EP_3 receptor agonist 17-phenyl trinor PGE_2. In these experiments, ONO-8130 caused a parallel shift to the right of concentration-response curve (no changes in E_{max} or Hill slopes). Schild plot analysis resulted in a slope of 1.04 [95% confidence interval (95% CI): 0.88–1.19] not different from unity (Figure 2B). Constraining the Schild slope to unity resulted in a pK_B value of 8.93 for ONO-8130 (95% CI: 8.83–9.04; Supporting Figure S3A).

The possible involvement of EP_3 and EP_4 receptors on the responses to PGE_2 were investigated using the EP_3 receptor antagonist ONO-AE5-599, or the EP_4 receptor antagonist ONO-AE3-208 (1 μM). Neither ONO-AE5-599 nor ONO-AE3-208 changed the maximal contractile response or the potency, for either the contractile (7.9 ± 0.1 for both) or the relaxant part (6.2 ± 0.1 and 6.1 ± 0.0, respectively) of the concentration–response curve for PGE_2, compared with controls (8.2 ± 0.2 for the contraction and 6.2 ± 0.1 for the relaxation; Figure 2C).

The absence of any effect attributable to the EP_3 receptor in the GPT was further supported by the observation that the EP_1/EP_3 receptor agonist sulprostone did not induce either a contraction or relaxation of the GPT in the presence of ONO-8130 (100 nM; n = 7). Moreover, ONO-AE5-599 failed to antagonize the contraction generated by 17-phenyl trinor PGE_2 (9.1 ± 0.2 and 8.7 ± 0.3, with and without antagonist respectively; Figure 2D).

PGE_2 mediates relaxation through the EP_2 receptor in GPT

To examine the role of the apparent relaxant receptor, the new selective EP_2 receptor antagonist PF-04418948 (Forselles *et al.*, 2011) was used. PF-04418948 caused a concentration-dependent increase of the peak contraction induced by PGE_2, concomitant with a rightwards shift of only the relaxation

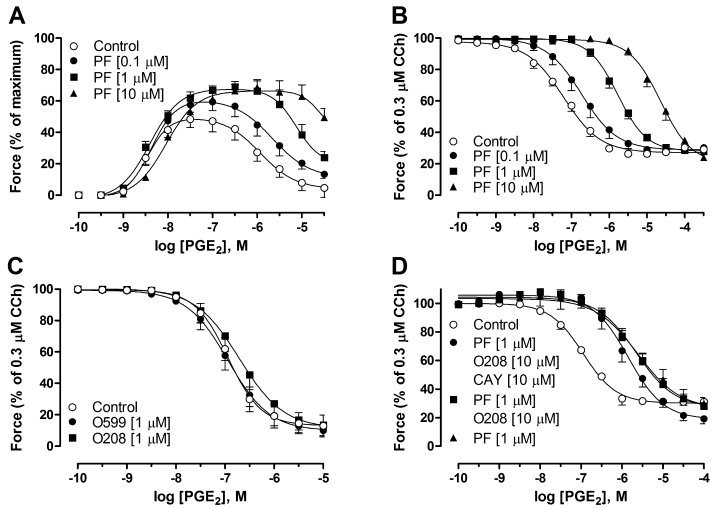

Figure 3

Concentration–response curves to PGE_2 in GPT. (A) In the presence of the selective EP_2 receptor antagonist PF-04418948 (PF) at different concentrations. (B) In segments pre-contracted with 0.3 μM carbachol (CCh) in the presence of ONO-8130 (100 nM) and SQ-29 548 (1 μM) and after treatment with PF-04418948 (PF) at different concentrations (0.1–10 μM). (C) In segments pre-contracted with 0.3 μM CCh after treatment with the selective EP_3 receptor antagonist ONO-AE5-599 (O599) or the selective EP_4 receptor antagonist ONO-AE3-208 (O208). (D) In segments pre-contracted with CCh after treatment with PF-04418948 (PF) together with the EP_4 receptor antagonist ONO-AE3-208 (O208) and selective IP receptor antagonist CAY 10441 (CAY), either alone or in combination. The contraction of each segment in all experiments was calculated as percentage of maximal contraction (histamine (1 mM), acetylcholine (1 mM) and KCl (60 mM)) or 0.3 μM carbachol in relation to maximal relaxation [theophylline (1 mM) or a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM)]. All experiments were performed in the presence of indomethacin (3 μM). Data represent mean ± SEM (n = 4–6).

part of the concentration–response curve (Figure 3A). In segments pre-contracted with carbachol (0.3 μM) in the presence of ONO-8130 (10 nM) and SQ-29548 (1 μM), PGE_2 caused a relaxation with a parallel rightwards shift of the concentration–response curves with increasing concentrations of PF-04418948 (Figure 3B). There was no difference in Hill slopes and E_{min}. Performing a Schild plot analysis revealed a slope of 1.04 (95% CI: 0.97–1.11), which was not different from unity. Constraining the Schild slope to unity resulted in a pK_B value of 7.48 for PF-04418948 (95% CI: 7.4–7.6; Supporting Figure S3B).

Further investigation of EP receptors revealed that PGE_2 induced a concentration-dependent relaxation with a pEC_{50} of 6.9 ± 0.1 and a maximal effect of 87.1 ± 6.9% that was neither affected by the EP_3 receptor antagonist ONO-AE5-599, nor the EP_4 receptor antagonist ONO-AE3-208 (Figure 3C). Experiments intended to achieve a combined blockade of the EP_2 receptor together with EP_4 and IP receptors using PF-04418948, ONO-AE3-208 and CAY10441, respectively, did not provide evidence for the presence of a relaxant PGE_2 effect, possibly mediated by EP_4 and IP receptors (Figure 3D).

The antagonistic effect of ONO-AE5-599 and ONO-AE3-208 was verified in assays displaying EP_3- and EP_4-mediated responses

Since no antagonistic effect was found for ONO-AE5-599 and ONO-AE3-208 in GPT, these antagonists were tested in assays known to display effects mediated by EP_3 and EP_4 receptors (Lydford and McKechnie, 1994; Jones and Woodward, 2011). As a positive control for the EP_3 receptor antagonist, it was shown that ONO-AE5-599 concentration-dependently antagonized the response to sulprostone in segments from endothelium-intact guinea pig aorta. These segments were pre-treated with ONO-8130 to abolish the EP_1 component of the response to sulprostone (Figure 4A). The selectivity of the EP_4 receptor antagonist was assessed in rat tracheal rings pre-contracted with carbachol. In this preparation, the relaxation induced by PGE_2 shifted to the right by more than two orders of magnitude, and the maximal relaxation was 54.3 ± 3.3% in the presence of ONO-AE3-208, as compared with 79.6 ± 3.8% in its absence (Figure 4B).

PGE_2 acting at EP_1 and EP_2 receptors maintains spontaneous tone in GPT

As described above, addition of the unselective COX-inhibitor indomethacin inhibited the spontaneous tone. This treatment resulted in a prompt relaxation with the force decreasing by 93.1 ± 1.6% in relation to the maximal relaxation induced by theophylline (Figure 5A–B). The relaxant effect of indomethacin was reproduced with two other non-selective COX-inhibitors, ibuprofen and diclofenac (n = 4 and 5 respectively). Addition of the selective EP_1 receptor antagonist ONO-8130, at concentrations of 10 nM and 1 μM, decreased the spontaneous tone (91.3 ± 1.2% and 90.4 ±

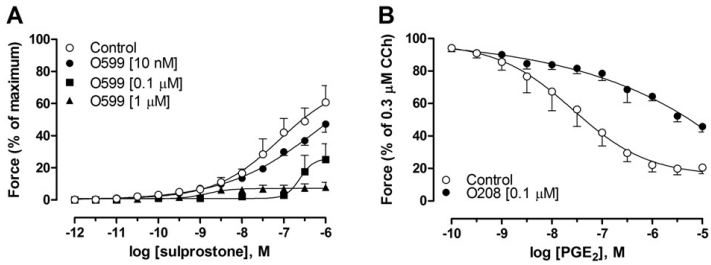

Figure 4

(A) Concentration–response curves to the selective EP_1/EP_3 receptor agonist sulprostone in endothelium-intact guinea pig aorta after treatment with the selective EP_3 receptor antagonist ONO-AE5-599 (O599). (B) Concentration–response curves to PGE_2 in rat tracheal segments pre-contracted with 0.3 μM CCh and treated with the EP_4 receptor antagonist ONO-AE3-208 (O208). All experiments were performed in the presence of 3 μM indomethacin and in panel A; 10 nM of the EP_1 receptor antagonist ONO-8130 was also added. Data represent mean ± SEM (n = 4–8).

2.2%, respectively) to the same level as indomethacin (Figure 5A–B). The onset of the effect was more rapid than that of indomethacin for the highest concentration of the EP_1 receptor antagonist. In contrast, treatment with 1 and 10 μM of the EP_2 receptor antagonist PF-04418948 resulted in a concentration-dependent increase in spontaneous tone, reaching 64.5 ± 3.7% and 75.5 ± 3.9% respectively, as compared with the maximal tissue contractility (Figure 5B).

To assess whether COX products other than PGE_2 might contribute to the spontaneous tone, segments were treated with the DP_1 receptor antagonist BWA868c, FP receptor antagonist AL-8810, IP receptor antagonist CAY10441 or TP receptor antagonist SQ-29548 under similar conditions. However, the relaxant effect of these four antagonists was negligible, 11.3 ± 4.3%, 6.4 ± 9.9%, 9.8 ± 4.7% and 12.3 ± 4.5, respectively, with no difference from the spontaneous decline observed in untreated segments (5.3 ± 5.1%) over the same time period (Figure 5B).

The claim that PGE_2 controls the spontaneous tone of these preparations obtained strong support when it was found that addition of a high-affinity neutralizing monoclonal antibody against PGE_2 relaxed the preparations (Figure 5A). The antibody concentration-dependently decreased the spontaneous tonus by 47.7 ± 5.5% (8 $\mu g \cdot mL^{-1}$) and 72.5 ± 7.4% (16 $\mu g \cdot mL^{-1}$), compared with untreated controls that relaxed 9.2 ± 6.5% during the same time (1 h) ($P < 0.05$; Figure 5C).

PGE_2 is mainly produced by the epithelium in the GPT

Evidence was obtained to support that a significant part of the biosynthesis of PGE_2, that maintains tone, originate from the tracheal epithelium. In preparations where the epithelium had been denuded, the spontaneous increase in active tone did not reach the same level as in segments with an intact epithelium. This attenuated active tone also resulted in a significantly reduced relaxant effect towards indomethacin, compared with segments with an intact epithelium ($P < 0.05$; Figure 5D). Thus, the effect of 3 μM indomethacin was only 40% of that observed in control preparations.

COX-2 is the major enzyme catalysing the formation of PGE_2 in the GPT

In an attempt to investigate which COX isoenzyme that mediated the response, the selective COX-1 inhibitors FR-122047 (1 μM) and SC-560 (1 μM), as well as the selective COX-2 inhibitors etoricoxib (1 μM) and lumiracoxib (1 μM) were applied during the spontaneous tone. It was found that both COX-2 inhibitors were superior in relaxing the spontaneous tone compared with COX-1 inhibitors. Lumiracoxib was the most efficient, inducing a reduction of 89.5 ± 3.1%, followed by etoricoxib causing a reduction of 54.7 ± 7.7%, SC-560 of 27.4 ± 9.6% and FR-122047 of 8.7 ± 3% (Figure 5E).

Homologous desensitization of the contractile, but not the relaxant response to PGE_2 in the GPT

To assess the how GPT responses are influenced by the endogenous production of PGE_2 over a longer time period, the tracheal segments were incubated in tissue culture for 4 days. After the incubation, it was found that the tracheal segments had lost responsiveness to the contractile effect of PGE_2. In contrast, when indomethacin was included in the medium during the incubation period, the responsiveness to exogenous PGE_2 was retained, and also showed an increase in potency compared with fresh GPT ($P < 0.05$; Figure 6A). The tracheal segments incubated in the presence of indomethacin responded identically to untreated segments, with respect to the relaxation induced by PGE_2, with an increased potency compared with fresh segments ($P < 0.05$; Figure 6B). The contraction evoked by histamine was not altered by indomethacin treatment during culture (Figure 6C). Since the decrease of the EP_1 receptor-mediated effect could be due to homologous desensitization, the levels of PGE_2 in the culture medium were measured. Indeed, very high levels of PGE_2 (5–20 $ng \cdot mL^{-1}$) were found during the 4 days of incubation, which were almost abolished after incubation with indomethacin (Figure 6D).

Treatment with another unselective COX inhibitor, ibuprofen, during the incubation period, mimicked the effect of indomethacin, causing both a maintained contractile

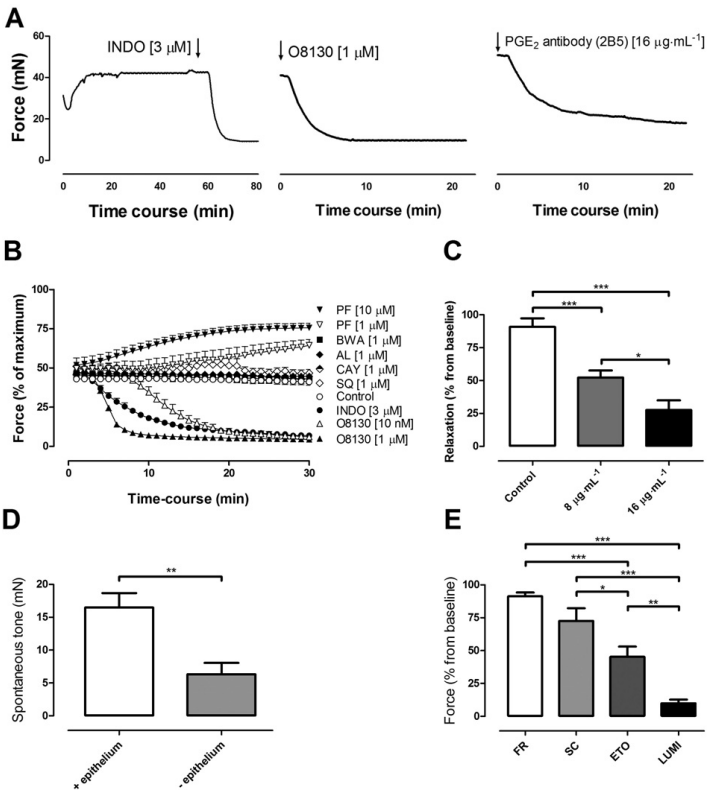

Figure 5

(A) Experimental trace showing the relaxation of tone in guinea pig trachea (GPT) induced by treatment with indomethacin (INDO), ONO-8130 (O8130) or monoclonal PGE_2 antibody (2B5) added at the arrow. (B) Time course of change in spontaneous tone in GPT after addition of the selective DP_1 receptor antagonist BWA868c (BWA), EP_1 receptor antagonist ONO-8130 (O8130), EP_2 receptor antagonist PF-04418948 (PF), FP receptor antagonist AL-8810 (AL), IP receptor antagonist CAY10441 (CAY), TP receptor antagonist SQ-29548 (SQ) or COX inhibitor INDO. (C) Relaxation of spontaneous tone in GPT subsequent to addition of monoclonal PGE_2 antibody (2B5) in the absence of indomethacin. (D) Removal of spontaneous tone in guinea pig tracheal segments with or without tracheal epithelium, treated with 3 μM indomethacin. (E) Relaxation of spontaneous tone in guinea pig trachea subsequent to addition of FR-122047 (FR; 1 μM), SC-560 (SC; 1 μM), etoricoxib (ETO; 1 μM) or lumiracoxib (LUMI; 1 μM). Data represent mean ± SEM (n = 4–6).

response to PGE_2 (Figure 6A) and an inhibition of PGE_2 biosynthesis (Figure 6D). In order to assess which pathway that was responsible for the biosynthesis of PGE_2 during the incubation, experiments were performed in the presence of the selective COX-1 inhibitor FR-122047 or the selective COX-2 inhibitor lumiracoxib during the 4 days of incubation. Under these conditions, both drugs somewhat unexpectedly caused complete inhibition of PGE_2 production (Figure 6D) and maintained the PGE_2 contractions (Figure 6A).

Discussion and conclusions

Using guinea pig-specific primers, we found mRNA expression of all four EP receptors in GPT. However, using new and selective pharmacological antagonists, we could only find evidence for functionally active contractile EP_1 receptors and relaxant EP_2 receptors. Furthermore, there was expression of mRNA for both COX-enzymes and experiments using a range of COX inhibitors suggest that both enzymes contribute to the biosynthesis of endogenous PGE_2, although the action of COX-2 seems to predominate. Epithelial denudation suggested that PGE_2 originates both from the smooth muscle and epithelial cells. Moreover, the experiments with COX-inhibitors, selective receptor antagonists for all prostanoids, and a specific antibody against PGE_2, together provided strong evidence that the level of spontaneous tone in the tracheal segments is maintained by PGE_2. Because of the relaxant effects of COX inhibitors, and previous data using unselective antagonists, it has been assumed that the basal

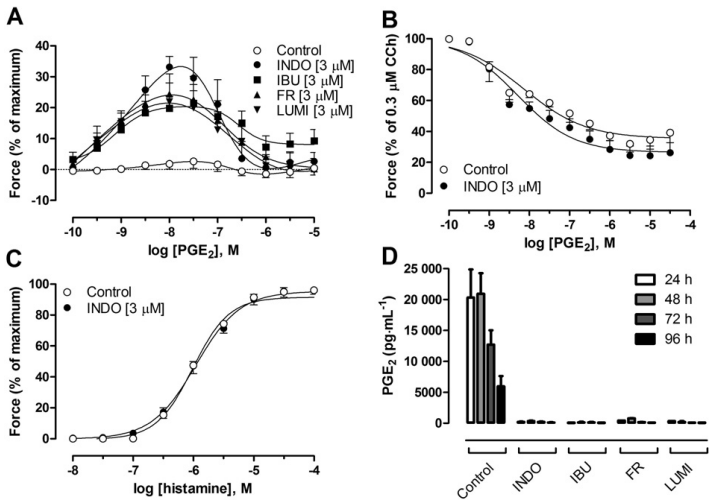

Figure 6

(A) Concentration–response curves to PGE_2 in GPT following culture for 4 days in the absence (Control) or presence of indomethacin (INDO; 3 μM), ibuprofen (IBU; 3 μM), FR-122047 (FR; 3 μM) or lumiracoxib (LUMI; 3 μM). (B) Concentration–response curves to PGE_2 in cultured guinea pig tracheal segments in the absence or presence of INDO (3 μM) and pre-contracted with 0.3 μM CCh. (C) Concentration–response curves to histamine in cultured guinea pig tracheal segments in the absence or presence of INDO (3 μM). (D) PGE_2 immunoreactivity measured in culture media from tracheal segments cultured for up to 96 h (n = 4–17).

tone is solely mediated by EP_1 receptors (Ndukwu *et al.*, 1997). This view seems to be in line with the observation that initial part of the concentration–response curve to PGE_2 is contractile, suggesting that relaxations only occur at higher and perhaps un-physiological concentrations of PGE_2. In the present study, we were able to test the new EP_2 antagonist PF-04418948 (Forselles *et al.*, 2011). It was revealed that basal tone immediately increased after EP_2 blockade. This leads to the new understanding that basal tone is maintained by a balance of the effects of PGE_2, and that both EP_1 and EP_2 receptors are active simultaneously.

This is the first report of mRNA expression for key enzymes and receptors in the PGE_2 pathway, analysed in a study that also examined functional responses of the same tissues, and furthermore, used primers that were designed based on actual guinea pig genetic sequence. The characteristics of the in-house designed primers were excellent (Supporting Figure S1), whereas we failed to get homogeneous melting curve data for previously published primers targeting human EP receptor mRNA (Rehal *et al.*, 2009). As described below, there were not only correlations between the mRNA data and functional experiments but also certain discrepancies suggesting that expression of the enzymes and receptors is regulated at several levels.

The expression of mRNA for EP_{1-4} receptors, in both tracheal smooth muscle and epithelium, suggested a possible involvement in functional responses. In line with previous observations (Coleman and Kennedy, 1980), PGE_2 produced a bell-shaped concentration–response curve with contraction at low, and relaxation at high concentrations, indicating activation of multiple signalling pathways. In the present study, ONO-8130 shifted the contractile part of the bell-shaped concentration–response curve for exogenous PGE_2 to the right and depressed the peak contractions at higher concentrations. The decrease in maximal effect was presumably due to simultaneous antagonism of the EP_1 receptor, in combination with PGE_2-mediated relaxation. When the antagonistic properties of ONO-8130 were further investigated using the selective EP_1/EP_3 receptor agonist, 17-phenyl trinor PGE_2, the analysis demonstrated a pK_B of 8.93 with a Schild slope not different from unity indicating a competitive effect at a single receptor site. The potency of ONO-8130 is in accordance with the earlier established binding affinity (K_i) of 1.9 nM, and antagonist activity (pIC_{50}) of 9.3 nM for the mouse EP_1 receptor (Data on file, Ono Pharmaceuticals Corp), which also showed that ONO-8130 exerts a more than 1000-fold selectivity for EP_1 compared with the other EP receptors. Thus, ONO-8130 is a far more potent and selective EP_1 receptor antagonist than the earlier used AH6809 with estimated pA_2 values of 6.4–7.0 for the EP_1 receptor and similar affinities for EP_2, EP_3, DP_1 and TP receptors (Abramovitz *et al.*, 2000). Further evidence in support of the PGE_2-induced contraction being solely mediated via EP_1 receptors was obtained since neither the EP_3 antagonist ONO-AE5-599 (Aihara *et al.*, 2007) nor the EP_4 antagonist ONO-AE3-208 (Ohinata *et al.*, 2006) had any effect on the concentration–response curves to PGE_2 or 17-phenyl trinor PGE_2. Accordingly, using the selective EP_1 receptor antagonist ONO-8130, we were able to conclusively confirm and extend the previous suggestions obtained using AH6809 (Ndukwu *et al.*, 1997).

The recently developed selective EP_2 receptor antagonist PF-04418948 (Forselles *et al.*, 2011) made it possible to characterize the EP-receptor-mediated relaxation. PF-04418948 did not antagonize the contractile part of the PGE_2-induced bell-shaped response. Instead, the contraction was further increased, due to PF-04418948 shifting the relaxant part of the PGE_2 induced bell-shaped curve to the right. In segments pre-contracted with carbachol in presence of both EP_1 and TP receptor antagonists, increasing concentrations of PF-04418948 caused a parallel rightwards shift of the concentration–response curve for PGE_2-induced relaxation. The Schild plot slope, which was not different from unity, indicated a competitive effect at a single receptor site and resulted in a pK_B value of 7.5. Interestingly, this value is lower compared with the pK_B value of 8.3 and 8.9 found in human myometrium and mouse trachea, respectively (Forselles *et al.*, 2011), suggesting species differences. Furthermore, the selective EP_2 receptor agonist ONO-AE1-259 relaxed carbachol pretreated segments in a concentration dependent manner that was unaffected by the combination EP_1, EP_3 and EP_4 receptor antagonists (Supporting Figure S2). The conclusion that the EP_2 receptor is the only relaxant receptor for PGE_2 in GPT was confirmed by the current findings, since neither the potency nor the maximal relaxation was affected by selective EP_3, EP_4 and IP receptor antagonists for exogenously applied PGE_2. Interestingly, in pre-contracted preparations, the relaxations occurred at lower PGE_2 concentrations than observed for the relaxant part of the bell-shaped concentration–response curve, suggesting an overlap in concentrations for the contractile and relaxant effects mediated by activation of EP_1 and EP_2 receptors, respectively.

To confirm the activity of ONO-AE5-599 and ONO-AE3-208, these compounds were tested in tissues known to express EP_3 and EP_4 receptors. In the presence of the EP_1 antagonist ONO-8130, ONO-AE5-599 concentration-dependently antagonized the contractions of guinea pig aorta induced by the EP_1/EP_3 receptor agonist sulprostone. This confirms and extends the evidence for functional EP_3 receptors in guinea pig aorta, previously identified using selective receptor antagonists (Jones *et al.*, 1998; 2011). Furthermore, we found high expression of the EP_3 receptor in guinea pig aorta. Moreover, in line with EP_4 receptors mediating relaxation of the rat trachea (Lydford and McKechnie, 1994; Buckley *et al.*, 2011), ONO-AE3-208 potently displaced the concentration–response curve for exogenous PGE_2 in rat trachea, a feature also observed in experiments using human bronchus (Buckley *et al.*, 2011). Thus, despite expression of mRNA for both EP_3 and EP_4 receptors in the smooth muscle, neither seems to be involved in contraction or relaxation of the GPT. As there are no guinea pig-specific antibodies for EP_3 and EP_4 available, we were not able to examine protein expression by Western Blot. The EP_3 receptor is implicated in the modulation of neural responses (Maher *et al.*, 2009) and activation has been linked to inhibitory responses on parasympathetic nerves innervating the GPT (Clarke *et al.*, 2004), whereas the EP_4 receptor has been reported to modulate secretory responses and cell growth (Pelletier *et al.*, 2001; Okuyama *et al.*, 2002; Rao *et al.*, 2007; Yao *et al.*, 2009). Future experiments are required to examine the functional relevance of the relatively high levels of EP_4 expression.

ONO-8130 was found to reduce the spontaneous tone of the GPT, and at the highest concentration (1 μM), the response to ONO-8130 was more rapid than that caused by indomethacin. This would suggest a mechanism involving the immediate blockade of receptors rather than a gradually diminished biosynthesis caused by indomethacin. In contrast, blocking other prostaglandin receptors (DP_1, FP, IP and TP receptors) did not affect spontaneous tone. Since EP_3 receptors are expressed in the trachea and have a possible neuronal link, this receptor could theoretically also be involved in the response to PGE_2. However, the cholinergic tone is essentially abolished by vagotomy or ganglionic blockade, suggesting that it is dependent upon ongoing preganglionic input arising from the CNS (Kesler and Canning, 1999) and thus is not relevant in our preparation. Unexpectedly, selectively blocking the EP_2 receptor induced a concentration-dependent increase of the spontaneous tone. This new and interesting finding clearly suggests that endogenously released PGE_2 also activates this relaxant receptor. The final piece of evidence that PGE_2 maintains spontaneous tone was shown by the concentration-dependent decrease in tone caused by addition of a specific high-affinity PGE_2 antibody, which has been shown to selectively block PGE_2 responses in cell models (Mnich *et al.*, 1995). Taken together, our experiments show that the tone in the GPT is mediated via endogenous PGE_2, and depends on the balance between EP_1-mediated contraction and EP_2-mediated relaxation. The finding that addition of either indomethacin or ONO-8130 sometimes did not produce complete 100% relaxation might suggest there is a small non-prostanoid mediator contributing to basal tension, or that there are small compensatory changes in length–tension relationship within the smooth muscle during the course of the experiment (Gunst *et al.*, 1995).

Using the optimized primers designed for this study, both COX-1 and COX-2 were expressed in all four tissues studied. COX-2 was predominantly expressed in the tracheal epithelium and smooth muscle, whereas COX-1 was more abundant than COX-2 in the lung parenchyma and thoracic aorta. Previous reports of COX-1 and COX-2 expression in the guinea pig lung have been conflicting. A Northern blot study using guinea pig-specific cDNA revealed constitutive expression of both enzymes in lung homogenates. This was confirmed by Western blot analysis, although the antibodies used were primarily raised against other species (Oguma *et al.*, 2002). An immunoblot study using rabbit antibodies detected COX-2 but not COX-1 in the smooth muscle and cartilage of GPT, and one selective COX-2 inhibitor had the same effect as indomethacin on spontaneous tone (Charette *et al.*, 1995). However, selective COX-1 inhibition was not tested in that study. We found that two different, but potent, COX-1 inhibitors (FR-122047 and SC-560) had a smaller effect on spontaneous tone compared with two selective COX-2 inhibitors (etoricoxib and lumiracoxib), confirming that COX-2 appears to be the quantitatively dominating enzyme catalysing formation of the PGE_2 that maintains tone under tissue bath conditions. Our study has the limitation that concentration–effect relations for the inhibitors not have been established in the tissue bath setting; however, the compounds were used in concentrations that in other models have shown high degree of selectivity (Ochi *et al.*, 2000; Esser *et al.*, 2005).

Since the epithelium has been suggested to be an important source of PGE_2 (Hay *et al.*, 1988), experiments were performed comparing the active tension between intact and epithelium-denuded segments. The active tension was lower in denuded segments than in intact segments, indicating that the epithelium is a major source of PGE_2. However, in line with the high expression of COX-2 in both smooth muscle and epithelial cells, the airway smooth muscle is also likely to contribute to the basal release of PGE_2.

Another aim of the current study was to examine how the endogenous production of PGE_2 affects the responses of EP_1 and EP_2 receptors over a longer period of time. We found that 4 days of culture resulted in a completely abolished contraction, yet maintained relaxation towards exogenously added PGE_2. This difference in response to PGE_2 is consistent with the described homologous desensitization of the EP_1 receptor, which has not been observed for the EP_2 receptor (Illes and Knoll, 1975; Vermue *et al.*, 1987; Penn *et al.*, 2001). Furthermore, the observed desensitization did not occur when the endogenous biosynthesis of PGE_2 was inhibited by indomethacin, ibuprofen, FR-122047 or lumiracoxib during the culture period. We cannot explain why under these conditions the inhibitors did not show selectivity. Both compounds (FR-122047 and lumiracoxib) were used at concentrations that in other models are very selective (Ochi *et al.*, 2000; Esser *et al.*, 2005); therefore, a lack of selectivity at the level of COX inhibition is an unlikely explanation of our results. It may also be that the kinetics of enzyme inhibition is altered during long-term incubation, enabling an inhibitor with a low potency to induce an effect. The mechanism underlying this phenomenon requires further investigation. Interestingly, after culturing, both the contractile and relaxant response towards PGE_2 was increased in potency. This type of hyperreactivity has been described previously for other excitatory and inhibitory stimuli in this culture model and is thought to be due to an enhanced coupling efficiency of the effectors involved (Morin *et al.*, 2005).

To put our main findings in perspective, using new, potent and selective pharmacological tools, we have not only confirmed that the EP_1 receptor is involved in the spontaneous tone of the GPT, but also discovered that the endogenous biosynthesis of PGE_2 simultaneously mediates relaxation through EP_2 receptors. We show for the first time that constitutively released PGE_2 induces a basal tone in GPT that depends upon the balance between the actions of these two opposing receptor functions. As the response of human airways towards prostanoids is known to be complex (Coleman and Kennedy, 1980; Brink *et al.*, 1981; Muccitelli *et al.*, 1987; Ressmeyer *et al.*, 2006; Canning and Chou, 2008; Ricciardolo *et al.*, 2008), and constitutively released PGE_2 can induce both contraction and relaxation, we suggest that PGE_2 may also play a similar regulatory role in human airways. Clearly, the net effect of PGE_2 regulation in human bronchi appears to be opposite to that in guinea pig airways, with indomethacin increasing tone, at least under tissue bath conditions (Bjorck and Dahlen, 1993; Coleman *et al.*, 1996; Watson *et al.*, 1997). Neither is the relative contribution of COX-1 or COX-2 to formation of PGE_2 in human bronchi known, whereas COX-1 appears to dominate in the upper airways (Harrington *et al.*, 2008). Further indications of species differences are the findings that in human bronchi relaxant EP_4 receptors appear to have a significant role (Buckley *et al.*, 2011; Benyahia *et al.*, 2012), although it remains to examine the effect of EP_2 receptor antagonism in the human tissue. The desensitization of EP_1 receptors by endogenous PGE_2 is also an observation with potential implications of relevance to human disease. One may speculate that this mechanism is important in situations where the local release of PGE_2 is increased, for example during chronic airway inflammation. This would change the balance between the contractile and relaxant response to PGE_2 in favour of a protective relaxation. In fact, there is good evidence that PGE_2 has a protective function in asthmatic airways (Melillo *et al.*, 1994; Pavord and Tattersfield, 1995; Gauvreau *et al.*, 1999).

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Conflict of interest

Kirk Maxey is employed by Cayman Chemicals from which we received the monoclonal PGE_2 antibody.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Target primers were designed in-house towards available sequences from the Ensemble database and the NCBI Genebank Sequences from the second scaffold of the guinea pig genome comprised by Broad Institute (Feb. 2008 *Cavia porcellus* draft assembly cavPor3). Validation included a melt curve analysis and dilution series of cDNA expressed as a slope regression line.

Figure S2 Concentration–response curves to selective EP_2 receptor agonist ONO-AE1-259 in guinea pig tracheal segments pre-contracted with 0.3 μM carbachol (CCh) and treated with selective receptor antagonists; ONO-8130 (O8130), ONO-AE5-599 (O599) and ONO-AE3-208 (O208). All experiments are performed in the presence of indomethacin (3 μM). Data represent mean ± SEM (n = 4–6).

Figure S3 (A) Schild plot derived from Figure 2B revealed a slope of 1.04 (95% CI: 0.88–1.19), which was not different from unity. Constraining the Schild slope to unity resulted in a pK_B value of 8.93 for ONO-8130 (95% CI: 8.83–9.04). (B) Schild plot derived from Figure 3B revealed a slope of 1.07 (95% CI: 0.97–1.11), which was not different from unity. Constraining the Schild slope to unity resulted in a pK_B value of 7.48 for PF-04418948 (95% CI: 7.4–7.6).

Table S1 Real-time PCR expression of mRNA in guinea pig tissue in relation to 10^5 mol·mol^{-1} β-actin.

Supplementary material and methods

Tissue preparation and organ culture

Animals used in the functional studies were sacrificed either by inhalation of CO_2 gas or overdose of sodium pentobarbital (Apoteket AB, Stockholm, Sweden) followed by removal of the heart-lung-package which was placed in ice-cold Tyrode's buffer. Animals from which mRNA was extracted were all sacrificed by an overdose of sodium pentobarbital and tissue preparations were removed and stored in RNAlater® (Applied Biosystems Foster City, Ca) at room temperature until use (0-4 days later). Trachea, lung parenchyma and thoracic aorta were gently dissected from the surrounding connective tissue, and for mRNA experiments, tracheal epithelium was denuded using a scalpel after which the airway smooth muscle layer was cut out. For functional studies, tracheal segments were cut along the cartilage into eight intact rings of equal length whereas the thoracic aorta was cut into four intact ring-segments. In experiments with denuded epithelium the tracheal lining was removed by gently rubbing the luminal surface with a scalpel. This was carried out under a preparation microscope (Lundblad *et al.*, 1988)

During organ culture, intact individual guinea pig tracheal rings were placed in separate wells containing Dulbecco's modified Eagle medium (DMEM)-F12 (Gibco, Auckland, NZ) supplemented with 1% penicillin (100 $IU \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) (Gibco) under sterile conditions. The culture plates were placed in a humidified incubator at 38 °C at 95% O_2 and 5% CO_2. Segments were incubated for 96 hours in total and transferred into new wells containing fresh media every 24 hours. Freshly made compounds were then added, as

described by Adner *et al*., 2002. All animals were housed in cages (5 animals together) under 12-hour light/dark cycle with food and water *ad lib*.

Tissue organ bath

Tyrode's buffer composition (NaCl [149.2 mM], KCl [2.7 mM], $NaHCO_3$ [11.9 mM], glucose [5.5 mM], $CaCl_2$ [1.8 mM], $MgCl_2$ [0.5 mM] and NaH_2PO_4 [0.4 mM] dissolved in demineralised water) solution at 37 °C, bubbled with carbogen (5% CO_2 in O_2) to maintain a pH of 7.4.

Measurements of mediator release

PGE_2 was measured by enzyme immunoassay (EIA, Cayman Chemical). The detection limit was 15 $pg \cdot mL^{-1}$and the PGE_2 monoclonal antibody displayed cross-reactivity towards PGE_3 (43%), 8-iso PGE_2 (37.4 %), PGE_1 (18.7%), 6-keto $PGF_{1\alpha}$ (1%), 8-iso $PGF_{2\alpha}$ (0.25%), 13,14-dihydro-15-keto prostaglandin E_2 (0.02%) and <0.01% to PGD_2, $PGF_{2\alpha}$ and TXB_2.

RNA preparation and real time-PCR

Guinea pig RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The concentration and purity of the RNA was measured using a Nanodrop 3300 (Thermo Scientific, Wilmington, U.S.A.). Aliquots of RNA were reverse-transcribed into cDNA using a QuantiTect® Reverse Transcription Kit (Qiagen) according to the instructions provided.

All PCR primers (Figure 1) were designed towards available guinea pig sequences from the Ensembl database (www.ensembl.org) and the NCBI Genebank Sequences (www.ncbi.nih.gov) from the 2nd scaffold of the guinea pig (*Cavia porcellus*) genome

comprised by the Feb. 2008 *Cavia porcellus* draft assembly (Broad Institute cavPor3) using the OligoCalc (Kibbe, 2007) and PrimerExpress 3.0 (ABI) software's. The oligonucleotides were synthesized at Cybergene, Stockholm Sweden according to standard procedures. Primers for the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) andβ-actin were adapted from previous studies (West *et al.*, 2005). All primers were titrated using increasing concentrations of cDNA to get a shift of the C_T values, expressed as a regression slope of 3.32 ($2^{3.32} = 10$) with 10% deviation. Furthermore, a melt curve analysis was performed to ensure the quality of the amplification product (Supplementary figure 1).

Real-time PCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems Foster City, Ca) according to the manufacturers' instructions. Each sample was analysed in duplicate in MicroAmp® Fast optical 96-well Reaction Plates (ABI) using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems Foster City, Ca). The program was set to 50 °C 2 min followed by a hot start at 95 °C for 15 min, followed by 6 cycles; 94 °C 30 sec, 66 °C 1 min, followed by 40 cycles; 94 °C 30 sec, 55 °C 1 min and followed by a melt curve analysis. mRNA levels were calculated using the comparative C_T method and normalized to the housekeeping gene β-actin and expressed as mol in relation to 10^5 mol β-actin.

Drugs and suppliers

NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, KH_2PO_4 and glucose were obtained from VWR (West Chester, PA). Histamine dihydrochloride, DMSO, acetylcholine, indomethacin, diclofenac, ibuprofen, phenylephrine, papaverine, sodium nitroprusside, carbachol and theophylline were purchased from Sigma-Aldrich (St. Louis, MO). Etoricoxib was purchased from Carbone Scientific (London, UK). Prostaglandin E_2, FR-122047, SC-560, monoclonal PGE_2 antibody

(2B5) 17-phenyl trinor PGE_2, CAY10441 and sulprostone were obtained from Cayman Chemical (Ann Arbor, MI). Lumiracoxib was obtained from SynphaBase AG (Switzerland). ONO-8130 (4-{[(6-{isobutyl[(4-methyl-1,3-thiazol-2-yl)sulfonyl]amino}-2,3-dihydro-1H-inden-5-yl)oxy]methyl}benzoic acid), ONO-AE5-599 (3-{2-({[(1R)-1-(3,5-dimethylphenyl)-3-methylbutyl] amino}carbonyl)-4-[(2-methylphenoxy)methyl] phenyl}propanoic acid), ONO-AE3-208 (4-[4-cyano-2-[2-(4-fluoronaphthalen-1-yl)propanoylamino]phenyl]butanoic acid) and ONO-AE1-259 ((Z)-7-[(1R,2R,3R,5R)-5-chloro-3-hydroxy-2-[(E,4S)-4-hydroxy-4-(1-prop-2-enylcyclobutyl)but-1-enyl]cyclopentyl]hept-5-enoic acid) were kind gifts from ONO Pharmaceuticals (Japan). PF-04418948 (1-(4-fluorobenzoyl)-3-{[(6-methoxy-2-naphthyl)oxy]methyl} azetidine-3-carboxylic acid) was a kind gift from Pfizer Central Research Division (Groton, CT). Stock solutions of indomethacin, PGE_2 (100 mM), and SC-560 were dissolved in 99% ethanol, ONO-8130, ONO-AE5-599, ONO-AE3-208, ONO-AE1-259, CAY10441, PF-04418948, 17-phenyl trinor PGE_2, FR-122047, lumiracoxib and etoricoxib were dissolved in DMSO. All stock solutions were stored at -20 °C. The other drugs were dissolved in Tyrode's solution or deionized H_2O on the day of the experiment. Dilutions of drugs were freshly made from stocks prior to each experiment.

Calculations and statistics

$$Response = Bottom + \frac{(Top - Bottom)}{1 + \{10^{LogEC50}[1 + ([B]/10^{-pA_2})^S]/[A]\}^{nH}}$$

The Top and Bottom denotes the highest and lowest, asymptote of the curves respectively, $LogEC_{50}$ denotes the logarithm of the agonist EC_{50} in the absence of the agonist [A], nH denotes the Hill slope of the agonist curve, S denotes the Schild slope and pA_2 denotes the

negative logarithm of the concentration of antagonist [B] that shifts the agonist EC_{50} by a factor of 2.

Supplementary results

Expression of mRNA for COX-1, COX-2, mPGES-1, mPGES-2, cPGES and EP_{1-4} in guinea pig ASM, airway epithelium, aorta and lung parenchyma

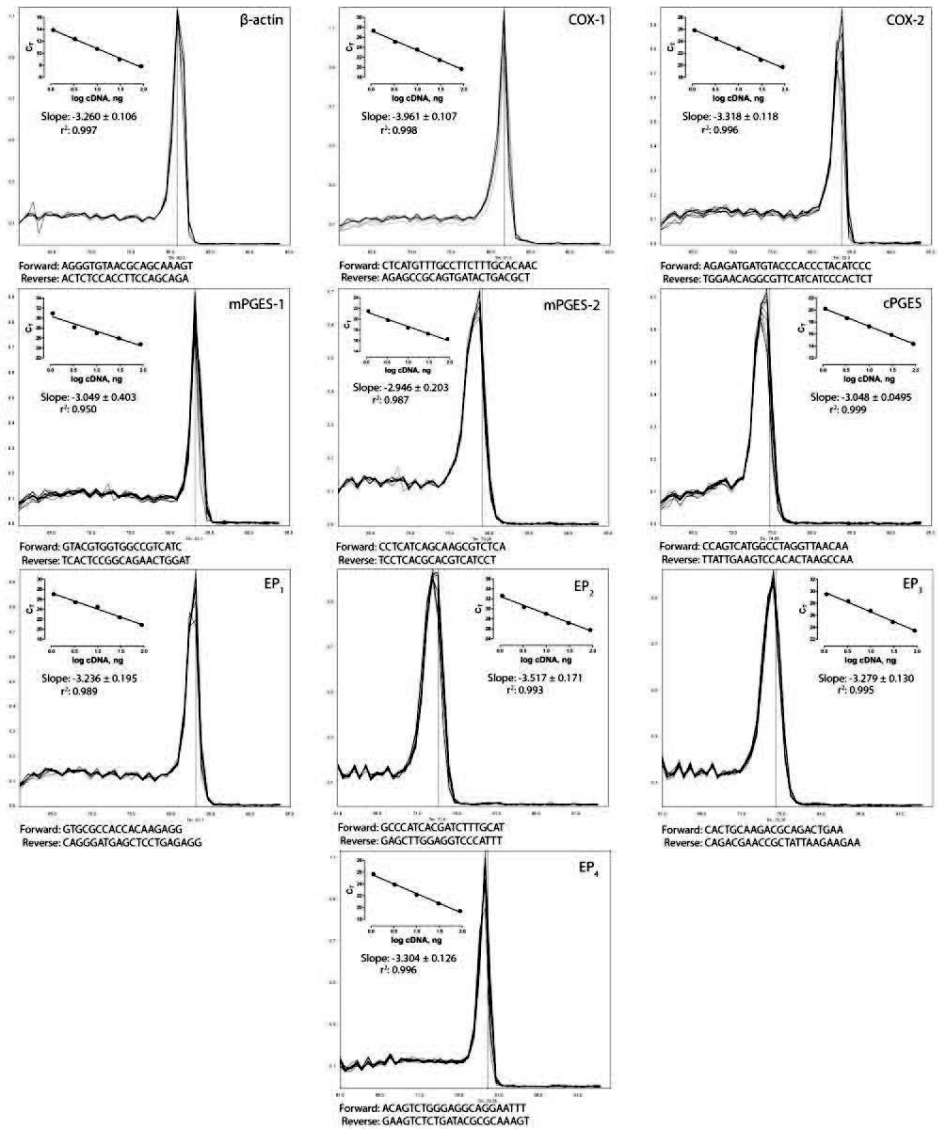
By the use of primers designed by alignment of the respective guinea pig gene sequences with their human counterparts, regression analysis with correlations and slope values together with melt curve analysis indicated a successful amplification of a single amplicon (Supplementary Figure 1).

The ratio of COX-2 to COX-1 mRNA in ASM and airway epithelium was 2±0 and 11±2, respectively. The ratio of COX-1 to COX-2 mRNA was 7±1 and 165±21 in lung parenchyma and aorta, respectively. The ratio of cPGES mRNA compared to mPGES-1 mRNA in ASM, airway epithelium, lung parenchyma and aorta was 130±22, 22±6, 2557±702 and 45±9, respectively. Furthermore, the ratio of cPGES mRNA to mPGES-2 was 24±3, 24±5, 5±3 and 40±10 for ASM, airway epithelium, lung parenchyma and aorta, respectively. The ratio between mPGES-2 and mPGES-1 in the parenchyma was 167±29.

Although the numerical expression of EP_1 mRNA in ASM was higher than that of either EP_2 or EP_3, neither difference reached statistical significance. The ratio of EP_4 mRNA to EP_1, EP_2 and EP_3 in ASM was 7±2, 32±3 and 39±5, respectively. The expression of EP_4 mRNA in the epithelium was 16±5 times higher than EP_3. The ratio of expression of EP_4 to EP_2 mRNA was 12±3 in the parenchyma. The ratio between EP_3 mRNA levels and EP_1, EP_2 and EP_4 mRNA was 104±16, 25±3 and 18±4, respectively.

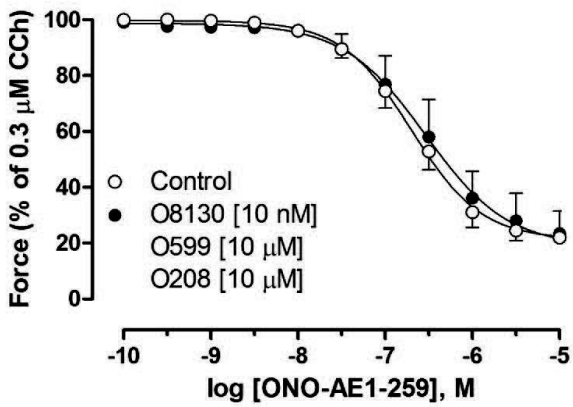
The EP_2 receptor agonist ONO-AE1-259 induced a concentration-dependent relaxation of segments pre-contracted with carbachol. The pEC_{50} for the relaxation was 6.7±0.1 and the amplitude of the relaxation reached at the highest tested concentration (10 μM) was 78.8±2.3%. Pre-treatment with ONO-8130, ONO-AE5-599 and ONO-AE3-208 did neither affect the sensitivity nor maximal relaxation in response to the EP_2 agonist (Supplementary figure 2).

Supplementary figures

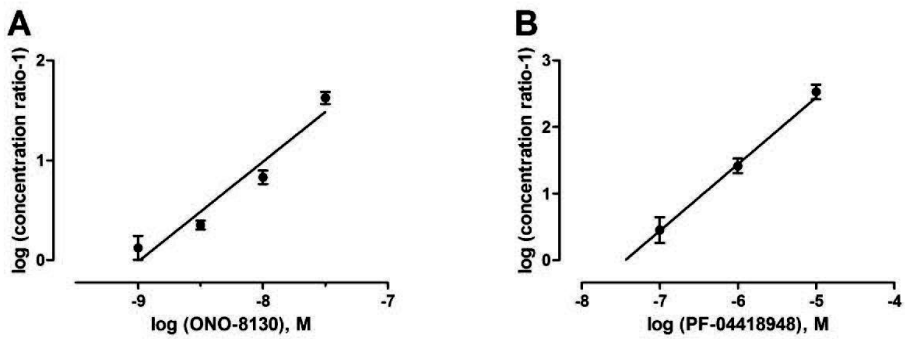


Supplementary figure 1: Target primers were designed in-house toward available sequences from the Ensemble database and the NCBI Genebank Sequences from the 2nd scaffold of the

guinea pig genome comprised by Broad Institute (Feb. 2008 *Cavia porcellus* draft assembly cavPor3). Validation included a melt curve analysis and dilution series of cDNA expressed as a slope regression line.



Supplementary figure 2: Concentration-response curves to selective EP_2 receptor agonist ONO-AE1-259 in guinea pig tracheal segments pre-contracted with 0.3 μM carbachol (CCh) and treated with selective receptor antagonists; ONO-8130 (O8130), ONO-AE5-599 (O599) and ONO-AE3-208 (O208). All experiments are performed in the presence of indomethacin (3 μM). Data represent mean ± S.E.M (n = 4-6).



Supplementary figure 3: (A) Schild plot derived from Figure 2B revealed a slope of 1.04 (95% CI: 0.88 to 1.19) which was not different from unity. Constraining the Schild slope to unity resulted in a pK_B value of 8.93 for ONO-8130 (95% CI: 8.83 to 9.04). (B) Schild plot derived from Figure 3B revealed a slope of 1.07 (95% CI: 0.97 to 1.11) which was not different from unity. Constraining the Schild slope to unity resulted in a pK_B value of 7.48 for PF-04418948 (95% CI: 7.4 to 7.6).

Supplementary tables

Supplementary table 1: Real-time PCR expression of mRNA in guinea pig tissue in relation to 10^5 mol·mol^{-1} β-actin

mRNA	ASM	Epithelium	Lung parenchyma	Aorta
COX-1	2004 ± 438	79 ± 19	456 ± 14[a]	3467 ± 1396[a]
COX-2	3683 ± 528[b]	691 ± 158[b]	68 ± 6	19 ± 5
mPGES-1	180 ± 14	460 ± 99	2 ± 1	110 ± 36
mPGES-2	993 ± 170	366± 57	197 ± 35[c]	109 ± 14
cPGES	22173 ± 1270[d]	7795 ± 723[d]	2536 ± 78[d]	3830 ± 398[d]
EP_1	181 ± 37	87 ± 46	73 ± 30	7 ± 1
EP_2	34 ± 4	116 ± 30	9 ± 1	27 ± 6
EP_3	28 ± 3	15± 4	38 ± 5	632 ± 97e
EP_4	1107 ± 203[f]	1878 ± 33[g]	103± 25[h]	51 ± 22

Real-time PCR expression of guinea pig mRNA for COX-1, COX-2, mPGES-1, mPGES-2, cPGES and $EP_{1\text{-}4}$ in airway smooth muscle (ASM), airway epithelium, lung parenchyma and aorta. Significant differences ($p < 0.05$) was found between [a]COX-1 vs. COX-2, [b]COX-2 vs. COX-1, [c]mPGES-2 vs. mPGES-1, [d]cPGES vs. both mPGES-1 and mPGES-2, [e]EP_3 vs. EP_1, EP_2 and EP_4, [f]EP_4 vs. EP_1, EP_2 and EP_3, [g]EP_4 vs. EP_3 and [h]EP_4 vs. EP_2. All values are represented as mean ± SEM ($n \geq 5$).

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The bitter taste receptor (TAS2R) agonists denatonium and chloroquine display distinct patterns of relaxation of the guinea pig trachea

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Pulkkinen V, Manson ML, Säfholm J, Adner M, Dahlén S. 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* 303: L956–L966, 2012. First published September 7, 2012; doi:10.1152/ajplung.00205.2012.—Activation of taste receptors (TAS2Rs) by bitter taste agonists has been reported to cause bronchodilation. The aim of this study was to extend the information on the effects of bitter taste agonists on responses induced by different contractile mediators in a standard airway physiology preparation. Isometric responses were assessed in guinea pig trachea (GPT). TAS2R agonists were administered either to segments precontracted with different agonists for contraction or given before challenge with the different contractile stimuli, including antigen in tissues from ovalbumin-sensitized animals. TAS2R mRNA expression on GPT epithelium and smooth muscle was measured with real-time PCR. Denatonium, chloroquine, thiamine, and noscapine induced concentration-dependent relaxations (R_{max}: 98.3 ± 1.6, 100.0 ± 0.0, 100.0 ± 0.0, and 52.3 ± 1.1% of maximum, respectively, in the presence of indomethacin) in segments precontracted with carbachol. The receptors for denatonium (TAS2R4, TAS2R10) and chloroquine (TAS2R3, TAS2R10) were expressed in GPT. Whereas denatonium selectively inhibited contractions induced by carbachol, chloroquine uniformly inhibited contractions evoked by prostaglandin E_2, the thromboxane receptor agonist U-46619, leukotriene D_4, histamine, and antigen. The effects of denatonium, but not those of chloroquine, were partly inhibited by blockers of the large Ca^{2+}-activated K^+ channels and decreased by an increase of the level of precontraction. In conclusion, TAS2R agonists mediated strong relaxations and substantial inhibition of contractions in GPT. Chloroquine and denatonium had distinct patterns of activity, indicating different signaling mechanisms. The findings reinforce the hypothesis that TAS2Rs are potential targets for the development of a new class of more efficacious agonists for bronchodilation.

bronchodilation; asthma; prostaglandins; airway smooth muscle

BITTER TASTE-SENSING TYPE 2 receptors (TAS2Rs) are G protein-coupled receptors (GPCRs) on cell surface that mediate gustatory taste perception on the tongue (16). The properties of TAS2Rs are different from many other GPCRs, as the receptors are promiscuous and capable of binding a wide range of compounds with relatively low specificity and affinity (16). TAS2Rs have recently been suggested to have important extraoral functions in the respiratory and gastrointestinal tracts (4, 11). In the human airway epithelium, TAS2Rs are expressed on the solitary chemosensory cells (25, 26) and ciliated epithelial cells (23), where they sense chemical irritation and promote ciliary beat frequency, respectively. Thus TAS2Rs may be protective and part of the defense against inhaled noxious compounds.

Recently airway smooth muscle cells were found to express TAS2Rs that mediated relaxations in both mouse and human isolated airway preparations (7). Stimulation of TAS2Rs increased intracellular Ca^{2+} signaling that was suggested to activate large conductance potassium channels (BK_{Ca}) and result in hyperpolarization of the cell membrane. However, in further studies BK_{Ca} blockers either failed to prevent (28) or only partially blocked (2) relaxation caused by the bitter tastant chloroquine, and direct recording of the channel current with patch clamp revealed that bitter tastants did not activate BK_{Ca} channels in mouse airway preparations (28). As the effects of TAS2R agonists appeared independent of the cyclic adenosine monophosphate (cAMP) and the protein kinase A (PKA) pathways (7), the mechanisms of TAS2Rs in causing airway relaxation are still unsolved. Nevertheless, the TAS2R agonists showed both greater relaxation and inhibition of airway hyperresponsiveness than a β_2-adrenoceptor agonist in mouse airways (7). Given the large selection of known natural and synthetic agonists recognized by the 25 TAS2Rs (16), the findings have introduced bitter taste receptors as a potential new family of targets for asthma pharmacotherapy. Moreover, we have recently found that leukocytes of patients with severe asthma have increased expression of TAS2Rs (Orsmark-Pietras C, James A, Konradsen J, Nordlund B, Söderhäll C, Pulkkinen V, Pedroletti C, Daham K, Kupczyk M, Dahlén B, Kere J, Dahlén SE, Hedlin G, Melén E, unpublished observations), suggesting also that TAS2Rs may represent a protective endogenous mechanism upregulated in disease.

The isolated guinea pig trachea (GPT) is a commonly used model in airway pharmacology, where the receptors for agonists and mediators of mast cell activation are closely similar to those of human airways (6). Accordingly, the antigen-induced contraction in guinea pig airways is caused by the concerted action of cysteinyl-leukotrienes, histamine, and prostanoids (24), mediators also known to cause allergen-induced bronchoconstriction in human subjects with asthma (20). In the present study, we characterized the expression of TAS2Rs in guinea pig airways, as well the effects of prototype TAS2R agonists on contractions induced by the different mediators of the antigen responses. The bitter taste agonists for the study were selected on the basis of the particular receptors expressed in the guinea pig genome. We examined both the "therapeutic" effects of TAS2R agonists on tracheal segments precontracted with different contractile agonists and the "prophylactic" effects of pretreatments with TAS2R agonists on agonist-mediated contractions. Our results extend the previous findings and

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provide a more detailed analysis of the mode of action of TAS2Rs in the airways. This includes assessment of interactions with different pathways for contractions and the relation between the response and the level of precontraction. As the basal tone of GPT is kept by prostaglandin (PG) E_2 (9, 21), we in particular compared the effects of several of the tested TAS2R agonists in the presence and absence of indomethacin, which inhibits spontaneous tone and biosynthesis of PGE_2.

MATERIALS AND METHODS

Tissue preparation. The study was approved by the Swedish Animal Experimentation Ethical Review Board (N257/09 and N323/11). Male albino guinea pigs (Dunkin-Hartley; 350–600 g) were killed by an overdose of pentobarbital sodium (Apoteket, Stockholm, Sweden), followed by rapid removal of the heart-lung-package and placement in ice-cold Krebs-Henseleit (KH) buffer solution (composition in mM: 118.5 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 11.1 D-glucose). For sensitization procedure, a single dose of 100 μg of ovalbumin (OVA) grade 5 and 0.1 g of aluminum hydroxide dissolved in 0.8 ml of sterile PBS was injected peritoneally, and animals were killed 14–28 days later. Trachea was gently dissected from the surrounding connective tissue. For mRNA expression, the epithelium in the trachea was scraped off with a scalpel, and the airway smooth muscle layer was cut out. Tissue preparations were extracted and stored in RNAlater (Applied Biosystems, Foster City, CA) at room temperature until use (0–4 days later). For the functional studies, tracheal segments were cut along the cartilage into eight intact rings of equal length. For denudation experiments, epithelium was removed by mechanical scraping with a scalpel.

Selection of the TAS2R agonists used for the study. Of the 25 human TAS2Rs identified, we found guinea pig orthologs for eight TAS2Rs, namely TAS2R1, TAS2R3, TAS2R4, TAS2R10, TAS2R14, TAS2R16, TAS2R40, and TAS2R41 by queries against the Ensemble database (www.ensembl.org). We selected agonists used in a previous study (7), namely chloroquine (TAS2R3 and TAS2R10 in guinea pig), denatonium (TAS2R4 and TAS2R10), and saccharin (as a negative control because the guinea pig genome lacks TAS2R8, TAS2R43, and TAS2R44 sequences). In addition, we selected agonists that bind to TAS2R1 (thiamine) and TAS2R14 (noscapine). No suitable agonists were available for TAS2R16 and TAS2R40, as well as the orphan receptor TAS2R41.

In vitro pharmacology. Intact GPT segments were set up in 5-ml organ baths with KH buffer solution at 37°C, bubbled with carbon gas (5% CO_2 in O_2). Changes in smooth muscle force were detected using an isometric force-displacement transducer linked to a Grass polygraph. During an equilibration period of 60 min, the resting force was adjusted to 30 mN. As a control of the tracheal reactivity, histamine (0.1 nM to 0.3 mM) was cumulatively added. Before the pharmacological studies, a further 30-min equilibration period was completed. Maximal relaxation was obtained by adding a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM), and maximal contraction was obtained by adding histamine (1 mM), acetylcholine (1 mM), and KCl (40 mM) at the end of the experiments. Parts of the experiments were performed in the presence of 3 μM indomethacin to abolish the constitutive release of prostaglandins.

GPT segments were precontracted with 100 nM carbachol, in both the absence and the presence of indomethacin. Precontraction by 100 μg/mL OVA was induced in the absence of indomethacin. To dissect the effects of PGE_2 and thromboxane A_2 (TXA_2) on TAS2R agonist-mediated relaxation, the EP_1 receptor antagonist ONO-8130 (10 nM) or the TP receptor antagonist SQ-29548 (1 μM) was added to the organ bath 30 min before precontraction. Experiments with different levels of carbachol precontraction (3 μM, 100 nM, and 30 nM) were performed in the presence of indomethacin. When a stable contraction was obtained after 40 min, the segments were exposed to the bitter taste agonists or salbutamol in a cumulative manner. In addition, a similar approach was used to study the effects of chloroquine and denatonium on stable precontractions induced by 0.1 μM U46619, 0.1 μM leukotriene D_4 (LTD_4), and 3 nM 17-phenyl trinor PGE_2, all in the presence of indomethacin.

To study the prophylactic effects of the bitter taste agonists, 300 μM denatonium or 300 μM chloroquine was given after 30-min incubation with indomethacin. The effects of 30-min denatonium and chloroquine prestimulation were studied on contractions induced by increasing concentrations of carbachol, U46619, PGE_2, LTD_4, or histamine.

To test whether denatonium could antagonize muscarinic receptors, acetylcholine-induced dilatation of guinea pig aorta was studied. During equilibration, segments were adjusted to a basal tension of 2 mN. The tissues were tested for viability by demonstrating a contractile response to 60 mM KCl and a relaxant response to 10 μM acetylcholine when precontracted with 10 μM phenylephrine. Thereafter, the segments were washed and exposed to 300 μM denatonium, precontracted with 3 μM $PGF_{2\alpha}$, and again exposed to 10 μM acetylcholine.

RNA preparation and real-time PCR. Guinea pig total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The concentration and purity of the RNA was measured using a Nanodrop 3300 (Thermo Scientific, Wilmington, DE). Aliquots of RNA were reverse-transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

All PCR primers were designed toward available guinea pig sequences from the Ensembl database (www.ensembl.org) and the NCBI Genebank Sequences (www.ncbi.nih.gov) from the second scaffold of the guinea pig (*Cavia porcellus*) genome comprised by the Feb 2008 Cavia porcellus draft assembly (Broad Institute cavPor3) using the Primer-BLAST software. Primers for TAS2R3 were: Fwd: 5′-GGTCAGTGGCAGCAGCTGGTT-3′ and Rev: 5′-TGGAGAGGGCCAGGTTGGTGA-3′, for TAS2R4: Fwd: 5′-TCACCAGACTTCTGCTGGGCTGT-3′ and Rev: 5′-ACTCCCAGGCCCATCCTTGCA-3′, and for TAS2R10: Fwd: 5′-TGCCAAGTGCAGTGGAAATCTTCC-3′ and Rev: 5′-ACCCATTCCCTAGAAGCCCCAGG-3′. The oligonucleotides were synthesized at Cybergene (Stockholm, Sweden) according to standard procedures. Primers for the housekeeping gene for β-actin were adapted from previous studies (27).

Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and in MicroAmp fast optical 96-well Reaction Plates (Applied Biosystems). Each sample was analyzed in duplicate. PCR reactions (20 μl) were performed with final concentrations of 100 nM each of forward and reverse primers. Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR system using default settings according to the manufacturers′ instructions. To ensure the quality of the amplification product, a melt curve analysis was performed as a final step. mRNA levels were calculated using the comparative C_T method, normalized to the housekeeping gene β-actin, and expressed as 10^5 mol/mol β-actin.

PGE_2 measurements. PGE_2 levels were determined by using an enzyme immunoassay (EIA) with a PGE_2 monoclonal EIA kit (Cayman Chemical, Ann Arbor, MI). Detection limit was 15 pg/ml with cross-reactivity to 8-iso PGE_2 (37.4%), 8-iso $PGF_{2\alpha}$ (0.025%), and 12,14-dihydro-15-keto PGE_2 (0.02%). Samples for these measurements were collected directly from the organ bath between 0 and 7 min at 1-min intervals after stimulation with denatonium and chloroquine. Bradykinin was used as a positive control for PGE_2 release.

cAMP measurements. Determination of cAMP levels was performed from tracheal segments as described previously (1). The segments were equilibrated for 60 min in tissue bath conditions as described above. This was followed by 30-min incubation of indomethacin (3 μM) and a further 30-min incubation of 200 μM 3-isobu-

tyl-1-methylxanthine, a nonspecific inhibitor of phosphodiesterases. Thereafter, the tracheas were precontracted with carbachol for 40 min and exposed to a single concentration of 300 μM denatonium, 300 μM chloroquine, or 300 nM salbutamol for 20 min to achieve stable and maximal effect for all three agonists. When the experiments were completed, the segments were snap-frozen in liquid nitrogen and stored in −80°C until preparation for the EIA (581002, Cayman Chemical). The tracheas were purified in accordance with the manufacturer's instruction for the EIA; the frozen tissues were placed in 5% trichloroacetic acid and homogenized on ice. After centrifugation at 1,500 *g* for 10 min, the trichloroacetic acid was extracted from the supernatant by water-saturated ether. The ether was removed by heating to 70°C before analysis by EIA (detection limit = 3 pmol/ml).

Calculations and statistics. All data are presented as means ± SE. The concentration-response curve values for pEC_{50}, E_{max}, and Hill slopes were calculated using nonlinear regression analysis. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple-comparison test or Mann Whitney *t*-test for comparison between two groups. For the analyses, GraphPad Prism 5.01 (GraphPad Software, San Diego, CA) was used.

Drugs and suppliers. Iberiotoxin, charybdotoxin, paxilline, H89, GF109203, and KT5823 were purchased from Tocris Bioscience (Bristol, UK). NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, KH_2PO_4 and glucose were obtained from VWR (West Chester, PA). Denatonium benzoate, dextromethorphan, chloroquine hydrochlorate, noscapine, thiamine, histamine dihydrochloride, DMSO, acetylcholine, indomethacin, papaverine, sodium nitroprusside, and salbutamol were purchased from Sigma-Aldrich (St. Louis, MO). Formoterol was a kind gift from AstraZeneca (Stockholm, Sweden). SQ-29548, PGE_2, 17-phenyl trinor PGE_2, U-46619, $PGF_{2\alpha}$, and LTD_4 were purchased from Cayman Chemical. ONO-8130 was a kind gift from ONO Pharmaceuticals (Tokyo, Japan). Stock solutions of indomethacin and U-46619 were dissolved in 95% ethanol, whereas ONO-8130 was dissolved in DMSO. All stock solutions were stored at −20°C. The other drugs were dissolved in KH solution or deionized H_2O or organic solvents on the experimental day. Dilutions of drugs were freshly made from stocks before each experiment, and parallel experiments with solvent controls were always performed.

RESULTS

Bitter taste agonists are effective bronchodilators against muscarinic contraction. To examine relaxant effects of the selected compounds, GPT was precontracted with 100 nM carbachol both in the absence and presence of indomethacin or prostanoid receptor antagonists. The segments were either exposed to cumulative concentrations of thiamine, denatonium, chloroquine, noscapine, or saccharin or kept untreated as controls for spontaneous relaxation. The partial β_2-adrenoceptor agonist salbutamol was used as a positive control for airway relaxation to allow for comparison with data previously obtained in mouse and human airways (7).

Although indomethacin prevented the spontaneous increase of basal tone in naïve preparations, carbachol induced a similar stable magnitude of precontraction in the presence or absence of indomethacin [precontraction levels: 73.5 ± 3.8% (n = 19) and 75.2 ± 4.2% (n = 18) of E_{max}, respectively]. Thiamine, chloroquine, denatonium, and noscapine evoked concentration-dependent relaxations, whereas saccharin had no effect (Fig. 1, Table 1). Indomethacin had no effects on the potency of the TAS2R agonists (Table 1). However, the magnitudes (R_{max}) of the relaxations induced by thiamine (72.7 ± 6.6%) and denatonium (57.5 ± 5.2%) were enhanced by indomethacin (100.0 ± 0.0 and 98.3 ± 1.6%, respectively). In contrast, chloroquine induced an almost complete relaxation also in the naïve preparation (98.2 ± 1.1%; n = 6) that was unaffected by indomethacin (100.0 ± 0.0%). Compared with denatonium, chloroquine was eightfold less potent (pEC_{50}: 3.8 ± 0.1 and 4.5 ± 0.1, respectively) and had a Hill slope that was significantly steeper (−5.5 ± 0.7 and −1.5 ± 0.1, respectively). The magnitude of the noscapine-induced relaxation was somewhat decreased by indomethacin (Fig. 1, Table 1).

Because cyclooxygenase inhibition with indomethacin enhanced airway relaxation for denatonium and thiamine, we tested the hypothesis that these two TAS2R agonists were opposed by PGE_2, the contractile prostanoid that is known to regulate tone in the preparation (9, 18, 21). The magnitude of denatonium-induced relaxation was thus indeed enhanced by the EP_1 receptor antagonist ONO-8130 (99.3 ± 0.7%) (Fig. 1*E*, Table 1) but not by the TP receptor antagonist SQ-29,648. Similar results were obtained with thiamine (Fig. 1*C*). Neither the EP_1 nor the TP receptor antagonist, however, had any influence on the relaxation induced by noscapine (Fig. 1*F*).

Expression of functional TAS2Rs in GPT. There was significant real-time PCR expression of receptors for TAS2R3, TAS2R4, and TAS2R10 in smooth muscle and epithelium of the trachea (Fig. 2*A*). The rank order of bitter taste receptor expression was TAS2R3 > TAS2R10 > TAS2R4. The expression was generally higher in the tracheal epithelium than in the smooth muscle cells.

In addition, we screened the expression pattern for other TAS2Rs, namely TAS2R1, TAS2R14, and TAS2R16 (data not shown). For TAS2R1, the lack of complete whole genome sequences for guinea hampered our efforts, and we were unable to design properly working primers. The expression levels of TAS2R14 and TAS2R16 were near the detection limit, and we did not receive consistent data. In further functional experiments, we, however, found that dextromethorphan (agonist for TAS2R1 and TAS2R10) induced almost complete relaxations (94.7 ± 1.5%, pEC50 4.4 ± 0.1, n = 6) of GPT segments precontracted by 100 nM carbachol in the presence of 3 μM indomethacin. The response to dextromethorphan closely resembled that of denatonium.

On the basis of these patterns of relaxation responses and expression of mRNA, we chose denatonium and chloroquine for the extended analysis of pharmacological activities.

Role of epithelium and smooth muscle in the relaxation. Both denatonium and chloroquine act on receptors that were found to be expressed (denatonium TAS2R4 and TAS2R10; chloroquine TAS2R3 and TAS2R10). In view of the expression data, we next determined whether the epithelial TAS2R expression was necessary for relaxation mediated by denatonium or chloroquine. The potency of chloroquine on airway relaxation was, however, only marginally changed and, in fact, enhanced by epithelial removal (pEC_{50} 3.8 ± 0.1 and 4.2 ± 0.1, respectively, P = 0.04), and there was no significant effect compared with the intact trachea for denatonium (Fig. 2, *B* and *C*).

Differential effects of denatonium and chloroquine on precontractions induced by agonists other than carbachol. First, because the cyclooxygenase inhibitor indomethacin as well as the EP_1 receptor antagonist ONO-8130 enhanced denatonium-mediated airway relaxation, the results suggested that either denatonium was unable to reduce the component of tone regulated by PGE_2 (18) or that denatonium stimulated release of PGE_2 that opposed its relaxant effect. To assess the latter possibility, PGE_2 was measured by EIA in serial samples from

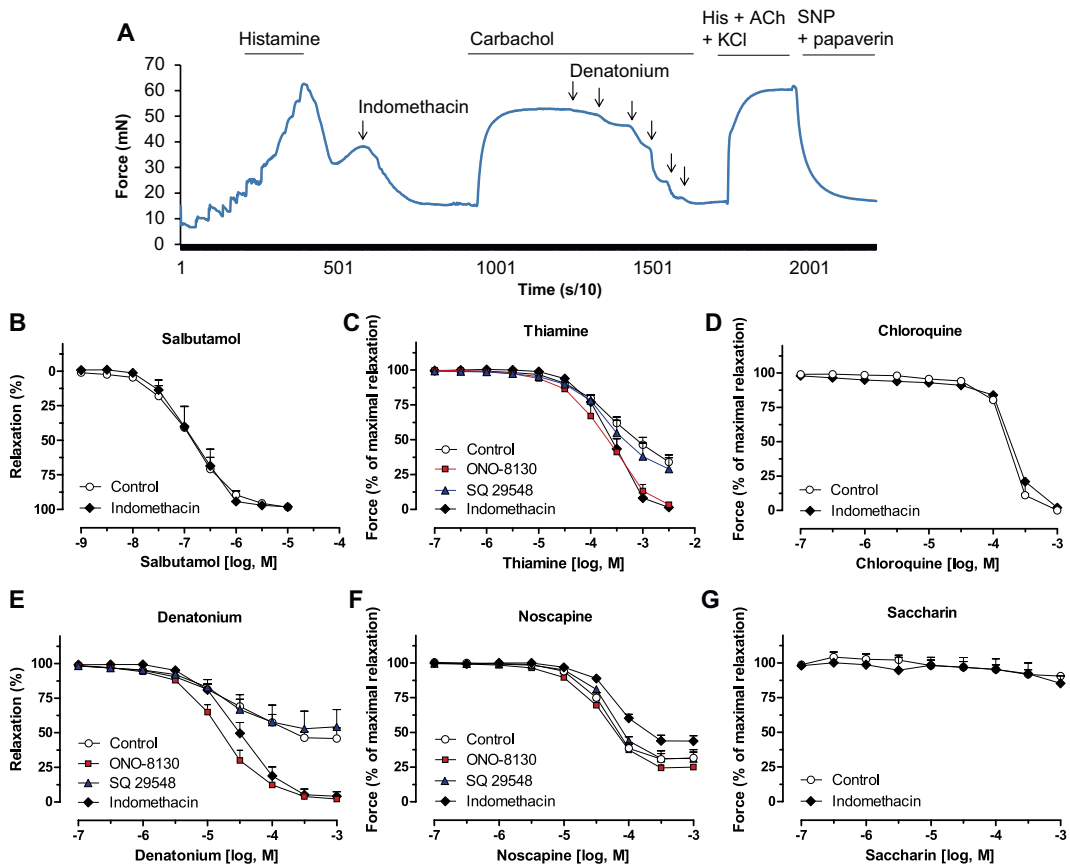

Fig. 1. *A*: a trace depicting denatonium-mediated relaxation in guinea pig trachea (GPT) precontracted with 100 nM carbachol in the presence of 3 μM indomethacin. Effects of salbutamol (n = 4) (*B*), and bitter taste agonists thiamine (n = 7); acting on TAS2R1 (*C*), chloroquine (n = 6–7; TAS2R3, TAS2R10) (*D*), denatonium (n = 5–9; TAS2R4, TAS2R10) (*E*), noscapine (n = 6–8; TAS2R14) (*F*), and saccharin (n = 4; no TAS2Rs in guinea pig) (*G*) on airway relaxation in GPT precontracted with 100 nM carbachol in the presence and absence of 3 μM indomethacin, 10 nM prostaglandin E_2 (PGE_2) receptor (EP_1) antagonist ONO-8310 and 1 μM thromboxane TP receptor antagonist SQ29648. Data are expressed as percentages of maximal relaxation obtained with a combination of papaverine (0.1 mM) and sodium nitroprusside (SNP) (0.1 mM) at the end of each experiment. ACh, acetylcholine.

the organ bath fluid after addition of denatonium to preparations precontracted with carbachol. Whereas the positive control bradykinin caused a marked increase of PGE_2 in the bath, there was no release of PGE_2 after exposure to denatonium or chloroquine (Fig. 3*A*). In contrast, when the preparation was precontracted with the EP_1/EP_3 receptor agonist 17-phenyl trinor PGE_2, denatonium failed to relax the preparation also in the presence of indomethacin (Fig. 3*B*). On the other hand, chloroquine induced complete relaxation of GPT precontracted with the EP_1/EP_3 receptor agonist 17-phenyl trinor PGE_2 (Fig. 3*B*).

A differential effect of the two tested TAS2R agonists was confirmed in subsequent experiments using several different means of producing a precontraction. Thus, whereas denatonium was unable to reverse the antigen-induced contraction in preparations from sensitized animals challenged with OVA, chloroquine induced complete smooth muscle relaxation also in this setting (Fig. 4*A*). Likewise, chloroquine was able to relax preparations precontracted with the thromboxane receptor (TP) agonist U-46619, LTD_4, or histamine, whereas denatonium was unable to relax contractions induced by any of these three agonists (Fig. 4, *B–D*).

Effects of pretreatment with denatonium or chloroquine on responses to contractile agonists. In the presence of indomethacin, the preparation was preincubated for 30 min with denatonium (300 μM) or chloroquine (300 μM) before establishment of concentration response relations for carbachol, U-46619, PGE_2, LTD_4, or histamine. Denatonium pretreatment partially inhibited (E_{max} 97.4 ± 1.7 vs. 65.6 ± 2.9%) and chloroquine pretreatment abolished (0.1 ± 0.1%) the contraction induced by the cholinergic agonist carbachol (Fig. 5, *A* and *B*). In line with the experiments on precontracted preparations,

Table 1. *Effects of TAS2R agonists and salbutamol on guinea pig tracheal segments precontracted with 0.1 μM carbachol with and without 3 μM indomethacin*

	Control	Saccharin	Thiamine	Chloroquine	Denatonium	Noscapine	Salbutamol
				No indomethacin			
n	6	6	7	6	8	8	4
R_{max}	10.7 ± 2.5	9.5 ± 2.1	72.7 ± 6.6	98.2 ± 1.1	57.5 ± 5.2	72.9 ± 4.4	98.0 ± 0.9
pEC50	n.a.	n.a.	3.58 ± 0.06	3.75 ± 0.07	4.60 ± 0.11	4.40 ± 0.05	6.91 ± 0.19
Hill slope	n.a.	n.a.	−0.91 ± 0.07	−4.03 ± 1.11	−0.92 ± 0.07	−2.27 ± 0.28	−1.43 ± 0.13
				Indomethacin			
n	7	4	7	7	9	8	4
R_{max}	13.1 ± 4.3	14.7 ± 5.3	100.0 ± 0.0*	100.0 ± 0.0	98.3 ± 1.6*	52.3 ± 1.1†	98.7 ± 1.0
pEC50	n.a.	n.a.	3.59 ± 0.10	3.82 ± 0.10	4.48 ± 0.10	4.17 ± 0.04†	6.85 ± 0.20
Hill slope	n.a.	n.a.	−1.61 ± 0.06	−5.51 ± 0.71	−1.54 ± 0.08	−2.12 ± 0.15	−1.74 ± 0.19
				ONO-8130			
n			7		5	6	
R_{max}			99.6 ± 0.4*		99.3 ± 0.7*	77.5 ± 1.8	
pEC50			3.95 ± 0.19		4.78 ± 0.12	4.40 ± 0.04	
Hill slope			−1.36 ± 0.05		−1.26 ± 0.09	−1.53 ± 0.08	
				SQ29548			
n			7		5	6	
R_{max}			87.4 ± 5.6		49.6 ± 13.6	70.5 ± 6.0	
pEC50			3.76 ± 0.06		4.74 ± 0.12	4.30 ± 0.05	
Hill slope			−1.22 ± 0.13		−1.11 ± 0.12	−2.06 ± 0.15	

The effects of denatonium, thiamine, and noscapine on precontracted segments were studied in the presence of 10 nM prostaglandin E_2 receptor (EP1) antagonist ONO-8130 and 1 μM thromboxane A_2 receptor TP antagonist SQ29548. *$P < 0.0001$, †$P < 0.005$ in comparison to the segments with no indomethacin.

chloroquine substantially inhibited the concentration-response relations for all other tested agonists (U-46619, PGE_2, LTD_4, and histamine), whereas denatonium had no significant effect (Fig. 5, *C–F*).

The effect of denatonium, but not that of chloroquine, is partly mediated through the Ca^{2+}-induced large-conductance potassium channels. To further investigate the mechanisms of the relaxation induced by denatonium and chloroquine, blockers of Ca^{2+}-induced large-conductance potassium channels (BK_{Ca} also known as MaxiK and Slo) were used. These included the scorpion venoms iberiotoxin and charybdotoxin that are blockers of the BK_{Ca} channels at 100 nM concentration (12) as well paxilline, an indole alkaloid mycotoxin from *Penicillium paxilli* that is a potent BK_{Ca} channel blocker at 1 μM (14). None of the BK_{Ca} channel blockers altered the maximal relaxation to the two TAS2R agonists in preparations precontracted by carbachol. However, the potency of denatonium (pEC_{50} 4.5 ± 0.1), but not that of chloroquine, was decreased by iberiotoxin, charybdotoxin, and paxilline (pEC_{50}: 3.5 ± 0.2, 3.7 ± 0.1, and 3.5 ± 0.2, respectively; Fig. 6, *A* and *B*). For all three BK_{Ca} blockers, there was thus approximately one log order of magnitude of shift in the concentration-response relation for the denatonium-mediated airway relaxation. In line with previous observations (12, 17), it was confirmed that the potency of salbutamol similarly was decreased by iberiotoxin without effects on the maximal relaxation (Fig. 6*C*).

To investigate the involvement of other pathways important for relaxation, the effects of kinase inhibitors for PKA, PKC, and PKG on denatonium-induced airway relaxation were examined. However, 30-min pretreatment with 10 μM H89 (PKA inhibitor), 1 μM GF109203 (a selective inhibitor of PKC), and

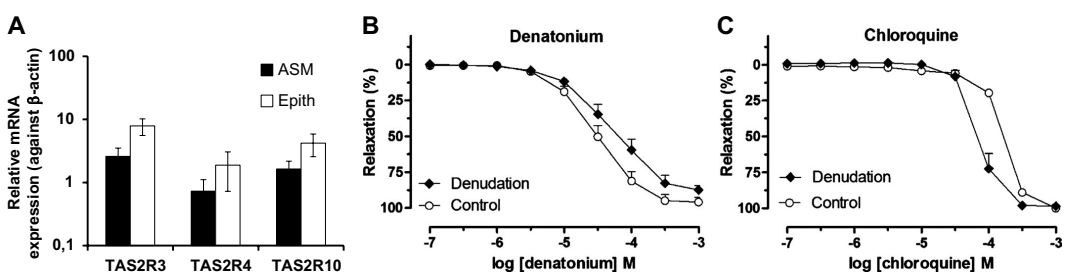

Fig. 2. *A*: real-time PCR analysis of TAS2R mRNA expression in smooth muscle (ASM) and epithelial cells from GPT. Expression levels were calculated using the comparative C_T method and normalized against the housekeeping gene β-actin and expressed as 10^5 mol/mol β-actin. Data are expressed as means ± SE (n = 7/group). Effects of epithelial denudation on denatonium (n = 4–9) (*B*) and chloroquine-mediated (n = 4–6) (*C*) airway relaxation in GPT precontracted with 100 nM carbachol in the presence of 3 μM indomethacin.

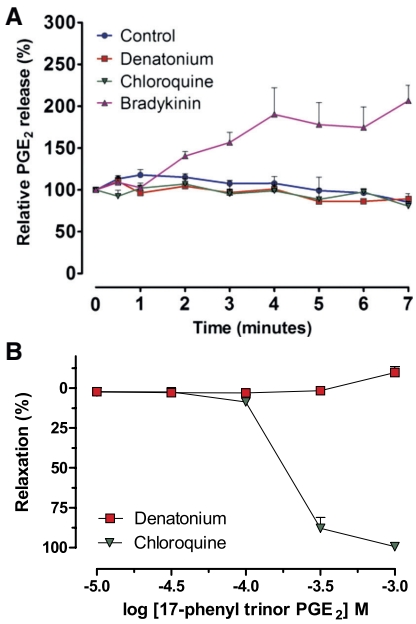

Fig. 3. *A*: serial samples were collected from the organ bath fluid at 1-min time intervals after 300 μM denatonium and 300 μM chloroquine stimulation of GPT precontracted with 100 nM carbachol. Bradykinin (3 μM) was used as a positive control and negative control samples were left untreated. The absolute PGE_2 release (pg/ml/mg dry weight) was measured with ELISA. PGE_2 values in each bath before the stimulations (timepoint 0) were set as a reference value of 100, and data are presented as relative values ($n = 3$). *B*: effects of denatonium and chloroquine on GPT precontracted with 3 nM 17-phenyl trinor PGE_2 in the presence of 3 μM indomethacin ($n = 3$–4).

1 μM KT5823 (a selective inhibitor of PKG) had no significant effects on denatonium-induced airway relaxation (Fig. 6*D*).

Because cAMP is essential for β_2-adrenoceptor agonist-mediated airway smooth muscle relaxation, we measured the levels of intracellular cAMP after denatonium and chloroquine stimulation with salbutamol as a positive control. Neither denatonium nor chloroquine had effects via the cAMP signaling pathway, however. As expected, salbutamol stimulation resulted in increase in intracellular cAMP levels (Fig. 6*E*).

In view of the apparent selectivity of denatonium against the cholinergic agonist carbachol, its effect on acetylcholine induced dilatation of phenyepinephrine precontracted guinea pig aorta was studied. However, the dilatation to acetylcholine was similar in the presence or absence of denatonium pretreatment (40.2 ± 1.2 vs. 35.2 ± 12.9%) (Fig. 6*F*).

Effect of different levels of precontraction on bitter taste agonist-mediated airway relaxation. The next set of experiments was performed to examine how the relaxation responses to denatonium and chloroquine were affected by different levels of precontraction, using salbutamol for comparison. Thus the preparations were stimulated with 30 nM, 100 nM, and 3 μM carbachol to produce precontraction levels of 47.1 ± 6.5% ($n = 11$), 73.5 ± 3.8% ($n = 19$), and 100.0 ± 0.0% ($n = 9$) of the maximal histamine-induced contraction, respectively. Both denatonium and chloroquine were able to induce maximal relaxations irrespective of the level of precontraction (Fig. 7, *A* and *B*; note that all levels of precontraction have been set to 100% to clearly illustrate the effect). The potency of denatonium, but not that of chloroquine, was, however, gradually decreased with increasing levels of precontraction. In contrast to both bitter taste agonists, salbutamol caused only a weak relaxation at the highest level of precontraction (Fig. 7*C*). Moreover, for the two lower levels of precontraction, where maximum relaxation still could be reached with salbutamol, the loss of potency with increasing level of precontraction was significantly greater for salbutamol than for denatonium (12 ± 2-fold and 2 ± 1-fold, respectively). In addition, we studied the effects of formoterol, which is a stronger agonist for β_2-adrenoceptors than salbutamol. As shown in Fig. 7*C*, formoterol failed to relax the highest level of precontraction. However, the loss of potency with the two lowest precontraction levels was similar to that of denatonium (Fig. 7*D*).

DISCUSSION

Our findings confirm and extend the concept that bitter taste receptors may represent a new target for bronchodilation in asthma and obstructive airway diseases. We show that bitter taste receptors are expressed in the GPT and that agonists for these receptors can induce strong relaxations. In fact, in carbachol-contracted preparations, denatonium and chloroquine relaxed maximally contracted preparations that were resistant to the β-adrenergic agonist salbutamol. Taken together, our results in another species confirm several of the key observations on the potential of agonists of TAS2Rs as bronchodilators (7). On the other hand, in our extended analysis, we found that the properties of the most potent agonist denatonium were distinct from those of chloroquine. Whereas denatonium only reduced the effects of cholinergic contractions, chloroquine uniformly inhibited contractions evoked also by histamine, contractile prostanoids, and cysteinyl-leukotrienes. This was observed when the mediators were given alone as agonists or when they were released together endogenously by the antigen challenge. In addition, although relaxations induced by denatonium were sensitive to inhibitors of BK_{Ca} activation, the effect was only partial and similar to the effect of that class of drugs on the β-adrenergic agonist salbutamol.

We identified expression of guinea pig TAS2Rs for denatonium (TAS2R4 and TAS2R10) and chloroquine (TAS2R3 and TAS2R10), consistent with the functional effects of these two agonists. In addition, we observed relaxations to thiamine (TAS2R1), dextramethorphan (TAS2R1 and TAS2R10), and noscapine (TAS2R14). All these agonists induced strong relaxation of GPT precontracted with carbachol. For denatonium, dextramethorphan, chloroquine, and thiamine, the magnitude of relaxation was comparable to those of salbutamol or formoterol, however, with markedly lower potencies. The low potency is expected and in accordance with the existing literature on agonists for bitter taste receptors (16). Furthermore, the potency values of salbutamol and formoterol obtained in this study are comparable with the results from previous studies in isolated GPT (pEC_{50}: 6.91 ± 0.19 and 6.89 ± 0.10 for salbutamol, respectively, and 9.32 ± 0.08 and 8.76 ± 0.09 for formoterol, respectively) (13). One of the challenges for

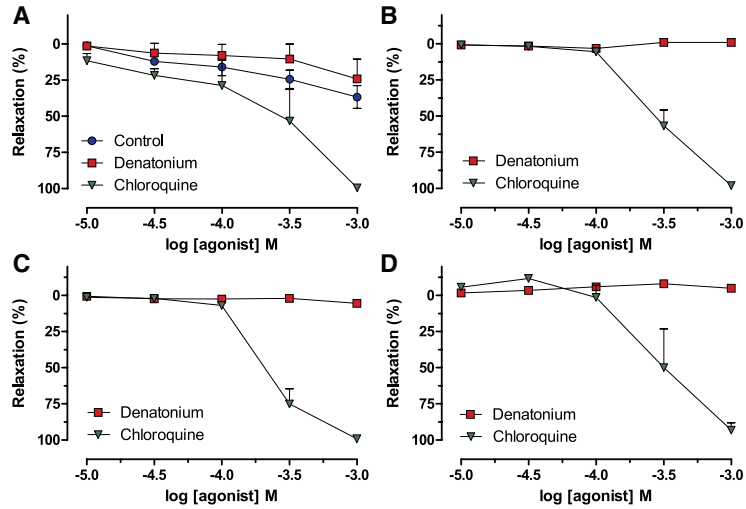

Fig. 4. *A*: effects of denatonium and chloroquine on airway relaxation of ovalbumin-sensitized guinea pig tracheal segments precontracted with ovalbumin. Data are expressed as percentages of maximal relaxation obtained with a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM) at the end of each experiment ($n = 4–6$). Effects of denatonium and chloroquine on 100 nM U46619 precontraction ($n = 4–6$) (*B*), 100 nM LTD_4 precontraction ($n = 4$) (*C*), and 10 μM histamine ($n = 3$) (*D*) in the presence of 3 μM indomethacin.

any future drug development is obviously to identify more potent ligands for TAS2Rs.

Another need is to define the TAS2Rs that are most suitable targets. Despite the lack of available antagonists for TAS2Rs, the effects of the tested agonists in the guinea pig tissues already provide relatively clear insight into which receptors have been activated. Thus the potency of denatonium, known to selectively activate TAS2Rs, was approximately fivefold higher in GPT compared with the potency reported for the same compound against hTAS2R10 in transfected human embryonic kidney (HEK)-293T cells (EC_{50}: 25 μM and 120 μM, respectively) (16). Because denatonium is 100-fold more potent for hTAS2R10 than for hTAS2R4, the data indicate that the relaxant effect of denatonium is mediated through the TAS2R10. This interpretation is further supported by the very similar potency and pattern of activity of dextromethorphan and denatonium. Dextromethorphan is known to activate both TAS2R1 and R10 (16), but, in absence of confirmed expression of TASR1 in GPT, we interpret our data to support that denatonium and dextromethorphan mainly evoke the observed relaxations via TASR10. One explanation for the observed higher potency of denatonium compared with the transfected human cells might be an interaction between TAS2R10 and a concomitant activation of TAS2R4. Although the potency of chloroquine was low, it was strikingly similar to that obtained for hTAS2R3 in transfected HEK-293T cells (EC_{50}: 178 μM and 173 μM, respectively) (16). Because chloroquine has a 1,000-fold lower potency for hTAS2R10 (16), it is assumed that the relaxant effect of chloroquine solely was mediated through TAS2R3.

Furthermore, the observation that the guinea pig does not have sequences for the known saccharin TAS2Rs is consistent with the observation that saccharin failed to relax GPT. Saccharin was active in mice airways, which also express its receptor (7). Although we could not study expression of TAS2R1, the potency of its agonist thiamine was similar to that of chloroquine in GPT. The potency of thiamine was higher than expected from data in transfected HEK-293T cells (16). For noscapine, which was as potent as denatonium but did not induce the same strong relaxation as salbutamol in GPT, the potency is in accordance with the effect on hTAS2R14 in transfected HEK-293T cells. The low efficacy may thus relate to limited receptor expression. Taken together, the analysis comparing our current data with those on human TAS2Rs supports that denatonium, chloroquine, dextromethorphan, thiamine, and noscapine cause relaxations of GPT through activation of specific TAS2Rs.

In this study, TAS2R3, TAS2R4, and TAS2R10 transcripts were detected both in epithelial and smooth muscle cells of GPT. Although denudation of the tracheal epithelium caused small shifts of the concentration response curves for the bitter taste agonists, it did not remove the relaxant effects. Thus these results support that the relaxations to denatonium and chloroquine are mediated almost exclusively through a direct effect on the airway smooth muscle.

The effects of denatonium and chloroquine differed though. Whereas relaxations induced by denatonium were enhanced by treatment with either indomethacin or the EP_1 receptor antagonist ONO-8130, the response to chloroquine was the same irrespective of whether spontaneous tone was abolished or not. The observations suggested that denatonium was unable to inhibit responses to PGE_2, which is established to maintain the spontaneous tone of the preparation (18, 21). This mode of action was confirmed because denatonium failed to inhibit the contraction response both to PGE_2 itself and the analog 17-phenyl trinor PGE_2. The finding that the TP receptor antagonist SQ-29548 did not mimic the effects of indomethacin or the EP_1 antagonist provided further strength to this conclusion. As we also found that denatonium did not induce release of PGE_2 from the GPT, we can attribute the reduced response to denatonium in naïve preparations solely to its inability to counteract the part of the preload contributed by constitutive release of PGE_2, and the data support that thiamine induces relaxation by the same PGE_2-sensitive mechanism. A similar

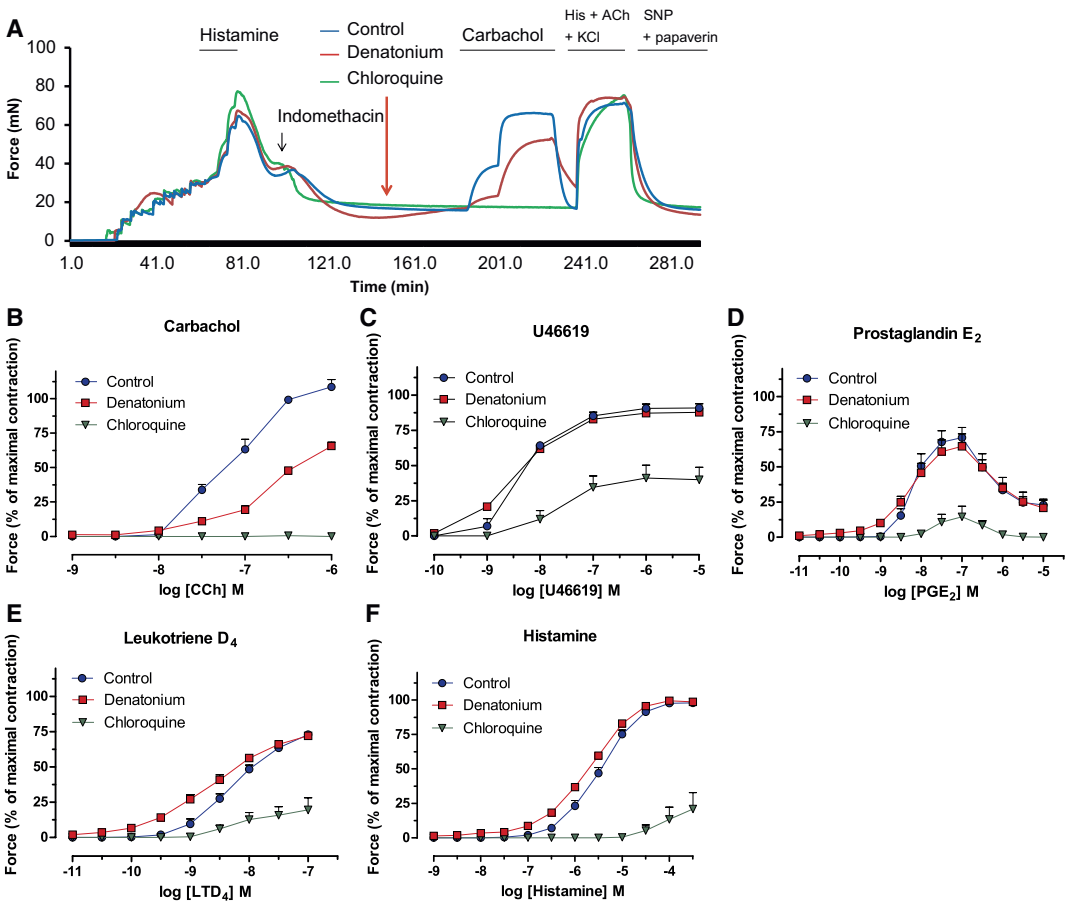

Fig. 5. *A*: trace depicting the effects of denatonium, and chloroquine prestimulation on contractions induced by increasing concentrations of carbachol. GPT was prestimulated with 300 μM denatonium or 300 μM chloroquine for 30 min in the presence of 3 μM indomethacin followed by increasing concentrations of carbachol (n = 5–6) (*B*), U46619 (n = 4) (*C*), PGE_2 (n = 5) (*D*), leukotriene D4 (LTD_4) (n = 5–6) (*E*), and histamine (n = 5–6) (*F*). Data are expressed as percentages of maximal contraction obtained with histamine (1 mM), acetylcholine (1 mM), and KCl (60 mM), which were added to the bath at the end of each experiment. In histamine experiments, the first histamine peak used as a control of the guinea pig trachea reactivity was used as a reference to avoid the effects of receptor desensitization.

decrease in relaxation has been shown for the β_2-adrenoceptor when the EP_1 receptor is activated (15).

Our data furthermore indeed implicate that the TAS2R agonists denatonium and chloroquine mediated relaxations through different signaling pathways. Whereas denatonium failed to reverse on-going antigen-induced contractions in the OVA-model, as well as the contractions induced by any of the mediators of that response (histamine, U-46619 for PGD_2/TXA_2, and LTD_4) (24), chloroquine was able to cause relaxations irrespective of contractile agonist. The difference in the effects of the two agonists was also shown when GPT was contracted with the different agonists after pretreatment with either denatonium or chloroquine. In these experiments, denatonium could again only reduce the effect of carbachol, whereas chloroquine decreased the effects of all contractile agonist used in the studies. The results further indicated that the denatonium-induced relaxation is dependent on the type of intracellular signaling induced by the particular agonist, whereas chloroquine-mediated relaxation responses appear to be signaling-independent, or at least via pathways that are able to counteract a broader range of signaling mechanisms.

As BK_{Ca} channels are shown to be involved in the relaxation mediated by β_2-adrenoceptors (12, 17) and TAS2Rs, we further investigated the importance of this mechanism in GPT by the use of three different BK_{Ca} channel blockers. The effects of chloroquine were not affected by iberiotoxin, charybdotoxin, and paxilline. With denatonium, roughly a one-log shift change was obtained with all channel blockers, but the maximal effect

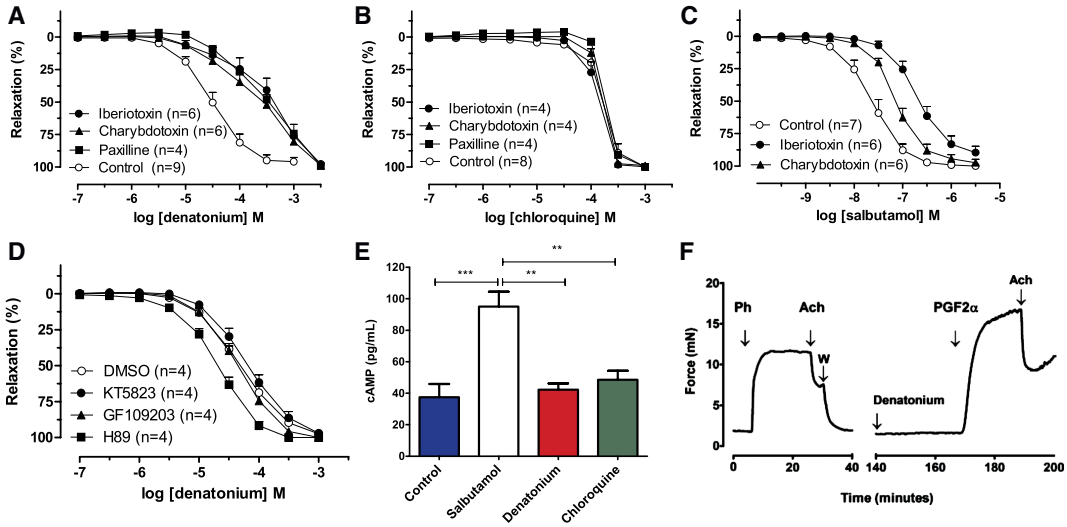

Fig. 6. GPT was treated with 3 μM indomethacin in the presence and absence of Ca^{2+} induced large K^+ channel blockers iberiotoxin (100 nM), charybdotoxin (100 nM), and paxilline (1 μM), and precontracted with 100 nM carbachol. The effects of denatonium (*A*), chloroquine (*B*), and salbutamol (*C*) on airway relaxation are expressed as percentages in relation to maximal relaxation. *D*: effects of kinase inhibitors on denatonium-induced airway relaxation. KT5823 = selective inhibitor of protein kinase G (1 μM), GF109203 = selective inhibitor of protein kinase C (1 μM), H89 = protein kinase A inhibitor (10 μM). *E*: samples were collected from the organ bath fluid 20 min after 300 nM salbutamol ($n = 4$), 300 μM denatonium ($n = 4$), 300 μM chloroquine ($n = 4$) and stimulation of GPT precontracted with 100 nM carbachol for 40 min followed by 30 min incubation of 200 μM 3-isobutyl-1-methylxanthine, a nonspecific inhibitor of phosphodiesterases. The absolute cAMP release (pg/ml/mg) was measured with enzyme immunoassay. $^{**}P < 0.01$, $^{***}P < 0.001$. *F*: guinea pig aorta was precontracted with 10 μM phenylephrine and exposed to 10 μM ACh. Thereafter, the segments were washed and exposed to 300 μM denatonium, precontracted with 3 μM $PGF_{2\alpha}$, and again exposed to 10 μM ACh.

was not altered. Similar results have been reported for the channel blockers against salbutamol (12). However, when the toxins were added, the level of the carbachol precontraction was increased, which per se was shown to induce a rightward shift of the concentration-response curve to denatonium (discussed below). This suggests that bitter taste agonists induce smooth muscle relaxation by more mechanisms than activation of BK_{Ca} channels. Our findings appear to be in line with the

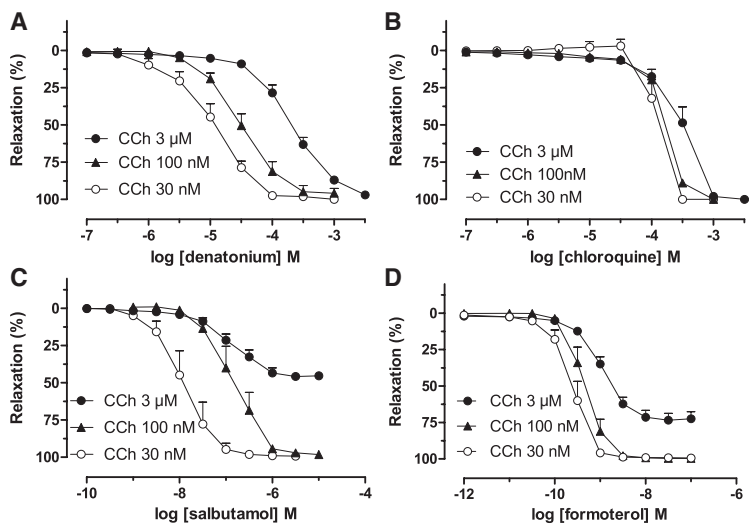

Fig. 7. Effects of denatonium ($n = 5$–9) (*A*), and chloroquine ($n = 4$–9) (*B*), salbutamol ($n = 4$) (*C*), and formoterol ($n = 6$) (*D*) on airway relaxation in GPT precontracted with 3 μM, 100 nM, and 30 nM carbachol (CCh) in the presence of 3 μM indomethacin. Data are expressed as percentages of maximal relaxation obtained with a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM) at the end of each experiment.

recent results of Zhang et al. (28), who did not find evidence of specific contribution of the BK_{Ca} channels to the relaxation caused by chloroquine in isolated mouse airways. However, An et al. (2) found the chloroquine-induced relaxation of mouse trachea and human airway smooth muscle to be partially sensitive to BK_{Ca} channel blockers. It is likely that methodological issues and species differences in bitter taste receptor homolog expression could contribute to different results in different systems. It is possible that BK_{Ca} channel activation may be a general process in smooth muscle regulation and not specific for responses mediated by TAS2Rs.

As discussed by others (5), the level of precontraction can influence both the magnitude and the potency of the agonist-induced relaxation. Indeed, when the preload was increased to supramaximal levels, both denatonium and chloroquine induced maximal relaxations, whereas neither of the β_2-adrenoceptor agonists did. This confirmed the observation that bitter taste agonists induced a stronger effect than β_2-adrenoceptor agonists (7). Furthermore, whereas the potency for denatonium increased with decreasing preload, the potency for chloroquine was unaffected. When the preload was decreased, β_2-adrenoceptor agonists, both with an increase of potency, induced a maximal relaxation similar to that of the bitter taste agonists. However, the increase in potency for salbutamol was greater than for formoterol, which probably is related to salbutamol being a partial agonist and more sensitive to the level of preload than formoterol, which is a full agonist. The finding that neither β_2-adrenoceptor agonists produced maximum relaxation at the highest preload indicates that the bitter taste agonists have a greater efficacy than the β_2-adrenoceptor agonists and induce smooth muscle relaxation by other mechanisms. These distinct mechanisms between bitter taste agonists and β_2-adrenoceptor agonists were recently shown also by the lack of interference in desensitization experiments (3).

In preliminary attempts to identify the signaling pathways for the denatonium-mediated relaxation, we used kinase inhibitors for PKA, PKC, and PKG, but none of the used inhibitors had any effects. We also measured the levels of cAMP, which is a central second messenger for the β_2-adrenoceptor-mediated relaxation that can also activate other pathways than PKA (8). However, we did not find any increase in intracellular cAMP after maximal stimulation of GPT with denatonium and chloroquine. Thus we could not identify the signaling mechanisms for the bitter taste agonist-mediated airway relaxations, and further search for this is currently hampered by the lack of molecular tools in the guinea pig.

The selective effect of denatonium on cholinergic responses in the GPT was obviously not due to muscarinic antagonism because acetylcholine-mediated relaxation in aorta was not inhibited by denatonium. Although the Hill slope values for denatonium suggest a GPCR-mediated mechanism for this agonist in our studies, it should be kept in mind that denatonium is able to activate both G protein-dependent and-independent pathways in the taste buds (22). In another study, intragastric administration of TAS2R agonists induced release of ghrelin and inhibited gastric emptying. However, only the former effect was abolished in mice lacking the α-gustducin G protein (11). For chloroquine, the Hill slope was consistently steep, which suggests an involvement of ion channel activation (10), which can either be linked through a TAS2R or due to other mechanisms. Because both agonists induce strong airway smooth muscle relaxation, it should be of importance to delineate the pathways further.

In conclusion, our results demonstrate that, in this particular preparation, denatonium may completely relax contractions induced by muscarinic agonists, but it does not inhibit responses induced by other contractile agonists. The selective effect of denatonium on muscarinic responses is most likely due to TAS2R10 activation, whereas chloroquine via activation of TAS2R3 and other yet unidentified additional actions display a pattern of global inhibition of different contractile responses in GPT. Although we could not find a specific G protein-coupled pathway in this first description of relaxation of guinea pig airways induced by activation of bitter taste receptors, the potency of the bitter taste agonists showed clear similarities to human expression systems (16). Taken together with previous findings in other species and models (3, 5, 7, 19), our findings reinforce that TAS2Rs are potential new targets for development of a novel class of bronchodilators. Priority should now be given to define the signaling mechanisms and to identify agonists, which are more potent and selective.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: V.P., M.L.M., J.S., M.A., and S.-E.D. conception and design of research; V.P., M.L.M., and J.S. performed experiments; V.P., M.L.M., J.S., M.A., and S.-E.D. analyzed data; V.P., M.L.M., J.S., M.A., and S.-E.D. interpreted results of experiments; V.P., M.L.M., J.S., and S.-E.D. prepared figures; V.P., M.L.M., J.S., M.A., and S.-E.D. drafted manuscript; V.P., M.L.M., J.S., M.A., and S.-E.D. edited and revised manuscript; V.P., M.L.M., J.S., M.A., and S.-E.D. approved final version of manuscript.

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III

Antagonising EP_1 and EP_2 receptors reveal the antigen associated TP receptor mediated contraction in the guinea pig trachea

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Abstract

The role of different cyclooxygenase (COX) metabolites released after antigen exposure has been difficult to assess due to simultaneous release of dominant constrictors such as histamine and cysteinyl-leukotrienes (CysLT). In addition prostaglandin E_2 (PGE_2) also has a powerful effect on basal tone. The aim was to exclude PGE_2, histamine and CysLTs from the antigen-induced contraction to define the possible involvement of remaining COX metabolites. Isometric force was measured in guinea pig trachea after exposure to cumulatively increasing concentrations of ovalbumin (OVA; 0.1 $ng \cdot mL^{-1}$ to 0.1 $mg \cdot mL^{-1}$) in the absence or presence of biosynthesis inhibitors and receptor antagonists.

Challenge with OVA induced a concentration-dependent contraction that reached 75% of maximal tissue response. COX-inhibition or a combination of EP_1 and EP_2 receptor antagonism (ONO-8130 and PF-04418948) completely abolished the tone, resulting in an augmented antigen response. COX inhibition in combination with either antihistamines or anti-leukotrienes (FLAP inhibitor or $CysLT_{1-2}$ receptor antagonist) displayed no difference compared to COX inhibition alone. However, a combination of all three classes reduced the contraction to 30%, revealing an unknown contractile component. Exchanging COX inhibition with EP_{1-2} receptor antagonists together with antihistamines and anti-leukotrienes could not decrease the contraction more than to 50%. However, when a TP receptor antagonist (SQ-29,548) was further included, the maximal antigen contraction reached 30%, similar as previously, clearly revealing a TP-mediated contractile component.

PGE_2 primarily regulate the basal tone via EP_1 and EP_2, whereas prostanoids, such as TXA_2 and PGD_2, contribute as mediator of the antigen-response by activation of the TP receptor.

Keywords: ovalbumin, guinea pig, eicosanoids, TP receptor, EP receptor, trachea

1 Introduction

The early allergic reaction (EAR) displays as an acute bronchoconstriction in asthmatic subjects minutes after antigen exposure (Boulet *et al.*, 2007). In humans and guinea pigs the predominant mediators responsible for airway smooth muscle contraction are histamine in combination with lipid-derived mediators such as cysteinyl-leukotrienes (CysLTs) and cyclooxygenase (COX) metabolites (Bjorck and Dahlen, 1993; Featherstone et al., 1990; Roquet et al., 1997; Selg et al., 2008). In addition to being potent bronchoconstrictors, these mediators also recruit inflammatory cells, induce mucus secretion and cause vasodilatation, factors aggravating the airflow obstruction (Bradding *et al.*, 2006; Jutel *et al.*, 2005; Matsuoka *et al.*, 2000).

Because several potent mediators are released concomitantly during the antigen-challenge, single or dual inhibition of the involved mediators have displayed limited effects in several guinea pig *ex vivo* models of antigen-induced airway contraction; such as parenchymal strips (Jonsson and Dahlen, 1994), isolated trachea (Bozkurt *et al.*, 2011), precision cut lung slices (Ressmeyer *et al.*, 2006) and the isolated, perfused and ventilated lung (Sundstrom *et al.*, 2003). However, combining antagonism of histamine receptors with inhibitors of COX and the 5-lipoxygenase pathways inhibits the major part of the antigen induced contraction in several guinea pig models (Riley et al., 2013; Sundstrom et al., 2003). Still, the precise roles and the interactions between the different mediators and their respective receptors have not been established. In guinea pig trachea, this has been particularly difficult due to a constitutive smooth muscle tone maintained by prostaglandin (PG) E_2 via EP_1 and EP_2 receptors (Safholm et al., 2013). It remains to determine the relative contributions of the large number of mediators that have been implicated in the antigen-induced airway constriction. In guinea pig airways PGE_2 contract by activation of EP_1 receptors, and by the use of a selective EP_2 receptor antagonist (Forselles *et al.*, 2011) we have recently shown that PGE_2 simultaneously evoke potent relaxation of the GPT by action on EP_2 receptors (Safholm et al., 2013). In particular, most previous studies have focused on the role of cysteinyl-leukotrienes and only studied the residual component of the contraction after pre-treatment with anti-histamines and different NSAIDs globally blocking formation of all COX products. Hence, this study aims for the first time to characterise the relative involvement of the individual COX-products under conditions when components in the antigen-induced contraction was defined by step-wise addition of selective inhibitors or antagonists for the main mediators involved. Using both selective EP_1 and EP_2 receptor antagonists, the role for the basal airway

smooth muscle tension could, for the first time, be assessed in relation to the antigen-induced contraction and thereby also delineate the role of other prostanoid receptors.

2 Methods

The study was approved by the Swedish animal experimentation ethical review board N257/09 and N323/11.

2.1 Sensitization procedure and tissue preparation

Male albino guinea-pigs (Dunkin-Hartley; 400-450 g) were sensitized by a single peritoneal injection containing 100 µg ovalbumin (OVA) grade V and 0.1 g aluminium hydroxide dissolved in 0.8 mL sterile PBS. Animals were sacrificed 14 to 28 days later by an overdose of sodium pentobarbital (Apoteket AB, Stockholm, Sweden) followed by the heart-lung-package rapidly removed and placed in ice-cold Krebs-Ringer PSS buffer (NaCl [118.5 mM], KCl [4.7 mM], KH_2PO_4 [1.2 mM], $MgCl_2$ [1.2 mM], EDTA [0.03 mM], $NaHCO_3$ [25 mM], glucose [11.1 mM], $CaCl_2$ [2.5 mM] dissolved in demineralised water). The trachea was gently dissected from the surrounding connective tissue, cut into eight intact rings of equal length containing between two and three cartilage rings each and placed in a 5 mL tissue organ bath containing Krebs-Ringer PSS and kept at 37 °C, constantly bubbled with carbogen (5% CO_2 in O_2) to maintain pH at 7.4).

2.2 Experimental procedure

Changes in smooth muscle force were detected using an isometric force-displacement transducer linked to a Grass polygraph. The response was displayed using LabChart 7.0.3 (ADInstruments, Australia). During an equilibration period of 60 min with washes every 15 min, the resting force was mechanically adjusted to 30 mN. As a control of guinea pig tracheal reactivity, histamine (0.1 nM) to (0.3 mM) was cumulatively added which was followed by a second wash period and a new 45-60 min equilibration period. To study the action of OVA, cumulative concentrations, from 0.1 ng/mL to 0.1 mg/mL, generating concentration-dependent contraction responses was elicited in the absence and presence of pharmacological tools which were given 45 min prior. Only one OVA response was investigated in each preparation and at the end of the experiment, histamine (1 mM),

acetylcholine (1 mM) and potassium chloride (60 mM) was added to the organ bath for 30 min which was used as a reference of the maximal contraction. As a reference for the maximal relaxation, a combination of papverine (0.1 mM) and sodium nitroprusside (0.1 mM) was added to the bath and left for 60 min. The different combinations of receptor antagonists and enzyme inhibitors did neither affect maximal contraction nor maximal relaxation.

2.3 Pharmacological tools

The unselective cyclooxygenase (COX) inhibitor indomethacin, 5-lipoxygenase (5-LOX) inhibitor zileuton, 5-LOX activating protein (FLAP) inhibitor MK-886 was used to investigate the involvement of the enzymes participating in producing the prostaglandins and leukotriene's. Furthermore, selective receptor antagonists for EP_1 (ONO-8130), EP_2 (PF-04418948), TP (SQ-29,548), H_1 (mepyramine), H_2 (metiamide), H_3 (ciproxifan), H_4 (JNJ 7777120), $CysLT_1$ (montelukast), $CysLT_{1-2}$ (BAY-u9773), M_{1-5} (atropine), $5\text{-}HT_{2A}$ (ketanserine) and B_2 (HOE-140) receptors were used to assess respective involvement during the antigen induced contraction.

2.4 Drugs and suppliers

NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, KH_2PO_4 and glucose were obtained from VWR (West Chester, PA). Ovalbumin (grade V), histamine dihydrochloride, DMSO, EDTA, metiamide, acetylcholine, indomethacin, ciproxifan, ketanserine, atropine, papverine and sodium nitroprusside and were purchased from Sigma-Aldrich (St. Louis, MO). SQ-29,548, montelukast, BAY-u9773 was obtained from Cayman Chemical (Ann Arbor, MI). Zileuton, MK-886, mepyramine, JNJ 7777120 and HOE-140 were purchased from Tocris (Ellisville, MO). ONO-8130 was a kind gift from ONO Pharmaceuticals (Osaka, Japan). PF-04418948 was a kind gift from Pfizer Central Research Division (Groton, CT). Stock solutions of indomethacin, SQ-29,548 and BAY-u9773 were dissolved in 95% ethanol. ONO-8130, PF-04418948, JNJ 7777120, ciproxifan, ketanserin, montelukast capsazepine, zileuton, MK-886 were dissolved in DMSO. All stock solutions were stored at -20 °C. The other drugs were dissolved in deionized H_2O on the experimental day. Dilutions of drugs were freshly made from stocks prior to each experiment in reducing concentrations of its solvent.

2.5 Calculations and statistics

The response generated by OVA is expressed as percentage compared to maximal contraction in relation to the total contractile response. All data are presented as mean ± SEM. Statistical analysis was performed using One-Way ANOVA followed by Bonferroni's Multiple

Comparison Test. For the analysis GraphPad Prism 5.01 (GraphPad Software Inc., SanDiego, CA) was used.

3 Results

3.1 PGE_2 modulates the response to antigen challenge via effects of EP_1 and EP_2 receptors on the basal tone

Following the initial resting period, washes and standard assessment of histamine responsiveness, tracheal segments developed a spontaneous contractile tone that stabilised within 30 min. In sensitised but otherwise untreated controls, the tonus reached a level of 33.7 ± 5.5% in relation to maximal tissue contractile response. Treatment with an unselective COX inhibitor (indomethacin), an EP_1 receptor antagonist (ONO-8130) or a combination of EP_1 and EP_2 receptor antagonists (PF-04418948) completely abolished the tone ($p < 0.05$; Figure 1A-B) whereas single treatment with the EP_2 receptor antagonist, in contrast, increased the basal tone to 67.1 ± 3.9% ($p < 0.05$; Figure 1B). Antagonising the TP receptor (SQ-29,548), $H_{1\text{-}2}$ receptors (mepyramine and metiamide), inhibiting the FLAP enzyme (MK-886) or combinations of these components did not affect the initial smooth muscle tone (Table 1). In contrast, zileuton which is considered to inhibit 5-LO enzyme caused an almost complete abolishment of the tone ($p < 0.05$).

Cumulative administration of OVA (0.1 ng/mL to 0.1 mg/mL) induced reproducible concentration-dependent contractions both in presence and absence of COX inhibition. However, in the presence of COX inhibition the maximal contractile force to OVA was significantly greater (87.1 ± 3.9%; $p < 0.05$) compared to sensitised untreated controls (74.6 ± 2.7%). Treatment with ONO-8130 significantly decreased the response (57.7 ± 1.6%; $p < 0.05$), whereas PF-04418948, alone or in combination with ONO-8130, increased the response to a similar level as COX inhibition (86.1 ± 4.5% and 84.8 ± 3.2%, respectively; $p < 0.05$; Figure 1A-B). On the contrary, in absence of COX inhibition, maximal OVA response was not affect by single or combined treatment with SQ-29,548, mepramine, metiamide and MK-886 (Table 1).

3.2 Antagonising the EP_1 and the EP_2 receptor unmask an antigen driven TP receptor contraction

In order to investigate the relative contribution of prostanoid receptors for the contraction, the influence of the constitutively produced PGE_2 was excluded by either antagonising both the EP_1 and EP_2 receptors or EP_1 receptor only. Further, since the strong contractions to histamine and CysLTs, which are known to be released by mast cells (Adams and Lichtenstein, 1979), can conceal the prostanoid effects, these experiments were also performed in presence of antagonists for $H_{1\text{-}2}$ and $CysLT_{1\text{-}2}$ receptors (BAY-u9773). In addition, as PGD_2 and TXA_2, also released by mast cells, both cause contraction in guinea pig lung through TP receptors (Featherstone *et al.*, 1990), this receptor was also antagonized. Thus, when $EP_{1\text{-}2}$, $H_{1\text{-}2}$, $CysLT_{1\text{-}2}$ and TP receptors were antagonized the antigen-induced contraction reached an E_{max} of 28.7 ± 0.7% that was significantly lower than using only $EP_{1\text{-}2}$ receptor antagonists ($p < 0.05$; Figure 2A). Keeping the similar set-up however exchanging BAY-u9773 with the FLAP-inhibitor MK-886 gave an identical E_{max} (28.0 ± 2.7%). Distinctly, when the TP receptor antagonist was excluded, a marked increase of the E_{max} was obtained (47.7 ± 6.2%; $p < 0.05$). The TP receptor component was also demonstrated in experiments when the EP_2 receptor antagonist was omitted. In these experiment the contractions with all other antagonists used was totally abolished regardless if BAY u9773 or MK-886 was used to block the Cys-LT component (Figure 2B). Yet, when the TP receptor antagonist was excluded a marked response was obtained with an E_{max} of 24.7 ± 3.4% ($p < 0.05$).

3.3 Inhibition of histamine and cysteinyl-leukotrienes in the presence of COX inhibition reduces antigen-induced contraction and unmasks an unknown residual contractile component

COX inhibition in combination with either $H_{1\text{-}2}$ antagonism or FLAP inhibition displayed no difference in contraction as compared to COX inhibition alone. However, segments treated with a combination consisting of COX inhibition, $H_{1\text{-}2}$ receptor antagonists and FLAP inhibitor clearly displayed a reduced maximal OVA-induced contraction, reaching an E_{max} of 32.4 ± 3.6% ($p < 0.05$; Figure 3). When the FLAP inhibitor was exchanged for a $CysLT_{1\text{-}2}$ receptor antagonist, a similar inhibition was observed, reaching an E_{max} of 29.6 ± 3.3% ($p < 0.05$). However, when the COX inhibition and antihistamines were used in combination with the $CysLT_1$ receptor antagonist montelukast, the reduction was absent, reaching an E_{max} of 79.5 ± 2.4%.

The residual response, that was observed regardless if the segments were treated with either $EP_{1\text{-}2}$ and TP receptor antagonists or COX enzyme inhibition in combination with $H_{1\text{-}2}$ receptor antagonists and FLAP inhibition, was further investigated by additional addition of antagonists for either the H_3 and the H_4 receptors (ciproxifan and JNJ 7777120, respectively), $M_{1\text{-}5}$ receptors (atropine), 5-HT_{2A} receptor (ketanserin) or B_2 receptor (HOE-140). Still, all combinations were found to be ineffective in reducing the remaining residual component unmasked in the antigen-induced contraction (Table 2).

4 Discussion

Analysis of the antigen-induced contraction in guinea pig trachea is confounded by the powerful airway smooth muscle tone maintained by PGE_2 acting on contractile EP_1 receptors and counteracted by relaxant EP_2 receptors (Safholm et al., 2013). However, with pharmacological interventions using selective receptor antagonists and enzyme inhibitors we have shown that the airway smooth muscle contraction induced by antigen is essentially mediated by the combined action of histamine, cysteinyl-leukotrienes and prostanoids that act on TP receptors. After complete inhibition of these three targets, only a small residual constriction component remained. This study shows that the airway smooth muscle antigen-induced response in guinea pig closely mimics the early allergic reaction (EAR) displayed in asthmatic subjects after antigen exposure (Bjorck and Dahlen, 1993; Roquet et al., 1997) and to achieve a strong inhibition, several receptors needs to be blocked simultaneously.

It has previously been reported that removing endogenously produced prostanoids, using non-steroidal anti-inflammatory drugs (NSAIDs), completely abolishes the basal airway tone of the guinea pig trachea (Orehek *et al.*, 1973). This inhibition has been shown to be due to reduced levels of endogenous PGE_2 resulting in a loss of EP_1 receptor-mediated contraction (Safholm et al., 2013). In the present study, this inhibitory effect was confirmed by both indomethacin and the selective EP_1 receptor antagonist ONO-8130, whereas antagonist for H_1, H_2, or TP receptors or the FLAP inhibitor MK-886 had no effect on the basal tone. On the other hand, when the EP_2 receptor was antagonised, the basal tone was increased, thus confirming the balance between EP_1 receptor mediated contraction and EP_2 receptor mediated relaxation in the isolated guinea pig trachea (Safholm et al., 2013). However, zileuton, which

generally is considered to be a 5-LOX inhibitor also reduced the basal tone. This may be explained by the recent discovery that zileuton in higher concentration also displays COX-inhibition in mouse peritoneal macrophages and in human whole blood (Rossi et al., 2010). Furthermore, the findings that the smooth muscle tone not was reduced by the FLAP enzyme inhibitor MK-886, the unselective CysLT receptor antagonist L-648,051 or by BAY-u9773 (Cuthbert *et al.*, 1991; Linden *et al.*, 1991), supports the hypothesis that zileuton also inhibits PGE_2 production in the guinea pig trachea. Thus, due to this additional effect, zileuton was not used further in the combinations.

Cumulative OVA exposure of the isolated trachea from sensitised guinea pigs caused a reproducible antigen-induced contraction irrespectively of initial smooth muscle tone. Addition of the unselective COX inhibitor, indomethacin amplified the maximum contractile response to the antigen. This augmented response after COX inhibition or unselective EP receptor antagonism AH-6809, seen both in human bronchi (Adams and Lichtenstein, 1985; Gorenne et al., 1994) and guinea pig trachea (Burka and Paterson, 1980; Hand and Buckner, 1979), has been hypothesised to be caused by enhanced release of histamine by removal of inhibitory cyclooxygenase products, such as PGE_2 (Undem *et al.*, 1987). Indeed, when the EP_2 receptor was blocked, the maximum contraction was increased both with and without EP_1 receptor blockade confirming that the increased response was due to inhibition of PGE_2. It is not possible to conclude from these experiments whether this is caused by reduced release of histamine, inhibition of the relaxation, or a combination of the two. However, we have demonstrated a role for the contractile EP_1 receptor by the reduced maximum effect of the antigen when this receptor was blocked.

To rule out a further prostanoid component in the contraction, experiments were performed comparing the effect of general COX-inhibition with selective blockade of PGE_2. The latter was assessed by selectively blocking EP_1 and EP_2 receptors which have been shown to be the EP receptors responsible for the contractile and relaxant effect of PGE_2 in guinea pig trachea, respectively (Safholm et al., 2013) . However, to isolate this component the effect of both histamine and Cys-LTs also had to be inhibited. Thus, in the presence of $H_{1\text{-}2}$ antagonism and FLAP inhibition with the tone removed by either an EP_1 receptor antagonist or both EP_1 and EP_2 receptor antagonists, a contraction approximately 20% greater was obtained compared to when prostanoid synthesis were inhibited. This effect was abolished the by the TP receptor antagonist SQ-29,548. It is established that TXA_2 and PGD_2 are released during antigen-induced degranulation (Turner and Dollery, 1988) exerting their main contractile effect in the

lung through the TP receptor (Beasley et al., 1989; Larsson et al., 2011). Thus, after removal of the tone in combination with antihistamines and FLAP inhibition, the contribution of the TP component could be identified.

As described previously, in both human and guinea pig, the main components of the antigen-induced contraction are mediated by histamine and Cys-LTs (Bjorck and Dahlen, 1993; Burka and Paterson, 1981) and combined inhibition of these mediators are needed to achieve substantial inhibition (Sundstrom *et al.*, 2003). Thus, neither blockade of $H_{1\text{-}2}$ receptors or inhibiting the response to Cys-LTs using the selective FLAP inhibitor MK-886 and nonselective $CysLT_{1\text{-}2}$ receptor antagonist BAY-u9773 affected the maximum antigen response. One possible reason for this is phenomenon is that the responses to both agonists are close to the maximum possible response so that any additive effect cannot be distinguished. Alternatively, the receptors may compete for the same intracellular signalling system. Here we have also shown that combined $CysLT_{1\text{-}2}$ receptor antagonism was required, which may be due to the same additive effect.

Irrespectively of whether the antigen-induced contraction was reduced using antihistamines in combination with complete inhibition of COX and 5-LOX metabolites, or by the use of antagonists targeting $EP_{1\text{-}2}$, TP or $CysLT_{1\text{-}2}$ receptors, the outcome was the same: an approximately 70% reduction of the antigen-induced contraction. Furthermore, atropine, ketanserine and HOE-140, all failed to alter this response indicating that muscarinic $M_{1\text{-}5}$ receptors, serotonin $5\text{-}HT_{2A}$ and bradykinin $BK\text{-}B_2$ receptors respectively, do not mediate antigen-induced contraction in the guinea pig trachea.

In conclusion, antigen-induced contractile responses to OVA in the guinea pig central airway appears to be due to a major release of histamine and 5-LOX products together with a significant effect induced by activation of TP receptors so that inhibition of all three classes of receptor is required to substantially reduce the effect. These findings are important for several reasons. Firstly, since the specific role of PGE_2 has not been established before, both a selective and potent EP_1 and EP_2 receptor antagonist was used revealing PGE_2 as a powerful modulator of the antigen-induced response. Secondly, by the use of several receptor antagonists, the specific contribution of the TP receptor could for the first time be identified. Thirdly, since the reduction of the antigen-induced contraction was minimal when the effect generated by two mediator classes was inhibited, it was necessary to investigate if inhibition by all three mediator classes, histamine, prostaglandins and leukotrienes, had any effect at

reducing the airway smooth muscle contraction. This study increases our understanding of the mediators released in conjunction with mast cell degranulation and has shown that inhibition of three classes of mediator is needed to achieve significant reduction of the antigen-induced contraction. As complete COX inhibition induces contractile amplification in both human and guinea pig (Adams and Lichtenstein, 1985; Bozkurt et al., 2011), an alternative therapeutic approach might be to combine selective receptor antagonists instead of global enzyme inhibition, which risks disrupting any potentially protective receptors such as EP_2 in guinea pig and EP_2 and EP_4 in human (Buckley et al., 2011; Safholm et al., 2013). From our findings, we conclude that PGE_2 primarily regulates basal tone whereas prostanoids, such as TXA_2 and PGD_2 contribute as mediators of the antigen-response by activation of the TP receptor.

Figures

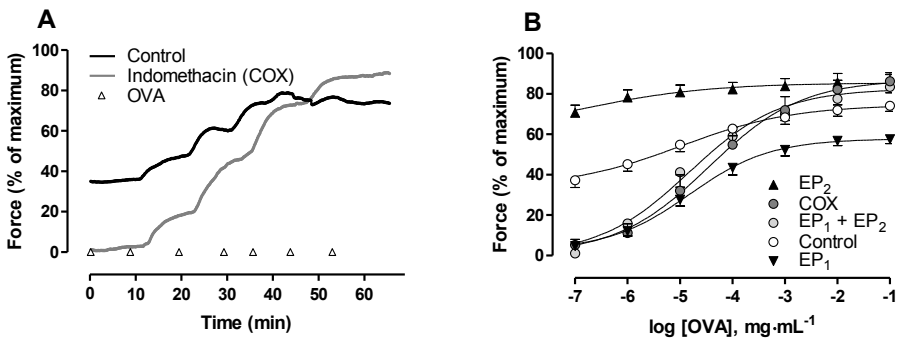

Figure 1(A) Representative trace of concentration-response curves to OVA in guinea pig trachea, in the presence or absence of indomethacin (COX). **(B)** Concentration-response curves to OVA in the presence of the COX inhibitor indomethacin (COX; 3 μM), EP_1 receptor antagonist ONO-8130 (EP_1; 10 nM) and EP_2 receptor antagonist PF-04418948 (EP_2; 10 μM). The contraction of each segment in all experiments was calculated as percentage of maximal contraction of 1 mM histamine, 1 mM acetylcholine and 60 mM KCl in relation to maximal relaxation, a combination of 0.1 mM papverine and 0.1 mM sodium nitroprusside. Data represent mean ± S.E.M (n = 8-23)

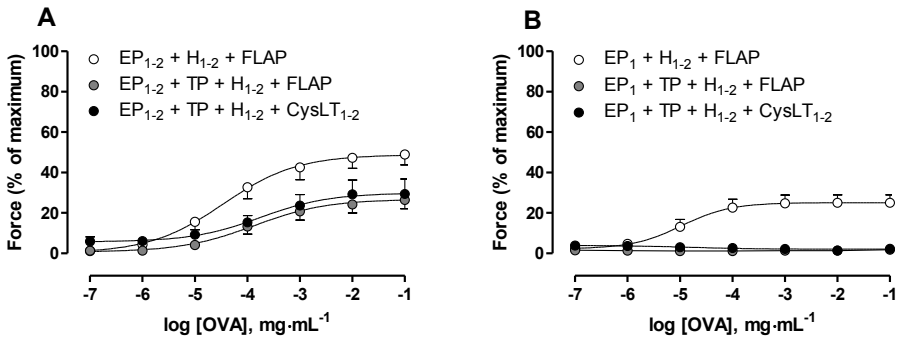

Figure 2 A-B Concentration-response curves to OVA in the presence of EP_1 receptor antagonist ONO-8130 (EP_1; 10 nM) and EP_2 receptor antagonist PF-04418948 (EP_2; 10 μM), TP receptor antagonist SQ-29,548 (TP; 1 μM), H_1 receptor antagonist mepyramine (H_1; 1 μM), H_2 receptor antagonist metiamide (H_2; 1 μM), FLAP enzyme inhibitor MK-886 (FLAP; 10 μM) and $CysLT_{1-2}$ receptor antagonist BAY-u9773 ($CysLT_{1-2}$; 1 μM).. The contraction of each segment in all experiments was calculated as percentage of maximal contraction of 1 mM histamine, 1 mM acetylcholine and 60 mM KCl in relation to maximal relaxation, a combination of 0.1 mM papverine and 0.1 mM sodium nitroprusside. Data represent mean ± S.E.M (n = 6-10).

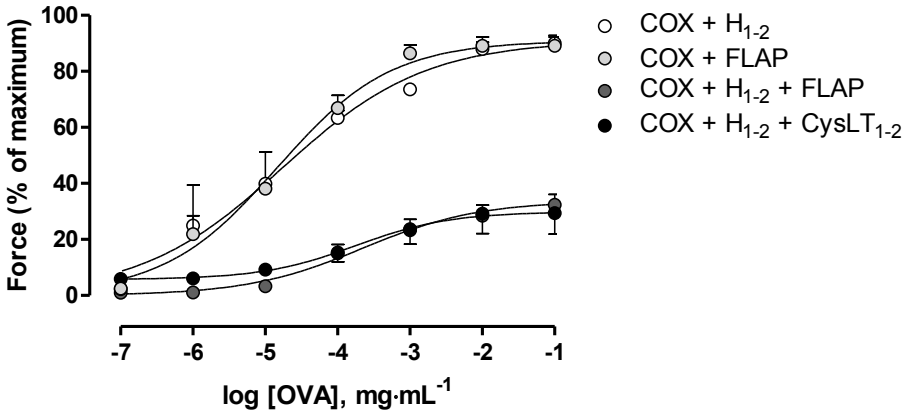

Figure 3 Concentration-response curves to OVA in the presence of the COX inhibitor indomethacin (COX; 3 μM), H_1 receptor antagonist mepyramine (H_1; 1 μM), H_2 receptor antagonist metiamide (H_2; 1 μM), $CysLT_{1-2}$ receptor antagonist BAY-u9773 ($CysLT_{1-2}$; 1 μM) and FLAP enzyme inhibitor MK-886 (FLAP; 10 μM). The contraction of each segment in all experiments was calculated as percentage of maximal contraction of 1 mM histamine, 1 mM acetylcholine and 60 mM KCl in relation to maximal relaxation, a combination of 0.1 mM papverine and 0.1 mM sodium nitroprusside. Data represent mean ± S.E.M (n = 5-12).

Tables

Table 1 E_{tone}, E_{max}, pEC_{50} calculated from a non-linear regression fit based on concentration-response curve to ovalbumin in isolated guinea pig trachea

Antagonist/inhibitor target	**E_{tone}**	**E_{max}**	**pEC_{50}**	***n***
Control	33.7 ± 5.5	74.6 ± 2.7	5.0 ± 0.4	*23*
TP	41.3 ± 6.0	72.5 ± 3.7	5.1 ± 0.5	5
TP + $H_{1\text{-}2}$	32.7 ± 6.0	73.8 ± 3.1	5.0 ± 0.5	5
TP + FLAP	40.9 ± 5.2	72.6 ± 4.6	4.2 ± 0.6	7
FLAP	45.4 ± 5.0	64.8 ± 2.7	5.3 ± 0.7	8
FLAP + $H_{1\text{-}2}$	36.1 ± 12.8	66.1 ± 5.9	5.1 ± 2.0	6
$H_{1\text{-}2}$	46.3 ± 5.1	68.8 ± 3.3	5.1 ± 0.6	5
5-LO	6.8 ± 3.6	80.6 ± 2.7	4.6 ± 0.2	5

TP receptor antagonist SQ-29,548 (TP; 1 μM), H_1 receptor antagonist mepyramine (H_1; 1 μM), H_2 receptor antagonist metiamide (H_2; 1 μM), FLAP inhibitor MK-886 (FLAP; 10 μM), 5-LO inhibitor zileuton (5-LO; 1 μM) and COX inhibitor indomethacin (COX; 3 μM). The contraction of each segment in all experiments was calculated as percentage of maximal contraction of 1 mM histamine, 1 mM acetylcholine and 60 mM KCl in relation to maximal relaxation to a combination of 0.1 mM papverine and 0.1 mM sodium nitroprusside. Data represent mean ± S.E.M.

Table 2 E_{tone}, E_{max}, pEC_{50} calculated from a non-linear regression fit based on concentration-response curve to ovalbumin in isolated guinea pig trachea

Antagonist/inhibitor target	**E_{tone}**	**E_{max}**	**pEC_{50}**	***n***
COX + $H_{1\text{-}2}$ + FLAP	1.7 ± 0.8	38.0 ± 2.9	3.6 ± 0.3	14
COX + $H_{1\text{-}4}$ + FLAP	0.7 ± 0.4	41.2 ± 2.9	3.8 ± 0.2	7
COX + $H_{1\text{-}2}$ + FLAP + $M_{1\text{-}5}$	0.7 ± 0.5	42.3 ± 8.7	3.3 ± 0.7	6
COX + $H_{1\text{-}2}$ + FLAP + 5-HT_{2A}	0.1 ± 0.0	37.4 ± 9.0	3.0 ± 0.8	6
COX + $H_{1\text{-}2}$ + FLAP + B_2	0.1 ± 0.0	32.9 ± 5.2	3.9 ± 0.6	6

COX inhibitor indomethacin (COX; 3 μM), H_1 receptor antagonist mepyramine (H_1; 1 μM), H_2 receptor antagonist metiamide (H_2; 1 μM), FLAP inhibitor MK-886 (FLAP; 10 μM), muscarinic $M_{1\text{-}5}$ receptor antagonist atropine ($M_{1\text{-}5}$; 1 μM), serotonin receptor 2A antagonist ketanserine (5-HT_{2A}; 100 nM), bradykinin B_2 receptor antagonist HOE-140 (B_2; 10 nM) and TP receptor antagonist SQ-29,548 (TP; 1 μM). The contraction of each segment in all experiments was calculated as percentage of maximal contraction of 1 mM histamine, 1 mM acetylcholine and 60 mM KCl in relation to maximal relaxation to a combination of 0.1 mM papverine and 0.1 mM sodium nitroprusside. Data represent mean ± S.E.M.

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IV

Prostaglandin E_2 responses in human small airways

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Abstract

Background: Prostaglandin E_2 (PGE_2), known to exert its effects through $EP_{1\text{-}4}$ receptors, is a potent mediator with the capability to induce both airway smooth muscle contraction and relaxation. The majority of studies regarding its role in airways are done in larger conduits, however current understanding in asthma spans the whole lung, thus leaving the role of PGE_2 in small airways at large.

Objective: The aim of the present study was to investigate the role of PGE_2 for basal tone and to characterise the receptors mediating responses in human small airways (⌀ < 2 mm).

Methods: Isometric force was measured in isolated human bronchial rings after treatment with pharmacological agents in the presence or absence of biosynthesis inhibitors and receptor antagonists.

Results: In segments pre-contracted with histamine, PGE_2 induced a concentration-dependent relaxation (E_{min}: 48%; pEC_{50}: 6.4±0.4) that was inhibited by EP_4 antagonism (ONO-AE3-208; 1 μM; E_{min}: 75%) whereas EP_2 antagonism (PF-04418948; 1 μM) had no effect. On basal tension, PGE_2 or the EP_1/E_3 agonist 17-phenyl trinor PGE_2 caused a contraction (E_{max}: 76%; pEC_{50}: 5.1±0.1 and 58%, respectively) that was inhibited by TP antagonism (SQ-29,548; 1 μM; E_{max}: 9% for both) whereas EP_1 antagonism (ONO-8130; 1 μM) had no effect. Furthermore, a COX- inhibitor (indomethacin; 3 μM) increased the spontaneous tone whereas a FLAP inhibitor (MK-886; 1 μM) decreased it. The tone was most effectively increased with a selective COX-2 inhibitor (lumiracoxib; 1 μM). Antagonists for TP, IP and $EP_{1\text{-}4}$ had no effect on the basal tension.

Conclusion: In human small airways, PGE_2 induced a concentration-dependent relaxation *via* the EP_4 receptor whereas at higher concentrations it caused a contraction mediated *via* the TP receptor, with no other receptors involved.

Introduction

In human airways, several clinical studies have described prostaglandin E_2 (PGE_2) as a potent smooth muscle relaxant in normal subjects (Kawakami *et al.*, 1973; Walters *et al.*, 1982b) as well as in asthmatics (Melillo et al., 1994; Pavord et al., 1993) where it also exerts anti-inflammatory properties (Gauvreau *et al.*, 1999; Sestini *et al.*, 1996). However, several *in vitro* and *in vivo* studies in airways have described potent, and sometimes opposing, responses after being exposed to PGE_2 (Haye-Legrand *et al.*, 1986; Walters *et al.*, 1982a; Walters *et al.*, 1982c). The reason for this is because PGE_2 have the possibility to mediate responses through several different receptors (Coleman *et al.*, 1984). Firstly, the primary $EP_{1\text{-}4}$ receptors with the capability to induce both contraction and relaxation and secondly, receptors normally activated by other prostanoids such as the contractile TP receptor (Armour *et al.*, 1989; Coleman *et al.*, 1989).

Initially it was described that EP_2 was the responsible relaxant receptor, based on a study using the poorly selective receptor antagonist AH6809 and the receptor agonist misoprostol (Abramovitz et al., 2000; Norel et al., 1999). However, recent studies reject the EP_2 hypothesis and implicate EP_4 as the responsible receptor (Benyahia et al., 2012; Buckley et al., 2011). Despite beneficial relaxing properties by inhaling PGE_2, occurrences of upper airway irritancy often displayed as cough have been reported (Gauvreau *et al.*, 1999; Pavord *et al.*, 1995) which recently was suggested to be mediated via activation of EP_3 receptors (Maher *et al.*, 2009).

The majority of studies regarding the role of PGE_2 have been performed in large airways (approximately 3-5 mm inner airway diameter correlating with 4-6^{th} airway generations. Today the current understanding in asthma spans the whole lung, from the central to peripheral parts (Tashkin, 2002; van den Berge *et al.*, 2011). Since the role of PGE_2 in the small bronchi is still uncharacterised, the aim of our study was, using selective receptor antagonists, to characterise the PGE_2 responses in human small airways (approximately 0.5-1 mm inner airway diameter correlating with 12-16^{th} airway generations) to ascertain through which receptors PGE_2 mediates their responses.

Methods

The study was approved by the Swedish ethical review board 2010/181-31/2.

Isolated tissue preparation

Macroscopically healthy human lung tissue was obtained from patients (consisting of 17 males and 19 females with mean age of 64 ± 7 years) undergoing lobectomy (after confirmed structural changes within the lobe) and placed immediately in ice-cold Krebs-Ringer PSS buffer (NaCl (118.5 mM), KCl (4.7 mM), KH_2PO_4 (1.2 mM), $MgCl_2$ (1.2 mM), EDTA (0.03 mM), $NaHCO_3$ (25 mM), glucose (11.1 mM), $CaCl_2$ (2.5 mM) dissolved in demineralised water). Within 3 hours, bronchial tissue was, using microscopy, gently dissected clear from lung parenchyma under ice-cold Krebs-Ringer PSS buffer condition, cut into intact rings (approximately 0.5-1 mm inner airway diameter and 3-5 mm in length) and placed in separate wells containing Dulbecco's modified Eagle medium (DMEM; Gibco, Auckland, NZ) supplemented with 1% penicillin (100 $IU \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) (Gibco) under sterile conditions. The culture plates were placed in a humidified incubator (37 °C at 95% O_2 and 5% CO_2) for 17-24 hours. On the experimental day, the rings were mounted horizontally on two metal prongs (200 µm in diameter) for recording of isometric tension (ADInstruments Ltd., Hastings, U.K.) using myographs (Organ Bath Model 700MO, DMT A/S, Aarhus, Denmark) containing Krebs-Ringer PSS and kept at 37 °C, constantly bubbled with carbogen (5% CO_2 in O_2) to maintain pH at 7.4). Preparations were allowed to equilibrate for 30 min with buffer changed every 10 min followed by a step-wise increase in tension over 60 min to a resting tension of 1.5 mN (Adner *et al.*, 1996) with buffer changed every 15 min.

Experimental procedure

After the equilibration period, as a control of bronchial reactivity and viability, KCl (60 mM) was added twice with a wash in-between, which was followed by a second 60 min equilibration period including washes. To study the involvement of prostanoids on the basal tone, unselective COX inhibitor indomethacin, selective COX-1 inhibitors FR-122047, selective COX-2 inhibitor lumiracoxib, selective FLAP inhibitor MK-886, IP receptor antagonist CAY10441, TP receptor antagonist SQ-29548, EP_1 receptor antagonist ONO-8130, EP_2 antagonist PF-04418948, EP_3 receptor antagonist ONO-AE5-599 or EP_4 receptor antagonist ONO-AE3-208 were given as a single concentration subsequent to the second equilibration period and the force was monitored over 30 minutes. The action of PGE_2 was investigated by two separate protocols. For the relaxation, segments were treated with indomethacin (3 μM) and SQ-29,548 (1 μM) and exposed to histamine (1 μM) to give a stable pre-contraction, prior to addition of PGE_2. For the contraction two separate concentrations of PGE_2 were added to segments treated with indomethacin (3 μM) and ONO-AE3-208 (1 μM). In both cases, receptor antagonists were added 45 min prior to addition of agonists. To study the TP receptor response, U-46619, PGD_2, PGE_2 and 17-phenyl trinor PGE_2 were given as two different concentrations in the presence of indomethacin (3 μM; 45 min) and ONO-AE3-208 (1 μM; 45 min) with or without SQ-29,548 (1 μM; 45 min). Only one response was investigated in each preparation. All experiments were ended by adding histamine (1 mM), acetylcholine (1 mM) and KCl (60 mM) as a reference for the maximal contraction.

Drugs and suppliers

NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, KH_2PO_4 and glucose were obtained from VWR (West Chester, PA). Histamine dihydrochloride, DMSO, acetylcholine, indomethacin, phenylephrine, sodium nitroprusside and papaverine were purchased from Sigma-Aldrich (St. Louis, MO). Prostaglandin E_2, Prostaglandin D_2, SQ-29,548, FR-122047 and 17-phenyl trinor PGE_2, CAY10441 were obtained from Cayman Chemical (Ann Arbor, MI). Lumiracoxib was obtained from SynphaBase AG (Switzerland). MK-886 was purchased from Tocris (Ellisville, MO). ONO-8130, ONO-AE5-599, ONO-AE3-208 were kind gifts from ONO Pharmaceuticals (Osaka, Japan). PF-04418948 was a kind gift from Pfizer Central Research Division (Groton, CT). Stock solutions of indomethacin, PGE_2 (100 mM), PGD_2 (100 mM), 17-phenyl trinor PGE_2 (100 mM) and MK-886 (100 mM) were dissolved in 99% ethanol, ONO-8130, ONO-AE5-599, ONO-AE3-208, ONO-AE1-259, CAY10441, PF-04418948, FR-122047, SQ-29,548 and lumiracoxib were dissolved in DMSO. All stock solutions were

stored at -20 °C. The other drugs were dissolved in Krebs-Ringer PSS buffer or deionized H_2O on the day of the experiment. Dilutions of drugs were freshly made from stocks prior to each experiment.

Calculations and statistics

The response generated by agonists is expressed as percentage compared to maximal contraction. All data are presented as mean ± SEM. Statistical analysis was performed using One-Way ANOVA followed by Bonferroni's Multiple Comparison Test. For the analysis GraphPad Prism 5.01 (GraphPad Software Inc., SanDiego, CA) was used.

Results

PGE_2 mediates relaxation through the EP_4 receptor in human bronchi

In segments pre-contracted with histamine (1 μM) in the presence of SQ-29,548 (1 μM), PGE_2 caused a concentration-dependent relaxation (E_{min}: 47.7±6.4; pEC_{50}: 6.4±0.4). To characterise the receptor responsible for the relaxation, the selective EP_2 receptor antagonist PF-04418948 and the selective EP_4 receptor antagonist ONO-AE3-208 was used. ONO-AE3-208 (1 μM) caused a rightward shift of the PGE_2 induced relaxation (E_{min}: 75.1±6.0) whereas PF-04418948 (1 μM) did not affect the relaxation **(Figure 1)**.

PGE_2 mediates contraction through the TP receptor and not EP_1 in human bronchi

To characterize the contractile receptors involved in the PGE_2 response, experiments were performed in presence of the EP_4 receptor antagonist ONO-AE3-208 (1 μM). PGE_2 induced a contraction (pEC_{50}: 5.1±0.1), reaching a maximum of 76.1±1.2% (100 μM). The selective TP receptor antagonist SQ-29,548 (1 μM) caused a marked inhibition reducing the contraction to 9.4±1.3% whereas the selective EP_1 receptor antagonist ONO-8130 (1 μM) had no effect **(Figure 2A)**. Also, the contraction induced by the EP_1/EP_3 receptor agonist 17-phenyl trinor PGE_2 (100 μM) was reduced from 58.3±5.7% to 9.4±1.3% by SQ-29,548 whereas ONO-8130 had no effect **(Figure 2B)**. The contraction caused by PGD_2 (100 μM) was reduced from 74.5±2.6% to 20.8±3.2% by SQ-29,548 **(Figure 2C)**. The potency and selectivity of SQ-29,548 was shown against the stabile TXA_2 mimetic U-46619. Constraining the Schild slope and Hill slope to 1 resulted in a pKB value of 7.7 for SQ-29,548 (95% CI: 7.6 to 8.1). **(Figure 2D)**.

Influence of cyclooxygenase inhibitors on the smooth muscle tone

After the wash and resting period, the unselective COX inhibitor indomethacin (3 μM; $p<0.05$) increased the tone with 23.0±3.6% whereas the FLAP inhibitor MK-886 (1 μM; $p<0.05$) decreased the tone with 15.5±5.1% **(Figure 3A)**.

To investigate which COX enzyme that is responsible for the increase, selective COX-1 inhibitor FR-122047 (1 μM) or the selective COX-2 inhibitor lumiracoxib (1 μM) were applied for 30 min. Lumiracoxib increased the tone with 66.5±5.1% and FR-122047 increased the tone with 24.6±3.7% ($p<0.05$) of maximal indomethacin response **(Figure 3B)**.

To explore which prostanoid receptor might contribute to the increased spontaneous tone segments were treated with the TP receptor antagonist SQ-29548 (1 μM), IP receptor antagonist CAY10441 (1 μM), EP_1 receptor antagonist ONO-8130 (100 nM), EP_2 receptor antagonist PF-04418948 (1 μM), EP_3 receptor antagonist ONO-AE5-599 (1 μM) or EP_4 receptor antagonist ONO-AE3-208 (1 μM) under similar conditions. The contractile effect of these antagonists was negligible, 1.1±0.8%, 2.6±0.1%, 1.9±1.1%, 0.8±0.7, 3.0±0 and 4.4±0.5 respectively, with no difference from the spontaneous decline in untreated segments (1.2±1.1%) observed over the same time **(Figure 3A)**.

Discussion

The aim of the present study was to clarify the receptors and responses regarding the action of PGE_2 in human small airways. These results suggest that PGE_2 have the capability to induce relaxation through the EP_4 receptor and a contraction through the TP receptor. Neither the EP_1, EP_2 nor EP_3 receptor seems to play a role regarding force regulation at the level of the the airway smooth muscle. Furthermore, experiments using COX inhibitors increased the airway tone whereas a FLAP inhibitor decreased it. Antagonising $EP_{1\text{-}4}$ receptors, as well as the TP and IP receptors, did not affect the tone, indicating that PGE_2 does not regulate the intrinsic tone in human small airways during normal conditions.

In our study, PGE_2 induced a concentration dependent relaxation in small airway segments contracted with histamine. The relaxation caused by PGE_2 in human primary bronchi and small airways was first suggested to be mediated by the EP_2 receptor (Norel *et al.*, 1999), similar as in the guinea pig trachea (Birrell *et al.*, 2013; Safholm *et al.*, 2013). However, recent studies, using potent and selective EP_4 antagonists and EP_2 and EP_4 agonists, have proposed that the relaxation in both primary bronchi and small airways is actually mediated through the EP_4 receptor (Benyahia *et al.*, 2012; Buckley *et al.*, 2011). Antagonising the EP_4 receptor clearly inhibited the relaxation, confirming recent findings with a different EP_4 receptor antagonist. A possible relaxant role for the EP_2 receptor was excluded by the use of a novel EP_2 receptor antagonist, also confirming previously observed lack of effect of a EP_2 receptor agonist on tone (Buckley *et al.*, 2011). Our study confirm recent studies showing that PGE_2 indeed relax small airways through the EP_4 receptor and that the EP_2 receptor is not involved in human small airway bronchorelaxation.

In the presence of an EP_4 receptor antagonist, higher concentrations of PGE_2 and EP_1/EP_3 agonist 17-phenyl trinor PGE_2 induced a strong contraction that reached 80% and 50%, respectively, of maximum tissue response. Antagonising the TP receptor almost completely abolished both contractile responses. This is in agreement with previously published results regarding the activation of TP receptors by PGE_2 and 17-phenyl trinor PGE_2 in human larger bronchi (Coleman *et al.*, 1989). Furthermore, antagonising the EP_1 receptor did not affect the contraction. This is different compared to the guinea pig trachea where the main contractile receptor to be activated by PGE_2 is the EP_1 receptor (Coleman *et al.*, 1985; Safholm *et al.*, 2013). As a verification of the TP antagonist effectiveness, a substantial reduction of the contraction of known TP receptor activators PGD_2 (Larsson *et al.*, 2011) and the 1000-fold

more potent U-46619 (Coleman *et al.*, 1989) was shown. In conclusion, PGE_2 induced bronchoconstriction in human small airway is mediated by TP receptors, and thus, use of selective TP receptor antagonists as anti-bronchoconstrictive therapy in asthma should block constrictive effects of TXA_2, PGD_2 and PGE_2.

Airway preparations of different species possess varying degrees of spontaneous tone (Linden *et al.*, 1991; Okazawa *et al.*, 1990; Schmidt *et al.*, 2000). In untreated human bronchial segments, a stable airway tone was maintained for at least 30 min. The unselective COX-inhibitor indomethacin caused a gradual increase over time that stabilised within 25 min. The role of cyclooxygenase products is somewhat unclear, with indomethacin previously displaying increased (Bjorck *et al.*, 1993; Coleman *et al.*, 1996; Hutas *et al.*, 1981), decreased (Ito *et al.*, 1989) or no effect on the tone (Brink *et al.*, 1980). To investigate which of the two COX enzymes that was responsible for the increase, selective COX-1 inhibitor FR-122047 and selective COX-2 inhibitor lumiracoxib were studied. Although neither of the two reached the same level of increase in tone as indomethacin alone both increased the tone, with lumiracoxib to a greater extent compared to FR-122047. Previously it could be argued that the increase is an indomethacin-specific effect, however our observations using selective COX inhibitors argue against that explanation.

In the guinea pig trachea, we have recently showed that the inherent tone is mediated and maintained by PGE_2, primarily released from the epithelium, by simultaneously activating both contractile EP_1 and relaxant EP_2 receptors (Safholm *et al.*, 2013). Since the increased contraction after COX inhibition could be the result of removal of PGE_2 responses (Watson *et al.*, 1997), selective antagonists for all four EP receptor as well as the TP and IP (Norel *et al.*, 1999) was used. However, antagonism of a single receptor did neither increase nor decrease the basal tone suggesting that the effect is not solely dependent on removal of one relaxant component. Furthermore, PGD_2 could potentially mediate a relaxant response (al Jarad *et al.*, 1994) through the DP_1 receptor (Boie *et al.*, 1995) that would be concealed by the contractile TP receptor (Coleman *et al.*, 1989). However, since no decrease was observed in the presence of the TP receptor antagonist we can assume that the DP_1 receptor is not involved.

Another possibility for the indomethacin-induced increase is removal of inhibition of leukotriene synthesis (Kuehl *et al.*, 1984), resulting in the greater effect of inherent contractile components, such as leukotrienes (Schmidt *et al.*, 2000). Indeed, the FLAP inhibitor MK-886

showed a decreased tone clearly displaying an important role for the leukotrienes in maintaining the tone.

Although previous experiments investigating the tone in human airways have been performed in large airways (⌀ > 2 mm) our study focused on the small airways. Nevertheless, we conclude that there are similarities in regulating the tone when comparing large and small airways, where both have the same increase in tone after removal of COX metabolites and decreased after removal of 5-LOX metabolites.

In summary, these data show that EP_4 receptor is responsible for the PGE_2 induced relaxation in human small airways whereas the TP receptor is solely responsible for the contraction evoked by PGE_2. Furthermore, neither $EP_{1\text{-}4}$, TP nor IP receptors seems to solely play a role in regulating the human airway smooth muscle tone during normal condition. Whether or not this is the same during inflammatory conditions needs to be further investigated. The findings support selective agonists of the EP_4 receptor as a possible novel bronchoprotective compounds for the treatment of asthma and other airway constrictive ailments.

Figures

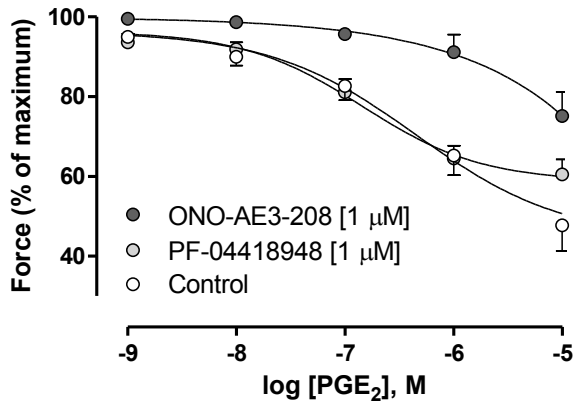

Figure 1 Concentration-response curves to PGE_2 in human small airways. Segments contracted with histamine (1 μM) in the presence of COX-inhibitor (indomethacin; 3 μM) and TP receptor antagonist (SQ-29,548; 1 μM) subjected to either EP_2 receptor antagonist (PF-04418948; 1 μM) or EP_4 receptor antagonist (ONO-AE3-208; 1 μM). Data represent mean ± S.E.M. (n = 7).

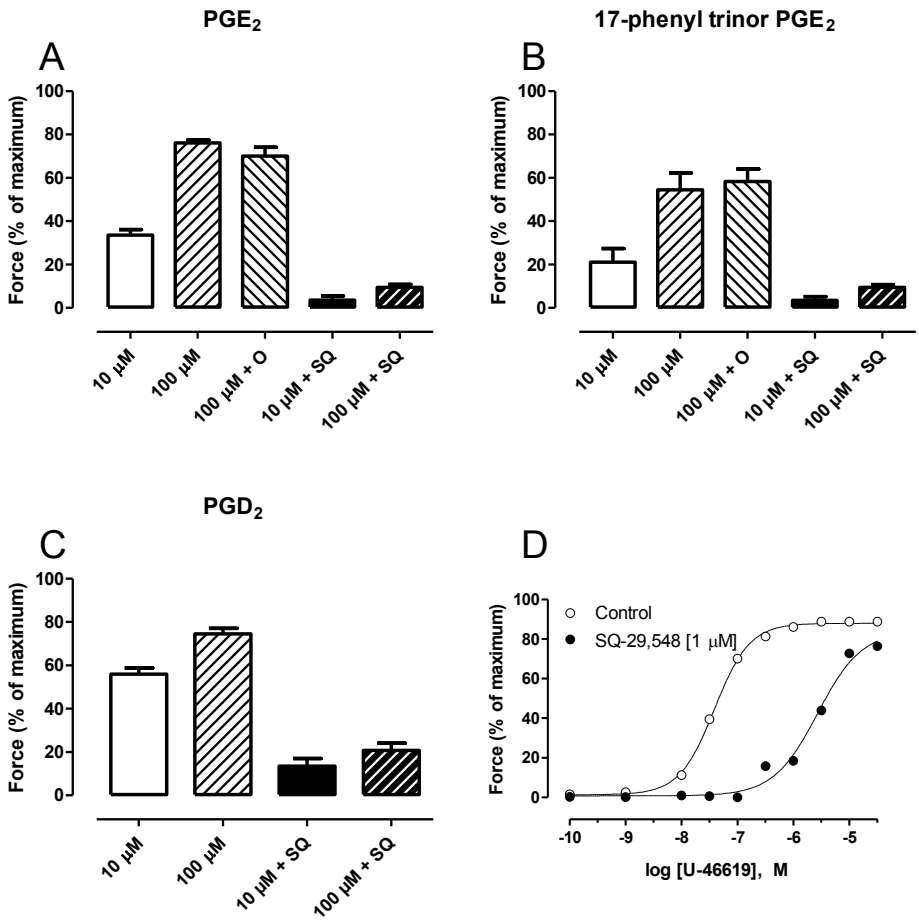

Figure 2 In the presence of COX-inhibitor (indomethacin; 3 µM) and EP_4 receptor antagonist (ONO-AE3-208; 1 µM) **(A)** PGE_2, **(B)** 17-phenyl trinor PGE_2, **(C)** PGD_2 or **(D)** U-46619 in the presence or absence of TP receptor antagonist (SQ-29,548; SQ; 1 µM). Data represent mean ± S.E.M. (n = 3-7) for A-C and a trace from one patient in D.

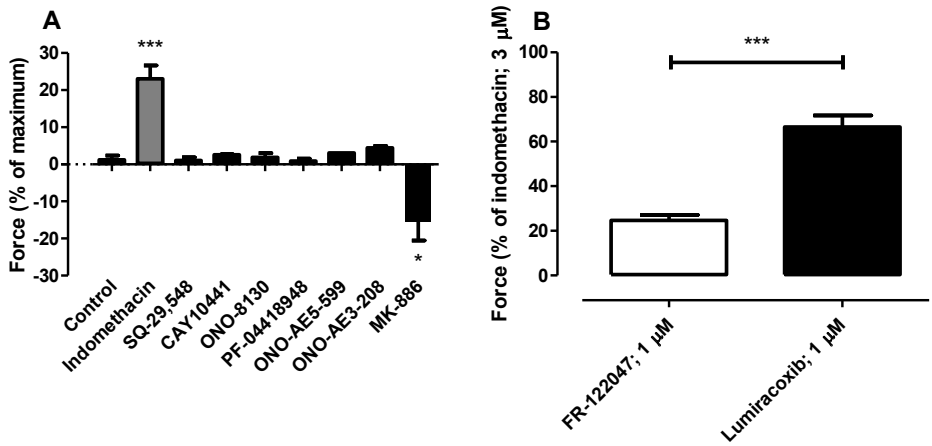

Figure 3 (A) Changes of spontaneous tone in human bronchi subsequent to addition of either inhibitor for COX (indomethacin; 3 μM) and FLAP (MK-886; 1 μM) or antagonists for TP (SQ-29,548; 1 μM), IP (CAY10441; 1 μM), EP_1 (ONO-8130; 1 μM), EP_2 (PF-04418948; 1 μM), EP_3 (ONO-AE5-599; 1 μM) or EP_4 (ONO-AE3-208; 1 μM) receptors. **(B)** Increased spontaneous tone in human bronchi subsequent addition of COX-1 inhibitor (FR-122047; 1 μM) or COX-2 inhibitor (lumiracoxib; 1 μM). Data represent mean ± S.E.M. (n = 3-10).

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