P2 Receptors in Airway Smooth Muscle

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Abstract

The hypertrophic airway smooth muscle (ASM) of asthmatic airways is hyperresponsive compared to that of healthy airways, although the underlying causes for this are unknown. P2 receptors, receptors for extracellular nucleotides including ATP, contribute to the regulation of smooth muscle contraction and proliferation. However, the roles of P2 receptors in ASM have not been fully elucidated. As the extracellular ATP concentration is increased in asthmatic airways, this could provide a mechanism by which P2 receptors contribute to the pathogenesis of ASM dysfunction in asthma.

In this thesis, I have demonstrated using qPCR the presence of P2 receptor transcripts in murine airways, and in ASM cells cultured from murine airways and those of both healthy and asthmatic donors. Biochemical and immunohistochemical approaches have revealed the expression of P2X1, P2X4 and P2X7 receptor proteins in murine airways which localises to the ASM specifically, and in ASM cells cultured from murine airways and those of healthy and asthmatic individuals. Using Ca^{2+} imaging, ASM cells cultured from murine airways were shown to express functional P2X7, P2Y1 and P2Y2 receptors. Additionally, ASM cells cultured from healthy airways expressed only functional P2Y2 receptors whilst those from asthmatic airways expressed both functional P2X4 and P2Y2 receptors. The application of extracellular nucleotides did not contract ASM of either murine or human airways. Yet, the pre-treatment of ASM from mouse, but not humans, with extracellular nucleotides appeared to enhance its contraction. In addition, extracellular nucleotides did not stimulate human ASM cell proliferation, although UTP (100 μM) induced the proliferation of murine ASM cells.

These data suggest that functional P2 receptors are expressed in ASM from mice and humans, but that P2 receptor function is comparable between healthy and asthmatic human ASM. The functional role of P2 receptors expressed by the ASM in asthma therefore remains unclear.

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I must first thank my supervisory team, Dr Catherine Vial and Professors Peter Bradding and Andy Wardlaw, for sticking with me and this project throughout the good and difficult times of this journey. I have learned a lot from you and, despite any difficulties encountered along the way, I am genuinely thankful and appreciative to all. I must also thank my colleagues at Glenfield for sharing this experience with me and for their contributions to my project, and particularly those who helped with the project by performing bronchoscopies and culturing and caring for the cHASM cells.

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Probably the most difficult part of this thesis to write is this paragraph. I have to thank those family members who have left us recently and can't see me complete my PhD: my Nan and Grandad, Annie and Bernie Burns, and my Aunt Sheila. Corny or not, I dedicate this thesis to you. CF will love you always.

Abstracts and prizes arising from this thesis

Abstracts:

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List of abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
0Ca-ES	Ca^{2+}-free epifluorescence solution
5-BDBD	5-(3-Bromophenyl)-1,3-dihydro-2H-Benzofuro[3,2-e]-1,4-diazepin-2-one
α,β-meATP	α,β-methylene adenosine-5'-triphosphate
ABC	Adenosine-5'-triphosphate-binding cassette
AC	Adenylyl cyclase
ADP	Adenosine-5'-diphosphate
AFB	Animal free blocker
AFB-W	Animal free blocker blocking solution
AHR	Airway hyperresponsiveness
AMP	Adenosine-5'-monophosphate
AP	Alkaline phosphatase
APS	Ammonium persulfate
ASL	Airway surface liquid
ASM	Airway smooth muscle
ATP	Adenosine-5'-triphosphate
AU	Arbitrary units
AUC	Area under the curve
BAL	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
BS-IF	Blocking solution for immunofluorescence
BS-W	Blocking solution for western blotting
BzATP	3'-O-(4-Benzoyl)benzoyladenosine 5'-triphosphate
Ca^{2+}_e	Extracellular Ca^{2+}
CaM	Ca^{2+}-calmodulin
cAMP	3',5'-cyclic adenosine monophosphate
CCR	CC chemokine receptor

cDNA	Complementary DNA
CFTR	Cystic fibrosis conductance regulator
cHASM cells	Cultured human airway smooth muscle cells
CICR	Ca^{2+}-induced Ca^{2+} release
cmT-ASM cells	Cultured mouse tracheal airway smooth muscle cells
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CPI-17	PKC-potentiated inhibitor protein
DAB	3,3'-Diaminobenzidine
DAG	Diacylglycerol
dH_20	Distilled water
DHPR	Dihydropyridine receptor
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EC_{50}	The concentration of a compound which gives 50% of a maximal response
EDTA	Ethylenediamine tetra-acetic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetra acetic acid
E-NPP	Ectonucleotide pyrophosphatase/ phosphodiesterase
E-NTDPase	Ectonucleotide triphosphate diphosphohydrolase
ER	Endoplasmic reticulum
ES	Epifluorescence solution
FBS	Foetal bovine serum
FEV1	Forced expiratory volume in 1 second
FS	Flexstation saline
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
GPCR	G-protein coupled receptor

GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
GWAS	Genome-wide association studies
HASM	Human airway smooth muscle
HBSS	Hanks Buffered Saline Solution
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
IC	Isotype control
IC_{50}	The concentration of a compound which gives 50% of maximal inhibition of a response
ICS	Inhaled corticosteroids
IFN	Interferon
IHC	Immunohistochemistry
IP_3	Inositol trisphosphate
IP3R	Inositol trisphosphate receptor
ITS	Insulin-transferrin-selenium
LABA	Long-acting β_2-agonist
LGIC	Ligand-gated ion channels
MLC20	20 kDa light chain of myosin
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mT-ASM	Mouse tracheal ASM
NCX	Na^+/Ca^{2+} exchanger
NEAA	Non-essential amino acids
O.C.T	Optimal cutting temperature compound
PA	Pluronic acid-F127
PBS	Phosphate buffered saline
PBSc	Commercial phosphate buffered saline
PC12	Pheochromocytoma
PG	Prostaglandin

PIC	Protease inhibitor cocktail
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma membrane Ca^{2+}-ATPase
PNS	Parasympathetic nervous system
PSS	Physiological saline solution
qPCR	Quantitative polymerase chain reactions
R	340 nm/380 nm ratio
ROI	Region of interest
RSV	Respiratory syncitial virus
RT	Room temperature
RyR	Ryanodine receptor
SABA	Short-acting β_2-agonist
SDS	Sodium dodecyl sulphate
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SNIC	Stretch-sensitive non-selective ion channels
SR	Sarcoplasmic reticulum
$t_{1/2}$	Half time
TBST	Tris-buffered saline and Tween 20 solution
TEMED	N,N,N',N'-Tetramethylethylenediamine
Th1	T helper 1 cell
Th2	T helper 2 cell
TLRs	Toll-like receptors
Tm	Primer melting temperatures
TM	Transmembrane
TNF	Tumour necrosis factor
Tris	Tris[hydroxymethyl]aminomethane
TRPV	Transient receptor potential vanilloid

TTBS	Tris-buffered saline/Tween solution
UDP	Uridine-5’-diphosphate
UTP	Uridine-5’-triphosphate
VGCC	Voltage-gated Ca^{2+} channel
WEM	Widefield epifluorescence microscopy

1 Introduction

1.1 The airways and respiratory diseases

The evolution of complex life in the form of eukaryotic cells has been linked to the introduction of oxygen into the atmosphere, and therefore to aerobic respiration (Dismukes et al., 2001). For humans, the development and function of healthy lungs and pulmonary vasculature are vital for efficient gas exchange to allow aerobic respiration to take place (Chinoy, 2003). Disorders of the lung, and the state of airway remodelling which is often induced by them, can impair respiration by obstructing or restricting ventilation (Fish and Peters, 1999, Tillie-Leblond et al., 2008). Therefore, it is vital that the mechanisms contributing to these disorders are elucidated so that effective therapies can be developed.

1.1.1 The structure of the airways

Mammalian lungs are typically made up of two and three lobes on the left and right side, respectively. The airways themselves can be categorized into conducting airways and respiratory airways (Jeffery, 1998). Human conducting airways trap particulates, and warm and moisten the air as it travels towards the alveoli. They begin at the mouth and nose, and are connected via the trachea to the primary bronchi. The bronchi descend the airway tree in a complex dichotomous branching network until the terminal bronchi are reached. These terminal bronchi then lead to the respiratory airways, which are composed firstly of respiratory bronchioles and then alveoli, where gaseous exchange occurs (Jeffery, 1998, Chinoy, 2003) (see figure 1.1.1).

In other mammals, such as mice, the bronchi do not branch in a dichotomous fashion but are monopodial, forming a daughter segment of airway which extends directly from the parent branch instead of creating two symmetric daughter segments (Patra, 1986). Mouse lungs also differ from human lungs in that they do not contain respiratory bronchioles, but the terminal bronchi are connected directly to the alveoli (Dixon, 1999, Cockayne et al., 2005).

1.1.2 Airway diseases

Diseases affecting the airways commonly narrow the airways producing airflow obstruction. This can be detected using simple techniques such as spirometry (Ranu et al., 2011). The key measurements recorded using spirometry are the forced expiratory

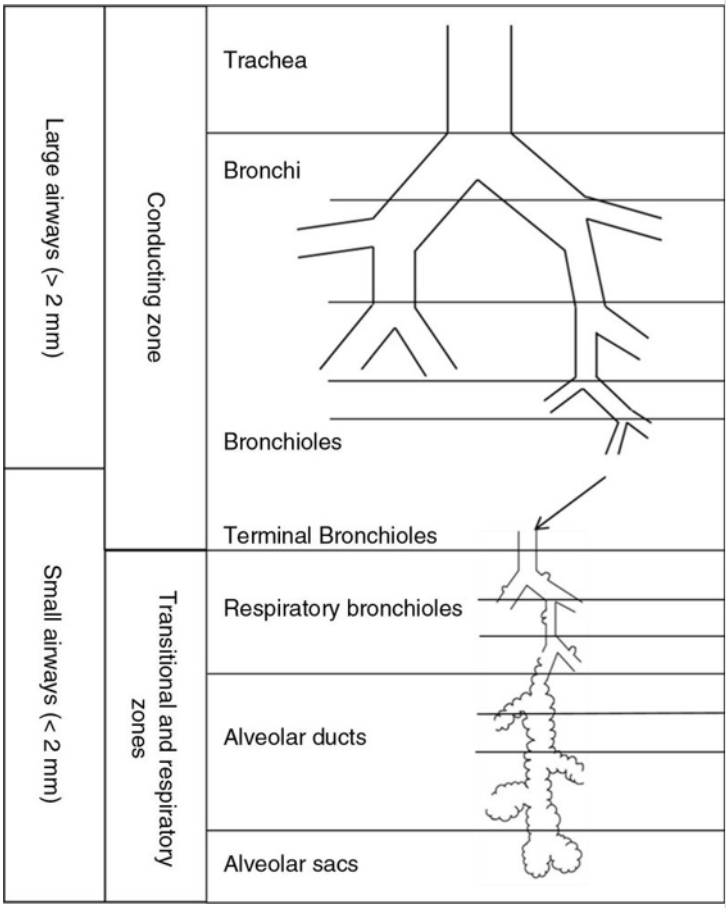

Figure 1.1.1. Human airway organisation.
The structure of the human airways, showing dichotomous branching from the large airways, such as the trachea, to the small airways including the respiratory bronchioles which ultimately transition into the alveoli.
Figure modified from McNulty and Usmani, 2014.

volume in one second (FEV1), the forced vital capacity (FVC) and the ratio of these two volumes (FEV1/FVC). For characterisation purposes, an FEV1/FVC <70% or below the lower limit of normal for the individual can indicate the presence of obstructive lung disease, whilst an FEV1/FVC >70% but accompanied by a large reduction in the predicted FVC value can indicate the presence of restrictive lung disease. Asthma and chronic obstructive pulmonary disease (COPD) are examples of obstructive lung diseases, whilst interstitial lung diseases including idiopathic pulmonary fibrosis are examples of restrictive lung diseases (Ranu et al., 2011). Diseases such as these are often difficult to treat and, with the prevalence of diseases such as asthma increasing (GINA, 2017), it is imperative that the mechanisms behind their pathogenesis are discerned to enable the development of effective treatments.

1.1.3 The definition of asthma and its subtypes

According to the World Health Organization, it was estimated that 235 million people suffered with asthma in 2013 (WHO, 2013). The Global Initiative for Asthma (GINA) defines asthma as 'a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation' (GINA, 2017). Factors contributing to asthma are often both genetic (Cookson, 1999, Yamamoto et al., 2006) and environmental (Umetsu et al., 2002, Martinez and Vercelli, 2013), and the induced airway remodelling/structural airway changes can obstruct the airways and reduce lung function (Fish and Peters, 1999, Tillie-Leblond et al., 2008). Asthma symptoms can be triggered by numerous exogenous stimuli including allergens such as house dust and Bermuda grass (Halonen et al., 1999, Ryten et al., 2002), viruses (Stein et al., 1999, Guo et al., 2013) and even non-allergic stimuli such as exercise and cold air (Simonsson et al., 1967, Surprenant et al., 1996).

Due to the complexity of this disease, its risk factors, triggers and symptoms, it is hard to fully define and categorize asthma. The above definition was reached only by consensus, and the following categories are just some of the more common clinical asthmatic phenotypes found in patients: allergic asthma, non-allergic asthma, adult-onset asthma, occupational asthma, aspirin-exacerbated airway disease, allergic bronchopulmonary mycoses, asthma with fixed airflow limitation and asthma with

obesity (Bel, 2004, Moore et al., 2010, Wenzel, 2012b, GINA, 2017). These classifications are based on observable characteristics seen in patients. However, these categories often overlap and the phenotypes are frequently unrelated to their causative pathogenic processes. Patients can also be categorized according to their clinical, physiological and inflammatory features using statistical tools such as cluster analysis (Haldar et al., 2008). These clusters can then be used to inform treatment plans (see figure 1.1.2).

A recent novel approach focuses on the molecular subtypes, often referred to as "endotypes" which contribute, either in whole or in part, to different pathological phenotypes (Anderson, 2008, Lotvall et al., 2011, Agache et al., 2012, Wenzel, 2012a), including eosinophilic versus non-eosinophilic asthma. Endotypes such as T helper 2 cell (Th2) cytokine-high or Th2 cytokine-low may underlie some of these pathological phenotypes (Anderson, 2008, Woodruff et al., 2009, Wesolowska-Andersen and Seibold, 2015), and the recognition of these is already enabling improved targeting of treatment (Haldar et al., 2009, Nair et al., 2009, Corren et al., 2011, Antohe et al., 2013).

1.1.4 The pathogenesis of asthma

1.1.4.1 The onset of asthma and its risk factors

The causes of the initial onset of asthma are unknown, but its pathogenesis is attributed to both genetic and environmental factors. Family and twin studies suggest a heritable component of up to 60% (Spain and Cooke, 1924, McKee, 1966, Edfors-Lubs, 1971, Gerrard et al., 1976, Duffy et al., 1990). The use of genome-wide association studies (GWAS) has enabled the discovery of numerous polymorphisms in different susceptibility genes for asthma, at many different loci. Among these are *ADAM33* (Van Eerdewegh et al., 2002), *IL18R1* (Zhu et al., 2008, Tulah et al., 2013), *IL33 (Tulah et al., 2013), SMAD3 (Tulah et al., 2013), CHI3L1 (Ober et al., 2008)* and *ORMDL3* (Moffatt et al., 2007), many of which are involved in the inflammatory response. Continuing with the use of GWAS could identify additional genetic risk factors for asthma in the future.

Many environmental factors are also known to play a role in asthma pathogenesis, and have been estimated to contribute ~40% of the risk (Duffy et al., 1990). For example, parental smoking increases the risk of children developing asthma (Mitchell et al., 2012), particularly if the mother smoked during pregnancy

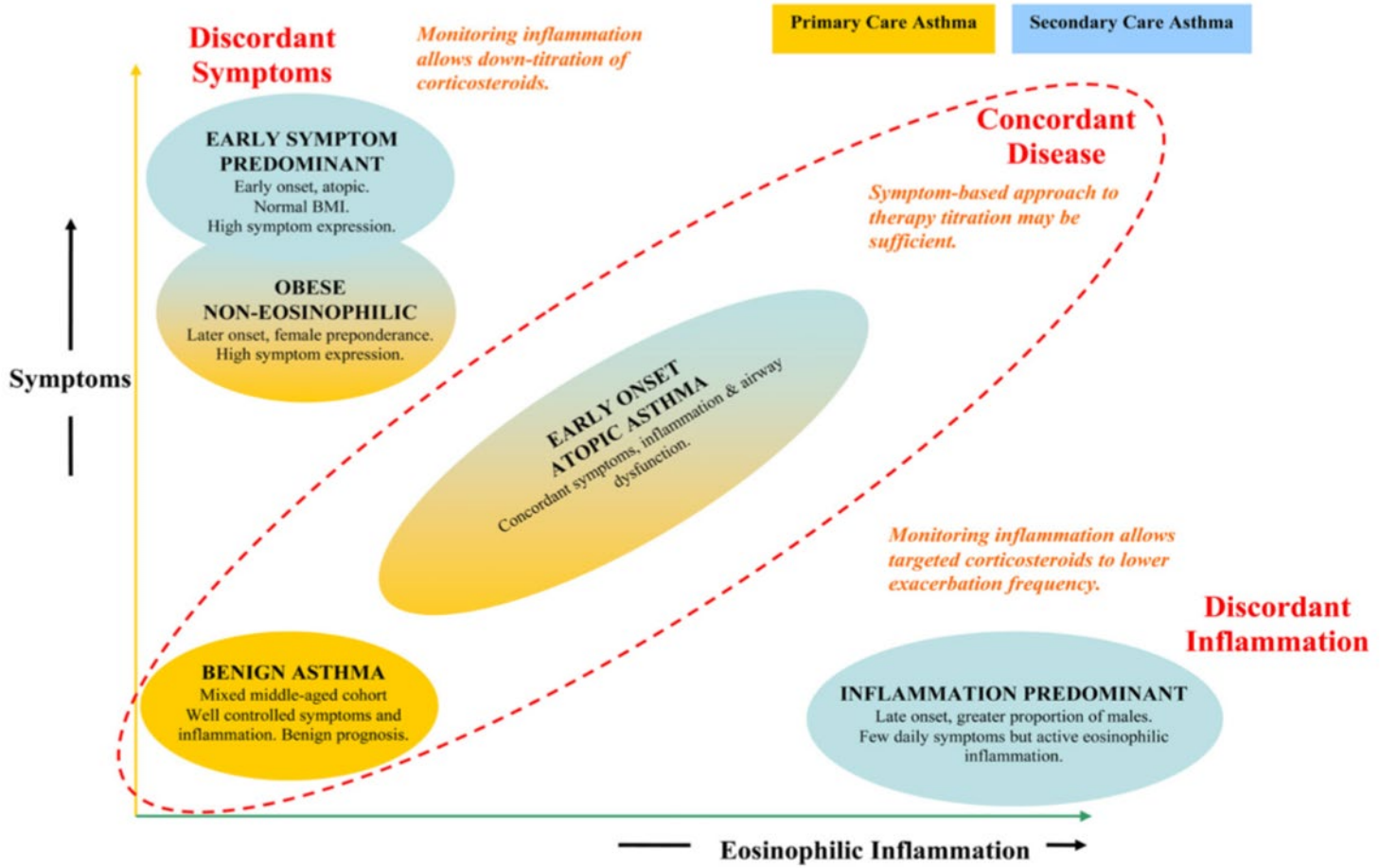

Figure 1.1.2. Clinical phenotypes of asthma.
Clusters of asthmatic patients can be observed when symptoms are correlated with levels of inflammation, such as eosinophilic inflammation. These clusters are then used to inform therapy programmes.
Figure taken from Haldar et al., 2008.

(Gilliland et al., 2006), whilst smoking by adolescents increases their risk of developing asthma (Gilliland et al., 2006).

Viral infections are also associated with asthma onset, particularly rhinovirus and respiratory syncytial virus (RSV) infection in early childhood (Kusel et al., 2007). Furthermore, viral infections are directly linked to ~85% and ~50% of asthma exacerbations in children (Johnston et al., 1995) and adults (Nicholson et al., 1993), respectively. In murine models, viral infections can induce aberrant adaptive immune responses which could predispose the host to asthma (Krishnamoorthy et al., 2012, Kaiko et al., 2013).

A lack of exposure to endotoxin is also associated with asthma risk. 'The Hygiene Hypothesis' was presented (Strachan, 1989, Strachan, 2000), suggesting that an increase in cleanliness or hygiene, along with fewer immediate family members to pass on infection, prevents exposure of individuals to pathogens and prevents the initiation of a T helper 1 cell (Th1)-mediated immune response which is known to inhibit Th2 responses (Romagnani, 1992, Holt, 1994) and so skews the immune system towards a Th2 response profile, predisposing individuals to the development of allergic diseases (Umetsu et al., 2002).

Indeed, the children of farmers have lower risks of atopic sensitization than other children, and those children whose parents farmed full-time as opposed to part time had an even lower risk of this (Braun-Fahrlander et al., 1999). Farming as an occupation could be associated with decreased hygiene and increased exposure to dirt and endotoxin. Not only are children of farmers less likely to develop asthma than those whose parents have very different occupations, but their risk of developing asthma appears to decrease as they interact more with animals (Von Ehrenstein et al., 2000). These data support the suggestion of The Hygiene Hypothesis, whereby the increase in allergic disease incidence could be due to an increase in cleanliness, and the subsequent reduction in exposure to dirt and pathogens.

Furthermore, the 'Old Friends Hypothesis' suggests that an increase in hygiene not only results in decreased exposure to pathogens but also to commensal bacteria that comprise our microbiota, which could impair the development of a healthy and functioning immune system (Rook et al., 2003). Gastrointestinal exposure to the commensals bifidobacteria and lactobacillus is associated with the reduction in the

incidence of allergy (Bjorksten et al., 1999), and the exposure of neonatal children to lactobacillus appears to protect against atopy (Kalliomaki et al., 2001). Additionally, hepatitis A infection is strongly associated with the impairment of asthma development (Matricardi et al., 1997), particularly because the gene for its cellular receptor, *Tim1*, has been identified as a susceptibility gene for asthma and has been linked, not only directly to airway hyperresponsiveness (AHR), but also to the development of Th2 lymphocytes and, therefore, to the development of atopic diseases (McIntire et al., 2001). This hypothesis could also explain why antibiotic use in early life is associated with an increased risk of asthma development (McKeever et al., 2002).

Clearly, asthma is a complex disease whose pathogenesis is linked to many genetic and environmental factors. Only by understanding its risk factors and underlying causes can the limited therapeutic options available be expanded and truly effective treatments be developed to ameliorate the asthmatic condition.

1.1.4.2 The pathophysiology of asthma

The defining pathophysiological feature of asthma is the presence of variable airway obstruction (Harris et al., 1962) characterised by a reduction in FEV1/FVC (Peat et al., 1987, Ulrik and Lange, 1994, Lange et al., 1998, Ranu et al., 2011). However, it has become apparent that this obstruction is often not completely reversible and that, despite clinical remission, some airflow obstruction may persist (Brown et al., 1984, Backman et al., 1997, Vonk et al., 2003, Zhang et al., 2016). AHR [*i.e.* the exaggerated bronchoconstrictor response to direct (methacholine) and indirect (including mannitol or exercise) stimuli] is another key abnormality of asthma (Postma and Kerstjens, 1998). Many pathophysiological features of asthma [including airway smooth muscle (ASM) contraction on a background of AHR, mucous hypersecretion and airway oedema] contribute to airway obstruction and lead to airway narrowing and airflow restriction (Fish and Peters, 1999, Tillie-Leblond et al., 2008, Doeing and Solway, 2013) (figure 1.1.3). Airway inflammation can directly influence many of these features (Flint et al., 1985, Eggleston, 1988, Frick and Busse, 1988, Mehlhop et al., 1997, Thomas et al., 2010), emphasizing the importance of airway inflammation in asthma pathogenesis.

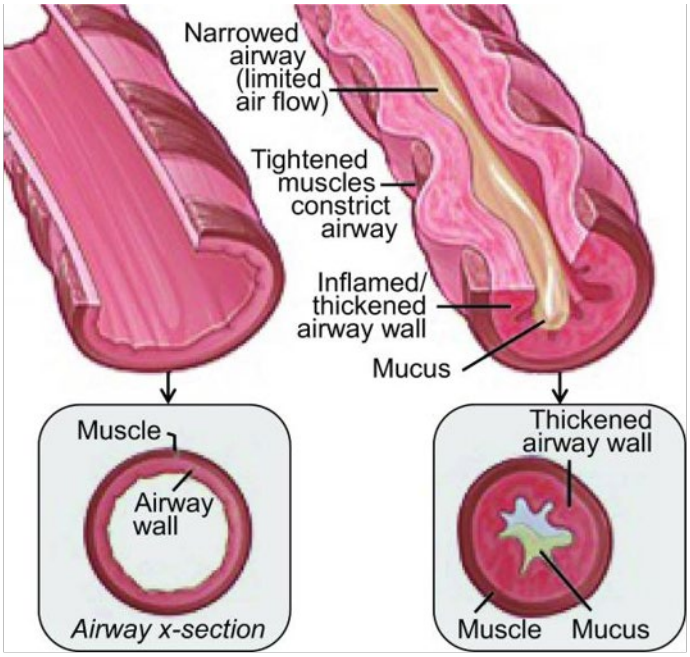

Figure 1.1.3. The pathophysiological features of asthma.
The pathophysiological features of asthma (shown in the right-hand panel) including inflamed and thickened airway walls, hyperresponsive and constricted airway smooth muscle and hypersecretion of mucous, which can lead to airway obstruction when compared to healthy airways (shown in the left-hand panel).
Figure taken from Doeing and Solway, 2013.

1.1.4.2.1 The inflammatory response in asthma pathogenesis

Asthma can have an allergic or a non-allergic basis (Holgate, 2008), yet despite 40% of Western populations being atopic, only ~7% of atopic patients develop asthma (Beasley et al., 2001). Although asthma is linked to atopy, comparing pathological features of asthma from non-atopic and atopic patients demonstrates that inflammation and airway remodeling can occur independently from one another (Turato et al., 2008). In further support of this, patients with eosinophilic bronchitis have a cough but normal airway calibre, accompanied by a severe Th2-driven mucosal eosinophilia (Brightling et al., 2002b, Brightling et al., 2003b). These data suggest that inflammation alone does not drive asthma pathophysiology, but exacerbates the underlying condition.

The epithelium is a natural physical and chemical barrier (Holgate, 2007, Holgate, 2010). However, the epithelium of asthmatic patients is dysfunctional in numerous ways, including defective tight junctions (Holgate, 2007), sensitivity to oxidative stress (Bucchieri et al., 2002) and impaired interferon (IFN) production post-viral infection (IFN-β and IFN-λ) (Wark et al., 2005). This promotes a chronic wound response to common environmental stimuli which can, in turn, exacerbate features of asthma (Holgate, 2000, Nelson et al., 2003, Swindle et al., 2009). Asthma could therefore originate from inappropriate epithelial responses (Holgate, 2000).

Allergen sensitisation involves dendritic cells, professional antigen-presenting cells (von Garnier et al., 2005, Hammad and Lambrecht, 2006). Allergen uptake and presentation by dendritic cells is facilitated by the presence of atopy (Kitamura et al., 2007), and promotes inappropriate Th2 responses in asthma (Hongjia et al., 2014, Vroman et al., 2015). CC chemokine receptor (CCR) 7 and its ligands mediate chemotaxis of dendritic cells to local lymph nodes where antigen presentation to naïve T cells occurs (Humrich et al., 2006, Pease and Williams, 2006) and T cell responses follow.

Two main subclasses of T cells exist, each determined by the expression of the cell surface markers CD8 (cytotoxic) and CD4 (helper). T helper cells can be further categorized into Th1 and Th2 cells which have their own 'killer' and 'allergic' cytokine response profiles, respectively (Berger, 2000). The type of T-cell response induced can be determined by antigen-presenting cells, including dendritic cells and their ability to generate IL-12, which can polarize T cell differentiation in favour of a Th1 response

(Kuipers et al., 2004). After antigen presentation, T cells take a large role in the propagation of immune responses by returning to the site of antigen presentation after sensitisation (Garcia et al., 2005, Kallinich et al., 2005). There, they produce several pro-inflammatory cytokines including the classical Th2 cytokines IL-4, IL-5 and IL-13 (Ying et al., 1995, Berger, 2000, Kay, 2006), and IL-2 which can augment antigen-induced T-cell proliferation and maturation (Anderson, 2002).

Optimal immune responses rely on balanced Th1 and Th2 responses (Berger, 2000). However, there is often a bias towards Th2 responses in patients with asthma (Umetsu and DeKruyff, 1997, Anderson, 2002, Woodruff et al., 2009), and a number of cell types in addition to T cells, including mast cells (Bradding and Arthur, 2016) and eosinophils, contribute to this bias (Barlow and McKenzie, 2014). A Th2 response profile correlates with increased serum IgE levels and eosinophil counts in peripheral blood (Woodruff et al., 2009) and is thought to contribute to atopy (Bellanti, 1998) and the recruitment and activation of immune cells, including mast cells (Burrows et al., 1989, Holgate, 2008).

Mast cells play a key role in asthma pathogenesis (Holgate et al., 1986, Bradding and Arthur, 2016). They produce many mediators including histamine, prostaglandin (PG)D_2, leukotriene C_4, IL-4, IL-5 and IL-13 (Bradding et al., 1994, Bradding et al., 2006, Wang and Lau, 2006). These mediators not only contribute to bronchoconstriction and vasodilation, in addition to AHR and ASM hyperplasia (Howarth et al., 1994, Berger et al., 2001, Bradding et al., 2006), but can modulate dendritic cell function to further propagate Th2 responses (McIlroy et al., 2006, Theiner et al., 2006). In asthmatic patients there is an increase in mast cell numbers in the airway epithelium (Bradding et al., 1994) and ASM bundles (Tomioka et al., 1984, Holgate et al., 1986, Brightling et al., 2002a) which correlates with elevated levels of mast cell-derived mediators (Casale et al., 1987, Wenzel et al., 1988, Hinks et al., 2015). However, it is not just higher mast cell numbers which account for the increase in mast cell-derived mediators as mast cells in asthmatic patients release mediators, including histamine, more readily (Flint et al., 1985). Indeed, mast cells appear chronically activated in asthmatic airways when analysed morphologically under the electron microscope (Bradding et al., 2006).

Mast cells can be activated in several ways including by the activation of toll-like receptors (TLRs) such as TLR3 and TLR4 (Nigo et al., 2006), IL-33 (Allakhverdi et al., 2007)

and the cross-linking of high affinity IgE receptors by allergens (Alber et al., 1992, Pribluda and Metzger, 1992, Turner and Kinet, 1999). These mechanisms demonstrate how innate immune responses to triggers such as viral infection can initiate and/or propagate asthma pathogenesis. After activation, in addition to contributing to airway obstruction by mucous plugging (Carroll et al., 2002a, Carroll et al., 2002b), mast cells infiltrate the ASM (Brightling et al., 2002a, Brightling et al., 2003a) where their mediators, including histamine and IL-13, can induce bronchoconstriction and AHR (Befus, 1987, Risse et al., 2011).

Eosinophils are another key effector cell in asthma pathogenesis (Gleich, 1990, Oddera et al., 1996). Their numbers in the sputum and bronchoalveolar lavage fluid (BAL) of asthmatic patients are raised (Kay, 2005, Lemière et al., 2006) and they produce Th2 cytokines (IL-4, -5 and -13) and cationic proteins such as major basic protein eosinophil cationic protein (Gleich and Adolphson, 1986), in addition to reactive oxygen species (Rothenberg and Hogan, 2006), all of which can cause local tissue damage. Eosinophils have been implicated both in profibrotic would healing responses (Al-Muhsen et al., 2013) and the regulation of ASM proliferation (Halwani et al., 2013), reaffirming their role in the propagation of airway remodeling.

Furthermore, a role for neutrophils in asthma pathogenesis is well documented if not completely understood. In patients with asthma, neutrophils can infiltrate mucous glands (Carroll et al., 2002b) and submucosal tissue (Sur et al., 1993). Moreover, in cases of severe asthma, exacerbations are often associated with mucosal neutrophilia (Qiu et al., 2007) and increased neutrophil numbers in peripheral blood and BAL (Cowburn et al., 2008). In addition to releasing numerous mediators including IL-8 and reactive oxygen species (Monteseirin, 2009), neutrophils produce oncostatin M which plays a role in airway remodeling by inducing the proliferation of ASM cells and fibroblasts (Simpson et al., 2009). From a clinical standpoint, it is important to note that corticosteroids prolong neutrophil survival by preventing their apoptosis (Cox, 1995), leading to persistent neutrophil-mediated inflammation. This also highlights the important role that a maintained immune response has in asthma pathogenesis; the importance is not just the initial recruitment and activation of immune cells.

Clearly the role of inflammation in the pathogenesis of asthma is extremely complex. Regarding it as a whole can be daunting and seem insurmountable. However,

by mapping clinical observations with patterns and trends such as potential triggers and the resulting dominant immune cells in play, the pathogenesis of asthma can be delineated.

1.1.4.2.2 Airway remodeling

The first evidence of structural changes to the airways (i.e. airway remodeling) in asthmatics was recorded in 1922 when asthmatic airway walls were observed to be thicker than those of healthy individuals, and often contained large numbers of infiltrating cells (figure 1.1.3). Furthermore, the airways themselves were often occluded by the presence of mucous plugs (Huber and Koessler, 1922). The airway epithelium was often denuded (Dunnill et al., 1969), whilst both the subepithelial and the ASM layers were thickened (Huber and Koessler, 1922, Dunnill et al., 1969, James et al., 1989).

1.1.4.2.2.1 Epithelial injury and shedding

Epithelial desquamation is a noted feature of asthma and is often observed histologically (Dunnill, 1960, Ordonez et al., 2000). In addition, creola bodies (clumps of epithelial cells) have been found in the sputum of asthmatics (Naylor, 1962, Grootendorst et al., 1997) and epithelial cells have been observed in BAL of asthmatics (Oddera et al., 1996, Grootendorst et al., 1997). Although it has been suggested that the observed desquamation could be caused by mechanical forces produced during the collection and preservation (such as embedding) of samples (Ordonez et al., 2000), dysregulated airway epithelial cell functions are thought to play a key role in asthma pathogenesis (Holgate et al., 2009, Fahy and Locksley, 2011, Holgate, 2011).

Epithelial shedding may exacerbate the underlying pathology further by enhancing sub-epithelial tissue exposure to environmental stimuli (Holgate, 2008, Holgate, 2010).

1.1.4.2.2.2 Mucous hypersecretion

Airway mucous, or airway surface liquid (ASL), is a thin fluid layer around the airways comprising water, salts and macromolecules such as mucins (Rogers, 2004, Fahy and Dickey, 2010). Its primary function is to protect the airways from foreign particles, including pathogens and pollutants, by mucociliary clearance. In this way, airway mucous prevents infection (Knowles and Boucher, 2002, Lillehoj and Kim, 2002).

Mucous hypersecretion refers to the hypersecretion of the mucin components of airway mucous, resulting in mucous with increased viscosity and elasticity that is more difficult to clear from the airways (Cone, 2009, Lai et al., 2009). Elevated mucin levels have been found in asthmatic airways (Aikawa et al., 1992, Sidebotham and Roche, 2003) and in sputum from asthmatics (Fahy et al., 1993, Kirkham et al., 2002, Jinnai et al., 2010). This can result in the formation of mucous plugs which are often observed in asthmatic airways (Huber and Koessler, 1922, Dunnill, 1960, Hogg, 1997).

In the airways, mucins are secreted by goblet cells in the epithelium (Rogers, 2003a) and mucous cells in the submucosal glands (Finkbeiner, 1999). The hypersecretion seen in asthmatic airways can be attributed to both increased mucin production (Young et al., 2007, Zhen et al., 2007) and goblet cell hypertrophy and hyperplasia (Aikawa et al., 1992, Jackson, 2001, Rogers, 2003b).

Mucous hypersecretion not only increases the risk of airway infection (Knowles and Boucher, 2002), but it can be linked to airway obstruction (Agrawal et al., 2007) and declining lung function (Lange et al., 1998, Evans et al., 2009, Thomson et al., 2013). Combined with other features of airway remodeling including AHR and airway wall thickening, mucous secretion contributes to airway narrowing and can ultimately block the airway lumen (James and Carroll, 1995).

1.1.4.2.2.3 ASM dysfunction in asthma

Asthmatic airways are typically hyperresponsive (Burrows et al., 1989, An et al., 2007, Holgate, 2008). Bronchoconstriction and narrowing of asthmatic airways occur in response to a wide range of both specific and non-specific stimuli including allergens (Pepys and Hutchcroft, 1975), cold air (Deal et al., 1980), and known bronchoconstrictors (Curry, 1947). Bronchoconstriction in asthma often occurs at concentrations of stimuli that would not typically induce narrowing in healthy airways (*i.e*. the stimuli have much lower provocation concentrations in asthmatics), and even in response to stimuli that would not contract healthy ASM at all (Boushey, 1982, Sterk, 1995, Bousquet et al., 2000). AHR is associated with airway obstruction (Van Schayck et al., 1991, An et al., 2007) and decreased lung function (Peat et al., 1987, Redline et al., 1989, Van Schayck et al., 1991, Sont et al., 1999).

An increase in ASM mass has been a cardinal feature of asthma for many years (Huber and Koessler, 1922, Dunnill et al., 1969, James et al., 2009) and, indeed, thicker

ASM in asthmatics correlates with a greater severity of the disease (Benayoun et al., 2003, James et al., 2009). One mathematical model suggests that increased ASM mass could increase the maximal force generated by the ASM (Lambert et al., 1993), which could therefore contribute to AHR and airflow obstruction.

The observed increase in ASM mass in asthma is thought to be a combination of numerous factors including increased extracellular matrix deposition (Hoshino et al., 1998, James et al., 2012) and the influx of cells, including mast cells, myofibroblasts, and fibrocytes which can migrate into ASM bundles (Gizycki et al., 1997, Sutcliffe et al., 2006, Saunders et al., 2009b). An increase in ASM cell survival (Martin and Ramos-Barbón, 2003) and their hypertrophy and hyperplasia are also implicated in the increase in ASM mass (Ebina et al., 1990, James et al., 2012). ASM cells from asthmatics have been found to intrinsically proliferate more than those from healthy individuals (Johnson et al., 2001), further supporting the contribution of hyperplasia in ASM thickening, particularly as ASM cells isolated from healthy donors proliferated more upon exposure to BAL from asthmatic patients (Naureckas et al., 1999). ASM hyperplasia in asthma could also explain the increase in ASM nuclei (Heard and Hossain, 1973) and cell number (Woodruff et al., 2004) observed in asthmatic ASM. Evidence suggests that, in large airways, hypertrophy of ASM cells is more prevalent, whilst hyperplasia of ASM dominates in smaller airways (Ebina et al., 1993, James et al., 2012). However, evidence points strongly against ongoing ASM proliferation in asthmatic airways (Benayoun et al., 2003, Woodruff et al., 2004, Begueret et al., 2007, Ward et al., 2008).

ASM dysfunction in asthma has been associated with inflammation. For example, inflammation enhances AHR (Eggleston, 1988, Frick and Busse, 1988) and the prolonged use of corticosteroids typically ameliorates AHR independently of the duration of asthma (Juniper et al., 1990, Sont et al., 1999, Boulet et al., 2000). It is thought that whilst the inflammatory response may play some role in initiating AHR, the inflammatory response itself is not sufficient, or necessary, for AHR development (Brusasco et al., 1998, Crimi et al., 1998, Brightling et al., 2002b, Brightling et al., 2003b). However, there is still conflicting evidence regarding the associations between airway wall structural remodeling and AHR (Siddiqui et al, 2008). It is possible that the variable results observed between studies could be due to the AHR being caused by different underlying mechanisms.

Many studies have investigated the effect of increased airway remodeling and ASM mass on the *in vivo* functions of ASM. The general consensus is that increased ASM mass results in increased force (James et al., 1989, Lambert et al., 1993). However, several studies have investigated the possibility that ASM cells from asthmatic and healthy airways differ at a cellular or even molecular level. ASM cells from asthmatic subjects are known to shorten (Ma et al., 2002) and contract (Matsumoto et al., 2007, Sutcliffe et al., 2012) to a greater extent than those from healthy individuals. Therefore, there could be differences in the contractile apparatus of the cells, or in regulatory aspects of ASM cells contraction. The comparison of ASM cells from asthmatic and healthy airways has typically found no differences in the expression of contractile proteins, including α-smooth muscle actin and myosin heavy chain isoforms (Benayoun et al., 2003, Woodruff et al., 2004). However, increases in the expression of positive regulators of cell contraction have been observed. For example, the transcript and protein expression of myosin light chain kinase (MLCK) (see section 1.2.4.2) is increased ASM cells from humans (HASM) and canines that had been sensitized (Kong et al., 1990, Ammit et al., 2000), in addition to ASM cells from asthmatic airways compared to those from healthy airways (Ma et al., 2002, Tillie-Leblond et al., 2008). However, some groups observed no differences in MLCK expression when comparing ASM cells from healthy and asthmatic subjects, although the methodologies and sample groups used in these papers could explain the lack of difference (Woodruff et al., 2004, Matsumoto et al., 2007).

It is evident that ASM is dysfunctional in asthmatic subjects, yet the literature is unclear on the causes of this dysfunction. It is most likely that different underlying mechanisms for ASM dysfunction in asthma exist and could contribute to contradicting data in the literature. ASM biology is discussed in more detail in section 1.2.

1.1.5 The treatment of asthma

The prevalence of asthma is growing (GINA, 2017), with the largest increase seen in industrialized countries (Pearce et al., 2007). Due to this, the burden of asthma on society and healthcare systems is rising. In 2011, the total estimated economic burden of asthma was €72.2 billion across Europe (Loddenkemper et al., 2013). It is clear that new measurements must be taken to reduce this burden.

Due to the lack of a cure for asthma, its treatment is intended to achieve control of symptoms and to prevent future exacerbations. Treatments are given in a step-wise manner depending on the severity and control of the observed symptoms (White et al., 2018). Firstly, patients are given short-acting β_2-agonist (SABA) reliever inhalers, such as salbutamol, for the relief of infrequent symptoms and use during an exacerbation by facilitating ASM relaxation (1997, GINA, 2017). For those who suffer more frequent symptoms or give any history of severe exacerbations, low doses of inhaled corticosteroids (ICS) are given and need to be used regularly (O'Byrne et al., 2001, Pauwels et al., 2003). ICS treatment suppresses inflammation, indirectly improving lung function (Adams et al., 1999) and reducing mortality rates from asthma (Suissa et al., 2000, Neffen et al., 2006). If symptoms persist, a long-acting β_2-agonist (LABA) is added to ICS treatment (Ducharme et al., 2010, GINA, 2017). Finally, patients are referred to an asthma specialist for alternative treatments if necessary (Chung and Adcock, 2004). Increases in severity can be an indicator that patients are not adhering to prescribed treatments and practices, and reaffirming these is recommended to reduce exacerbation rates and disease severity (de Marco et al., 2005). Alternative treatments are given depending on patient suitability. For example, adult patients with a history of exacerbations can be given long-acting muscarinic antagonists, such as tiotropium (Rodrigo and Castro-Rodríguez, 2015).

Although the above-prescribed treatments often aim to reduce inflammation, further modulation of the immune response is associated with improved asthma symptoms. For instance, long-term ICS use is associated with improved AHR (Juniper et al., 1990). Atopic patients with a prominent allergic disease component can benefit from allergen immunotherapy, where attempts to modify inappropriate immune responses to common allergens are made by injecting allergens subcutaneously (Abramson et al., 2010). However, this is not used in the UK as there is a high risk of anaphylaxis during treatment and the results are mixed. Furthermore, targeting an IgE-mediated response with humanized anti-IgE monoclonal antibodies, including Omalizumab (Corne et al., 1997), can neutralize aberrant allergic responses and decrease the rate of severe exacerbations (Humbert et al., 2005). Mepolizumab is an anti-IL-5 treatment which has shown promise by decreasing eosinophil counts in blood and sputum and reducing severe exacerbations (Haldar et al., 2009). However, Mepolizumab has limited effect on

AHR (Nair et al., 2009), and any observed benefits in clinical trials were lost within 3 months of treatment cessation (Haldar et al., 2009). Additionally, anti-IL-13 antibodies such as including Lebrikizumab (Corren et al., 2011) and Tralokinumab (Antohe et al., 2013) have been shown to increase lung function in asthmatic subjects.

Despite the above descriptions, asthma treatments are limited. No cure for asthma is known, and there is a subset of patients with severe asthma that do not respond well to ICS treatment (Chung et al., 1999a). Until the underlying mechanisms contributing to each asthma endotype are completely understood, the best approach for treating asthma is arguably to treat specific, problematic features of asthma in addition to the typical therapies described above. For example, if a patient was observed to have a particularly high increase in ASM mass, in addition to AHR, bronchial thermoplasty could be used to decrease this AHR for up to 2 years (Cox et al., 2006). It is undeniable that the future of asthma therapeutics lies in the personalisation of treatments, either to individual patients or clusters of patients, by determining their specific endotype(s) of asthma and treating the underlying mechanisms (Chung et al., 1999a, Hartley et al., 2014).

1.2 Airway Smooth Muscle biology

1.2.1 The origins of ASM

The origins of ASM cells have not yet been fully elucidated, although data suggests that embryonic ASM cells differentiate from cells in the early embryonic lung mesenchyme, particularly as local and circulating mesenchymal progenitor cells have been observed to mature into smooth muscle cells *in vitro* (Simper et al., 2002, Kobayashi et al., 2004, Summer et al., 2007, Murphy et al., 2008). Due to their phenotypic plasticity (see section 1.2.2), smooth muscle cells (including ASM cells) are not considered terminally differentiated (Halayko and Solway, 2001).

1.2.2 The physiological roles of ASM in healthy airways

The primary function of ASM in healthy airways has long been considered to be the regulation of bronchomotor tone via contraction and relaxation (Einthoven, 1892, Dixon and Brodie, 1903). However, the physiological relevance of ASM to healthy airways is largely unknown. ASM is even considered to be vestigial, or the 'appendix of the lung', due to a lack of definitive function (Mitzner, 2004). Some have therefore argued that ASM is not required in the airways, particularly as there are no known disease states associated with the loss of ASM (Seow and Fredberg, 2001). However, due to the complexity of ASM, it has long been considered to have functional importance (Macklin, 1929). To this end, numerous possible functions of ASM in healthy airways have been suggested (Mitzner, 2004, Janssen, 2012).

As the primary ASM function is suspected to be to regulate bronchomotor tone, it would follow that ASM contraction/peristalsis aids exhalation. This peristaltic wave was thought to begin in the peripheral airways (Miller, 1921, Macklin, 1929). This idea is still relevant today and research suggests that ASM, and its rhythmic/phasic constrictions (Lewis, 1924), facilitates gaseous exchange by reducing expiratory flow and anatomical dead space (Kondo et al., 2003). ASM could also contribute to pulmonary function through the regulation of the mechanics of coughing by increasing airflow velocity (Leith, 1977). This would also increase the airflow velocity at flow-limiting segments, supporting mucous clearance indirectly (by coughing) (Mead et al., 1967) and indirectly (by peristalsis) (Bullowa and Gottlieb, 1922). However, the mechanism of mucous clearance by peristalsis has been disputed (Widdicombe, 1963),

and it is presumed that the primary route of mucous clearance is mucociliary (Fleischner, 1949), although more recent data using foetal lung explants has observed the apparent displacement of lung fluids by spontaneous peristaltic airway contractions (Schittny et al., 2000). This could lend more support to the idea that prenatal and postnatal ASM behave differently, as contractions tend to be phasic in the former and tonic in the latter, and that perhaps ASM presence and function is more important prenatally than postnatally (Schittny et al., 2000).

The numerous putative roles of ASM are not limited to physical processes such as contraction/relaxation. ASM is now known to have secretory functions, and can therefore modulate inflammatory responses by secreting inflammatory mediators, including those produced via lipid metabolism such as PGE_2 (Belvisi et al., 1997, Pang and Knox, 1997). Following stimulation by inflammatory mediators such as tumour necrosis factor (TNF)-α, cultured HASM cells (cHASM cells) release chemoattractants for immune cells including eotaxin (Chung et al., 1999b) and RANTES (Berkman et al., 1996, John et al., 1997), both of which are chemokines for eosinophils (Alam et al., 1993, Griffiths-Johnson et al., 1993, Jose et al., 1994, Garcia-Zepeda et al., 1996, Venge et al., 1996). RANTES is also a chemokine for neutrophils, monocytes and memory T-cells (Schall et al., 1990, Venge et al., 1996). Stimulation of ASM cells with IFN-γ also induces the production of the chemokine CXCL10, which regulates mast cell migration (Hardaker et al., 2004, Brightling et al., 2005).

Moreover, in addition to attracting immune cells, ASM cells can release pro-inflammatory mediators *per se* following stimulation, including GM-CSF (Saunders et al., 2009a), IL-1β and IL-6 (Hedges et al., 2000), IL-8 (John et al., 1998, Pang and Knox, 1998, Hedges et al., 2000) and IL-11 (Elias et al., 1997). Subsequently, stimulation of ASM cells *in vivo* can propagate local inflammatory responses both through the direct release of pro-inflammatory cytokines and via the recruitment of numerous immune cell types (see figure 1.2.1), and can therefore have drastic implications for inflammatory airway diseases (Halayko and Amrani, 2003, Hirst, 2003, Oliver and Black, 2006).

The physiological importance of ASM becomes particularly noticeable in disease states such as asthma (see section 1.1.4.2.2.3), where the role of ASM changes (see figure 1.2.2). This modulation of ASM behaviour is, at least in part, due to its remarkable degree of phenotypic plasticity which was first noted in canine ASM cells. In culture they

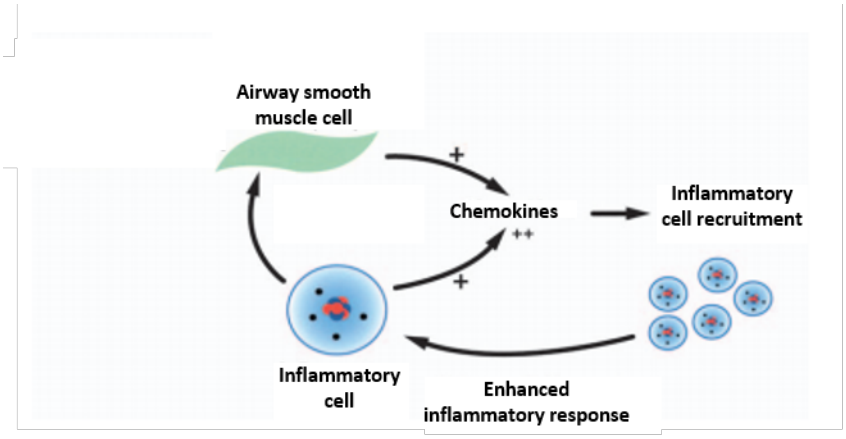

Figure 1.2.1. The role of ASM cells in the propagation of the inflammatory response in asthma.
Activated ASM cells release inflammatory mediators, such as cytokines, which recruit immune cells and propagate the local inflammatory response in the airways.
Figure modified from Howarth et al., 2004.

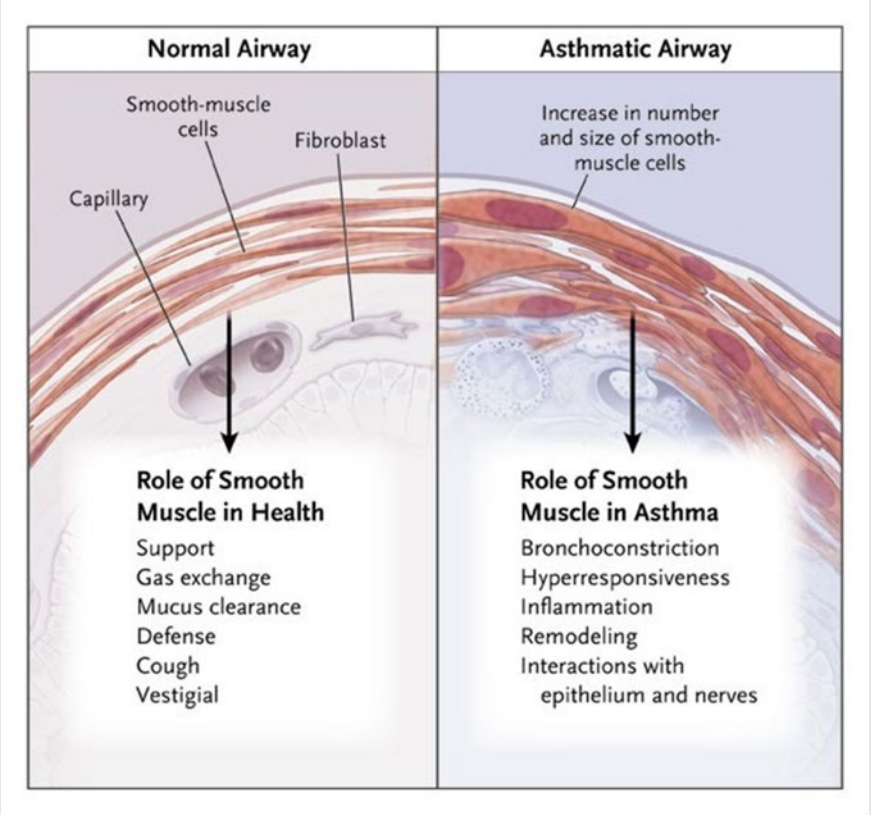

Figure 1.2.2. The physiological and pathophysiological roles of ASM in health and asthma.

An asthmatic phenotype (right-hand panel) not only changes the airway structure, but also the recognised roles of ASM when compared to healthy airways (left-hand panel). Figure taken from Solway and Irvin, 2007.

became proliferative after ~5 days and, from then, the expression of contractile markers including α-smooth muscle actin, smooth muscle heavy chain and desmin, were reduced (Halayko et al., 1996). When cell confluence was reached, the expression of these markers was restored. It is now generally accepted that ASM cells are plastic in relation to their mechanical, secretory and proliferative phenotypes (Halayko and Solway, 2001, Amrani and Panettieri, 2003). For example, the contractile apparatus of ASM is known to change in response to media serum content and seeding density in cultures of rat ASM cells (Wong et al., 1998). In addition, a change in the local constitution of inflammatory mediators can regulate the release of mediators by ASM, further increasing the plasticity of ASM cells in response to their environment (Chung, 2000).

It is evident, therefore, that ASM is a very important component of the airway wall, particularly when considered in the context of inflammatory airway diseases such as asthma. The main foci in relation to ASM dysfunction tend to be contraction (see section 1.2.4) and proliferation (see section 1.2.6). However, both processes are heavily regulated by the concentration of calcium ions (Ca^{2+}) in the cytoplasm. Therefore, the regulation of Ca^{2+} flux in smooth muscle will be described first.

1.2.3 Calcium flux in smooth muscle

In smooth muscle generally, Ca^{2+} enters the cytoplasm either by influx from external solutions or by Ca^{2+} release from intracellular stores, via pathways summarised in figure 1.2.3. The resulting Ca^{2+} flux can increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in smooth muscle cells from a resting concentration of ~100 nM to ~1 μM, which is sufficient to induce physiological effects in the cell (Williams et al., 1985, Sommerville and Hartshorne, 1986, Williams and Fay, 1986, Bootman, 2012). In ASM specifically, increases in $[Ca^{2+}]_i$ typically occur in oscillations (Prakash et al., 1997, Perez and Sanderson, 2005).

As discussed previously, ASM in asthma is dysfunctional and it is plausible that, as ASM processes are so heavily dependent on $[Ca^{2+}]_i$, dysregulation of Ca^{2+} flux could result in tissue dysfunction. To this end, the mechanisms behind $[Ca^{2+}]_i$ rises will be discussed next.

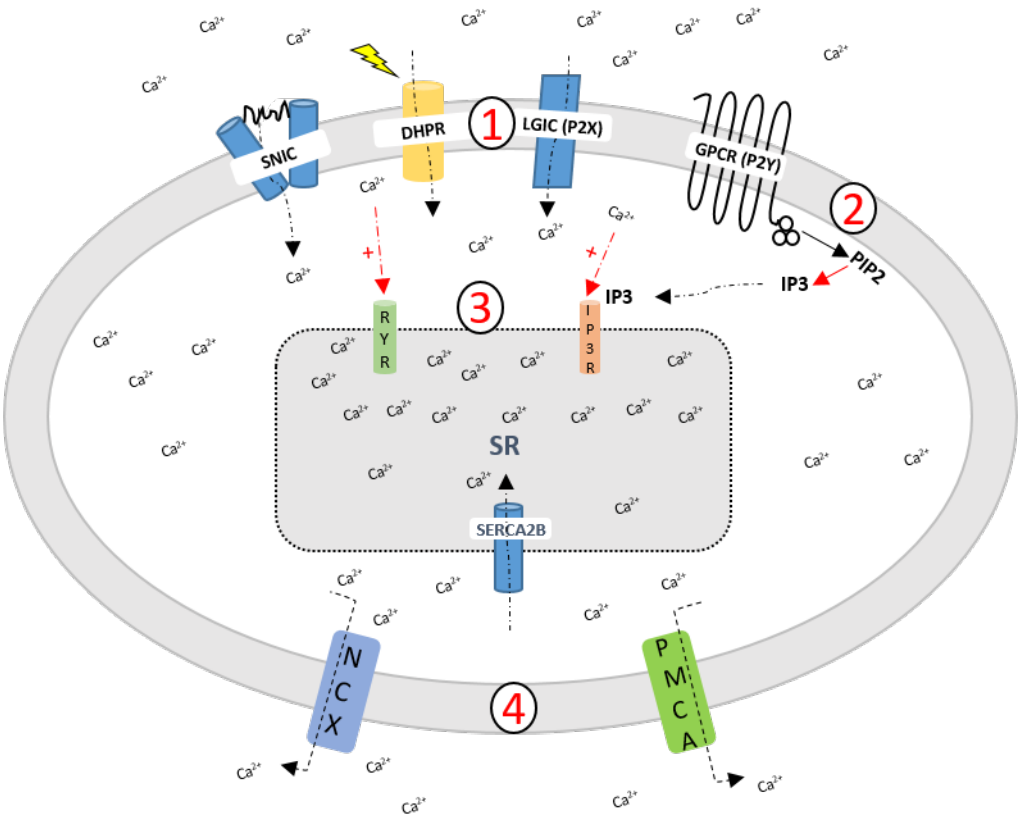

Figure 1.2.3. The pathways mediating cytosolic calcium changes in smooth muscle cells.

The calcium signalling pathways in smooth muscle cells are summarised. 1) The activation of cell surface calcium channels including stretch-sensitive non-selective ion channels (SNIC), the L-type voltage-gated calcium channel (DHPR) and the ligand-gated ion channels (LGIC) such as P2X receptors, allows Ca^{2+} influx into the cell. 2) The activation of GPCRs such as P2Y receptors induces the activation of their associated G proteins. Gq-coupled GPCRs activate PLC to cleave PIP2 and produce IP3. IP3 then binds to the IP3 receptor (IP3R). 3) Ryanodine receptors (RYR) and IP3Rs are positively modulated by Ca^{2+}, activating and releasing Ca^{2+} from the sarcoplasmic reticulum (SR) into the cytoplasm. 4) The SERCA2B pump in smooth muscle pumps Ca^{2+} back into the SR, whilst the sodium/calcium exchanger (NCX) and the plasma membrane calcium-ATPase (PMCA) pump Ca^{2+} out of the cell.

1.2.3.1 Routes of calcium influx

Ca^{2+} influx can be triggered in many different cell types by numerous stimuli. For example, The Bayliss effect demonstrated that stretching of muscles induced contraction (Bayliss, 1902), and this was confirmed to be mediated by stretch-sensitive non-selective ion channels (Guharay and Sachs, 1984, Sigurdson et al., 1992). Although these preliminary data were obtained using chick skeletal and cardiac muscle, similar stretch-sensitive channels have also been found in the smooth muscles of toad stomach (Kirber et al., 1988) and guinea pig urinary bladder (Wellner and Isenberg, 1993, Wellner and Isenberg, 1994), and even in cHASM cells (Ito et al., 2008). Although it has been further confirmed that stretching of smooth muscle can initiate contraction (Himpens and Somlyo, 1988), the exact mechanism is not understood. A candidate stretch-sensitive receptor of the transient receptor potential vanilloid (TRPV) channel family has been suggested in cHASM cells (Ito et al., 2008). Nevertheless, the attenuation of ASM contraction by the use of a stretch-sensitive ion channel inhibitor, Gd^{3+} (Ito et al., 2006), demonstrates the importance of these channels in ASM physiology.

The activation of ligand-gated ion channels can also trigger Ca^{2+} influx into smooth muscle. For example, members of the P2X receptor family, which are activated by the extracellular binding of the nucleotide adenosine-5'-triphosphate (ATP) (Burnstock, 1978, Burnstock and Kennedy, 1985, North, 2002), have been directly implicated in ASM function (Mounkaila et al., 2005, Oguma et al., 2007, Nagaoka et al., 2009). Furthermore, the activation of P2X4 receptor specifically has been shown to induce contraction in porcine ASM (Nagaoka et al., 2009).

Physiologically, the influx of Ca^{2+} into the cell due to the activation of stretch-sensitive and ligand-gated ion channels can induce membrane depolarisation and activate voltage-gated ion channels, (Davis et al., 1992, Hamill and Martinac, 2001, Povstyan et al., 2011), of which there are several types (Lodish et al., 2000). Due to the important physiological roles of Ca^{2+}, the voltage-gated Ca^{2+} channels (VGCCs) are typically more important here (Catterall, 2011). There are 6 subtypes of these VGCCs including L-, N-, P-, Q-, R- and T-types (Karaki et al., 1997, Catterall, 2011). However, the L-type VGCC, also known as the dihydropyridine receptor (DHPR), is the most important concerning muscle contraction (Reuter, 1979), and particularly that of smooth muscle (Ganitkevich and Isenberg, 1991, Vogalis et al., 1991, Karaki et al., 1997, Knot and

Nelson, 1998). It follows that L-type VGCCs are expressed in ASM specifically, and are the main functional VGCC in this tissue too (Janssen, 2002). The resting membrane potential of smooth muscle cells is typically between -50 mV and -40 mV (Fleischmann et al., 1993, Xiansheng et al., 2003, Kuo and Ehrlich, 2015) and depolarisation of the membrane opens the channel. However, this resting membrane potential lies between the activation and inactivation potentials for L-type VGCCs, and so a small population of these channels is constitutively open (Kuo and Ehrlich, 2015). L-type VGCCs also have an additional level of regulation beyond simple membrane potential changes; they are characterized by Ca^{2+}-mediated inactivation, adding a negative-feedback loop to their function (Ganitkevich and Isenberg, 1991).

1.2.3.2 Calcium release from intracellular stores

Ca^{2+} influx is not the only mechanism by which the $[Ca^{2+}]_i$ can be increased. A large pool of Ca^{2+} at ~1 mM is stored in the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR) of striated and smooth muscle cells, for example (Somlyo and Somlyo, 1971, Shannon and Bers, 1997, Wray and Burdyga, 2010). In various cell types, Ca^{2+} can be released from these intracellular stores into the cytoplasm by the activation of one of two (or both) intracellular receptors which are expressed on the sarcoplasmic reticulum: ryanodine receptors (RyR) (Inui et al., 1987, Lai et al., 1988, Otsu et al., 1990) and inositol trisphosphate (IP_3) receptors (IP3R) (Nixon et al., 1994), respectively. Ca^{2+} flux through these receptors can either lead to global changes in Ca^{2+} concentration within the cell, or to (spontaneous transient) local Ca^{2+} concentration changes, which are also known as Ca^{2+} sparks (Zucchi and Ronca-Testoni, 1997, Bolton, 2006).

1.2.3.2.1 RYR-mediated calcium release from the SR in smooth muscle

It was first proposed as early as 1965 that Ca^{2+} influx could induce further release of Ca^{2+} from intracellular stores (Weiss and Bianchi, 1965, Bianchi and Bolton, 1966), and evidence supporting this was later published (Endo, 1968, Ford and Podolsky, 1970, Zucchi and Ronca-Testoni, 1997), suggesting that Ca^{2+} influx through VGCCs activates RYRs on the SR.

RYRs are large, homotetrameric ion channels (Inui et al., 1987, Lai et al., 1988). There are 3 isoforms (RYR1, RYR2 and RYR3) found in mammals and their expression is

thought to be tissue-specific. RYR1 and RYR2 are found primarily in skeletal and cardiac muscle, respectively, whilst RYR3 is more widely expressed (Marks et al., 1989, Nakai et al., 1990, Otsu et al., 1990, Zorzato et al., 1990, Ledbetter et al., 1994, Giannini et al., 1995, Zucchi and Ronca-Testoni, 1997). RYR3 is also expressed in smooth muscle (Giannini et al., 1995, Mironneau et al., 2002). Concerning ASM specifically, all 3 RYR isoforms are expressed in murine ASM (Du et al., 2005), whilst only RYR1 and RYR2 are expressed in rat ASM (Du et al., 2006). Although RYR2 is thought to be the dominant isoform expressed in (porcine tracheal) smooth muscle (Kannan et al., 1997), only RYR3 appears to be expressed in HASM (Hyvelin et al., 2000). Further data using freshly isolated ASM cells from mouse trachea actually suggests that RYR1 and RYR2 are mainly expressed in the SR membrane, whilst RYR3 is typically expressed around the nucleus (Lifshitz et al., 2011). Therefore, differential expression and localisation between muscle types, and even between the same tissues from different species, could account for functional differences.

RYR activation leads to the release of Ca^{2+} from the SR into the cytoplasm (Zucchi and Ronca-Testoni, 1997, Lanner et al., 2010). The mechanisms through which RYR-mediated Ca^{2+} release occurs were primarily investigated using striated muscle. Evidence for the same mechanisms in smooth muscle cells is conflicting, which could be due to the differential tissue expression of RYRs. However, the 2 mechanisms which were proposed in striated muscle, involving either direct or indirect contact with RyRs, will be discussed here.

Firstly, it has been proposed that there is a direct interaction, or a mechanical coupling, between L-type VGCCs on the sarcolemmal membrane (the cell membrane of a muscle cells) and RYRs on the SR membrane. Mutation of the DHPR abolished excitation-contraction coupling in skeletal and cardiac muscles while its restoration to the cell via complementary DNA (cDNA) injection reversed the effects (Beam et al., 1986, Tanabe et al., 1988, Tanabe et al., 1990a, Tanabe et al., 1990b). Morphological (Block et al., 1988) and biochemical (Marty et al., 1994) data have since corroborated this idea of a mechanical coupling between DHPR and RYRs. If not associated directly, a linker protein such as triadin could physically couple the DHPR and RYR (Brandt et al., 1990, Kim et al., 1990). Evidence for a similar physical coupling in ASM cells, specifically, has also been presented (Du et al., 2006).

Secondly, it is logical that the activity of RYRs is modulated by Ca^{2+} to establish feedback mechanisms. It has been discerned that a $[Ca^{2+}]_i$ of ~10 μM opens RYRs (Ma et al., 1988), and the EC_{50} (the concentration of a compound which gives 50% of a maximal response) of RYRs has been estimated at ~2 μM in physiologically-relevant buffers (Meissner et al., 1986). Therefore, as the $[Ca^{2+}]_i$ increases (due to the opening of ligand-gated or voltage-gated ion channels for example), RYRs open and augment this increase. This is Ca^{2+}-induced Ca^{2+} release (CICR), which is an indirect positive-feedback mechanisms that, physiologically, works to potentiate a small increase in $[Ca^{2+}]_i$ (Endo, 1977, Fill and Copello, 2002). It is of physiological importance, therefore, that there is also a negative-feedback mechanism. Indeed, RYRs appear to be inhibited by Ca^{2+} concentrations ~250 μM (Ma et al., 1988), with estimated IC_{50} values (the concentration of a compound that gives 50% of maximal inhibition of a response) of ~120-150 μM (Meissner et al., 1986, Nagasaki and Kasai, 1983). However, others have suggested that RYRs are only inhibited by Ca^{2+} concentrations of 1 mM or higher (Meissner, 1994). Furthermore, global $[Ca^{2+}]_i$ of even 10-20 μM have been shown to inactivate L-type VGCCs, removing the stimulus of CICR in an additional layer of regulation (Romanin et al., 1992, Haack and Rosenberg, 1994, Sham et al., 1995).

The precise mechanisms of CICR and its physiological relevance to different muscle types is still not fully determined. For example, membrane depolarisation of cardiac myocytes opens VGCCs, inducing Ca^{2+} influx and the subsequent activation of closely associated RYR2 receptors, suggesting that CICR is the main contributor to RYR-mediated Ca^{2+} release in these cells (Fabiato, 1983, Cleemann and Morad, 1991, Bers, 2002, Fill and Copello, 2002). However, in skeletal muscle, although membrane depolarisation does also activate Ca^{2+} influx and RYR-mediated Ca^{2+} release, data suggests that Ca^{2+} influx is not essential for this process and so perhaps the mechanical coupling is a more physiologically relevant mechanism in this muscle type (Frank, 1960, Lüttgau, 1963, Armstrong et al., 1972, Fill and Copello, 2002).

As mentioned, evidence for the existence and exact mechanisms of CICR in smooth muscle is confusing. CICR is thought to exist as a mechanism in smooth muscle through loose coupling of RYRs to VGCCs (Ganitkevich and Isenberg, 1995, Collier et al., 2000, Kotlikoff, 2003), and Ca^{2+} influx via L-type VGCCs appears to activate CICR in

smooth muscle from both guinea pig and rat (Ito et al., 1991, Ganitkevich and Isenberg, 1992, Gregoire et al., 1993). Although, recent evidence has suggested that CICR mediates the relaxation of smooth muscle and not its contraction (Nelson et al., 1995, Perez et al., 1999, Jaggar et al., 2000). Confusion regarding these data in smooth muscle could be due to differences in expression and localisation of RYRs, as described above. For now, the existence and mechanisms of CICR in ASM must be inferred from data established in other smooth muscle types, until a time when they have been fully elucidated in ASM. However, it appears safe to conclude that CICR does contribute to Ca^{2+} changes in ASM cells, although to what extent is unknown.

1.2.3.2.2 IP_3-induced calcium release in smooth muscle

IP_3 was first shown to induce Ca^{2+} release from the ER in 1983 (Streb et al., 1983). IP_3 production is typically mediated by the activation of Gq-coupled G-protein coupled receptors (GPCRs) which are functionally coupled to phospholipase C (PLC), an enzyme involved in lipid metabolism (Berridge et al., 2000, Bootman et al., 2001). Upon activation, PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to its second-messenger products diacylglycerol (DAG) and IP_3 (Putney and Bird, 1993, Foskett et al., 2007, Berridge, 2009). IP_3 is the endogenous ligand for IP3Rs (Foskett et al., 2007, Berridge, 2009) which, as described, are intracellular ligand-gated Ca^{2+} channels expressed on the SR membrane (Nixon et al., 1994). IP3Rs are similar to RYRs in that they are also modulated by Ca^{2+} and, as such, are implicated in CICR where $[Ca^{2+}]_i$ < 300 nM induce positive feedback, whilst those above 300 nM activate the negative feedback mechanism (Iino, 1990, Bezprozvanny et al., 1991, Finch et al., 1991, Adkins and Taylor, 1999). However, IP3Rs are different from RYRs in that they require the presence of both IP_3 and Ca^{2+} in order to be activated, and not just Ca^{2+} (Finch et al., 1991, Marchant and Taylor, 1997, Adkins and Taylor, 1999).

There are 3 known isoforms of IP3Rs: IP3R1, IP3R2, and IP3R3 (Sudhof et al., 1991, Ross et al., 1992, Blondel et al., 1993, De Smedt et al., 1994, Maranto, 1994, Foskett et al., 2007). Each is encoded by a different gene and, typically, are ubiquitously expressed in all mammalian cell types, with overlapping expression in numerous tissues (Foskett et al., 2007). Despite the wide expression of the 3 isoforms, differential patterns of expression with varied ratios of expressed isoforms could lead to differential functional contributions by IP3Rs to Ca^{2+} signalling in each cell type.

All IP3R isoforms are expressed in smooth muscle but, again, there are expression differences between smooth muscle types (Foskett et al., 2007). IP3R1 is the predominant isoform expressed in vascular smooth muscle, including those from thoracic aorta (Islam et al., 1996, Wang et al., 2001, Morel et al., 2003, Grayson et al., 2004, Zhou et al., 2008). Furthermore, IP3R2 and IP3R3 expression levels were found to be threefold higher in proliferating cultures of aortic smooth muscle cells than in isolated tissue (Tasker et al., 2000). Indeed, IP3R3 expression is implicated in the regulation of cell development and proliferation (Tasker et al., 1999, Tasker et al., 2000), suggesting that differential isoform expression could be due to the nature of the tissue and its differentiation state. All 3 isoforms are expressed in equine tracheal ASM (Wang et al., 2004).

These data suggest that all 3 isoforms are expressed in smooth muscle cells, and in ASM cells particularly, and therefore could contribute to Ca^{2+} flux in ASM. Indeed, IP_3-mediated Ca^{2+} release is thought to be the main intracellular store release mechanism in smooth muscle cells (Chalmers et al., 2007, Bai et al., 2009).

Only by studying the different mechanisms of Ca^{2+} flux (influx into the cell or release from intracellular stores) at the cellular level can an appreciation of the complex interplay that occurs be gained. For example, the activation of ligand-gated ion channels, such as P2X receptors, or stretch-activated channels can activate voltage-dependent channels by causing membrane depolarization (Davis et al., 1992, Hamill and Martinac, 2001), which could then activate CICR as discussed above. This mechanism could then be potentiated even further as the mechanical stretching of cHASM cells has been shown to release ATP (Takahara et al., 2014), which could possibly activate other P2 receptors. Therefore, it is vital to understand these mechanisms by which the $[Ca^{2+}]_i$ can be increased.

1.2.4 Smooth muscle contraction

In order to understand how the contraction of ASM can contribute to disease pathogenesis, it must first be understood how this contraction is triggered, signalled into and effected by the cell. To this end, this section will discuss the triggers of and mechanisms behind ASM contraction.

1.2.4.1 Regulation of ASM resting tone

Unlike skeletal muscle, cardiac and smooth muscle are not under the voluntary control of the somatic nervous system, but are regulated by the autonomic nervous system. In fact, the airways are highly innervated and the parasympathetic nervous system (PNS) is a large contributor to bronchoconstriction (Barnes, 1992). Of the PNS, it is mostly cholinergic innervation that regulates basal airway tone (Widdicombe, 1963) by activating post-junctional M3 muscarinic receptors at neuromuscular junctions (Roffel et al., 1990, Eglen et al., 1996). Sympathetic innervation of human lungs is thought to be sparse and functional evidence regarding the regulation of ASM tone by these nerves is lacking (Barnes, 2012). However, afferent (sensory) nerves are found in human lungs and release mediators, such as substance P, which contract ASM (Corson et al., 1990, Barnes, 2012). Indeed, airway innervation and substance P expression is increased in moderate persistent asthmatics compared to both mild persistent asthmatics and healthy controls, and therefore could contribute to ASM hyperresponsiveness in asthma (Drake et al., 2018).

ASM tone/contraction is also regulated by extracellular mediators released from resident cells, including epithelial and inflammatory cells. For example, histamine, cysteinyl leukotrienes and PGD_2 released from mast cells contract HASM *ex vivo* and *in vivo* (Schild et al., 1951, Hanna et al., 1981, Hardy et al., 1984). Furthermore, even extracellular ATP has been shown to contribute to ASM contraction in both healthy control and asthmatic human lungs (Pellegrino et al., 1996), although the precise role of the nucleotides was unconfirmed.

These mediators signal via their cell-surface receptors and regulate Ca^{2+} flux in the manners described above. Once the $[Ca^{2+}]_i$ is increased, contraction occurs as outlined below.

1.2.4.2 Mechanisms of smooth muscle contraction

Smooth muscle is comprised of three main contractile proteins which are arranged into filaments: thick myosin filaments, and thin F-actin filaments arranged in a double-helix with tropomyosin bound in the furrow along the helix. These thin filaments also contain associated caldesmon and calponin proteins (Marston and Smith, 1985, Roux et al., 2012). The thick myosin filaments are anchored to 'dense bodies' found on the plasma membrane and the actin filaments are found between myosin ones

(Roux et al., 2012). Intermediate filaments made predominantly of either desmin or vimentin are also present in smooth muscle, typically being found in visceral (Bennett et al., 1978) and vascular smooth muscle (Frank and Warren, 1981, Tang et al., 2005), respectively, although both are often expressed simultaneously (Johansson et al., 1997). In ASM specifically, intermediate filaments of both desmin and vimentin have been reported (Halayko et al., 1996, Wang et al., 2006). These intermediate filaments are thought to play a more structural role in smooth muscle, connecting the dense bodies with the contractile filaments (Roux et al., 2012, Ouedraogo and Roux, 2014).

The myosin filaments contain dimeric globular myosin-head protrusions at regular intervals. Each monomer consists of 2 identical myosin heavy chains and 2 light chains, one at 17 kDa and the other at 20 kDa. The heavy and light chains form the head and neck domains, respectively (Ouedraogo and Roux, 2014). In addition to catalytic sites for ATP hydrolysis, the head domains also contain actin-binding domains which can form cross-bridges with actin filaments. For this to occur, the 20 kDa light chain (MLC20) of both myosin heads must be phosphorylated (Adelstein and Eisenberg, 1980, Hartshorne, 1980, Wingard et al., 2001, Harnett and Biancani, 2003). This phosphorylation is performed by MLCK (Dabrowska et al., 1977) and induces a conformational change in the neck region of myosin, rotating the head domain and pulling the filaments along one another, shortening the cell (Roux et al., 2012). The phosphorylation of MLC20 also activates the ATPase activity of the myosin head (Adelstein and Eisenberg, 1980, Kamm and Stull, 1985), allowing the hydrolysis of ATP which is required for the breaking of the cross-bridges and the detachment of myosin from actin (Roux et al., 2012). The repetitive cycling of these cross-bridges causes the cell to contract and shorten. The force of the contraction is dictated by the number of cross-bridges that form between the filaments, whilst the rate of the cross-bridge cycling indicates the speed of contraction. These events are summaries in figure 1.2.4.

1.2.4.3 Mechanisms of smooth muscle relaxation

As described, smooth muscle contraction occurs when a contraction stimulus leads to the activation of MLCK and the phosphorylation of MLC20, typically mediated by an increase in $[Ca^{2+}]_i$. For relaxation to occur, by the removal of the contraction stimulus or by direct stimulation of relaxation, there must be a decrease in $[Ca^{2+}]_i$ and MLC20 must be dephosphorylated (Webb, 2003). Evidently, a decrease in cytosolic Ca^{2+}

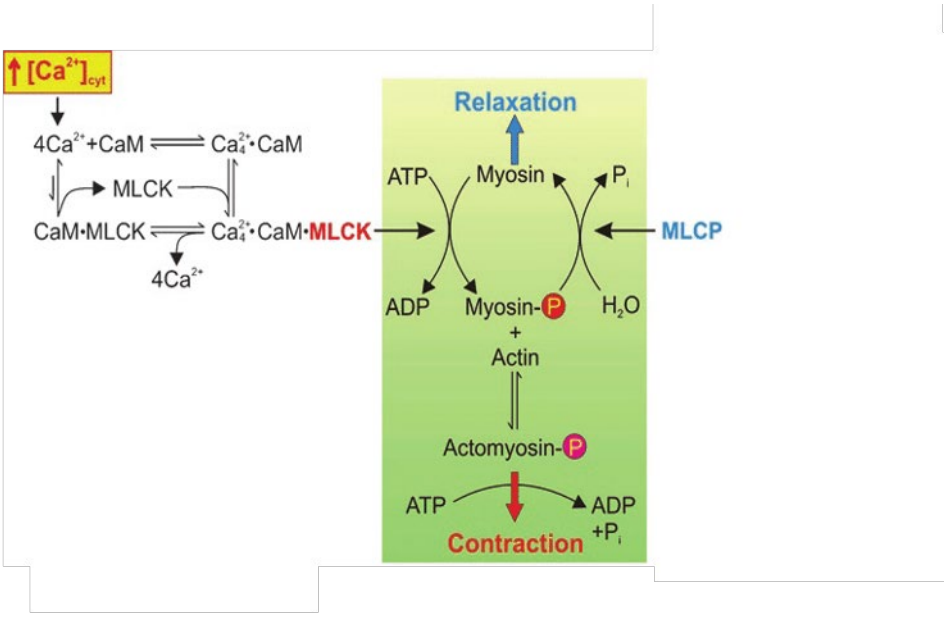

Figure 1.2.4. Summary of the mechanism of smooth muscle contraction and relaxation.

Rises in cytosolic calcium concentrations lead to the formation of the Ca^{2+}-calmodulin (CaM) complex which activates myosin light chain kinase (MLCK). This induces the phosphorylation of myosin (light chain 20) and the subsequent binding of phosphorylated myosin to actin. This allows cross-bridge cycling and contraction to take place. Myosin light chain phosphatase dephosphorylates myosin, preventing actomyosin formation and preventing cross-bridge cycling, resulting in relaxation. The depletion of cytosolic calcium levels leads to the inactivation of MLCK, favouring relaxation.

Figure modified from Firth and Yuan, 2011.

levels will not favour activated MLCK and contraction. The dephosphorylation of MLC20 is carried out by myosin light chain phosphatase (MLCP) (Ito et al., 2004) which, unlike MLCK, is regulated independently of cytosolic Ca^{2+} levels. Instead, it is regulated by GPCR-mediated signalling pathways which typically result in its phosphorylation and the inhibition of its phosphatase activity (Somlyo and Somlyo, 1994, Trinkle-Mulcahy et al., 1995, Ichikawa et al., 1996). This then results in higher levels of phosphorylated MLC20 and an increase in the force generated by a stimulus, and so enhances the effect of the contractile Ca^{2+} levels in a process known as Ca^{2+} sensitization (Kitazawa et al., 1991a, Kitazawa et al., 1991b, Kubota et al., 1992). This process will be discussed more fully in section 1.2.4.4.2.

There are two main mechanisms for the removal of Ca^{2+} from the cytoplasm; re-uptake into intracellular organelles such as the SR or Ca^{2+} efflux from the cell (see figure 1.2.3). For the former, specialised pumps are expressed on the SR/ER membranes, known as sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps. There are three SERCA pumps in the family, known as SERCA1, SERCA2 (made up of SERCA2A and SERCA2B isoforms) and SERCA3. SERCA2 (and particularly the SERCA2B isoform) appears to be widely expressed whilst SERCA1 and SERCA3 typically show more restricted expression patterns (Burk et al., 1989, Lytton et al., 1989, Wu et al., 1995). SERCA2 is the dominant family member expressed in vascular smooth muscle, with SERCA2B being the most abundant isoform in both this tissue (Wu et al., 2001) and in ASM (Mahn et al., 2009). The SERCA pumps have a high affinity for Ca^{2+} and, upon ATP hydrolysis, undergo a conformational change and transport Ca^{2+} into the SR, up their concentration gradient (MacLennan et al., 1997).

Ca^{2+} efflux is mediated by the low affinity but high capacity Na^+/Ca^{2+} exchanger (NCX) and the high affinity but low capacity plasma membrane Ca^{2+}-ATPase (PMCA) (Brini and Carafoli, 2011). NCX is functional in ASM (Hirota and Janssen, 2007, Liu et al., 2010). The NCX uses the electrochemical gradient of Na^+ across the membrane to drive the extrusion of one Ca^{2+} per three Na^+. However, the NCX is also known to operate in reverse and so can, if conditions allow, contribute to Ca^{2+} influx (Blaustein and Lederer, 1999). This reverse mode is known to occur in ASM cells (Liu et al., 2010, Rahman et al., 2011).

The PMCA has four different isoforms (PMCA1-4), and whilst PMCAs 1 and 4 are ubiquitously expressed, PMCAs 2 and 3 are more prominently expressed in the nervous system (Strehler and Zacharias, 2001, Brini and Carafoli, 2011). It follows, therefore, that PMCAs 1 and 4 are expressed in ASM specifically (Chen et al., 2014). PMCAs contribute to Ca^{2+} efflux by using ATP hydrolysis to pump Ca^{2+} and protons out of and into the cell, respectively (Marín et al., 1998).

Relaxation of ASM can also be stimulated directly. For instance, the activation of β2-adrenoreceptors, which are abundantly expressed in the airways (Carstairs et al., 1984, Carstairs et al., 1985) including ASM (Mak et al., 1994), induces ASM relaxation through a presumably 3',5'-cyclic adenosine monophosphate (cAMP)-mediated pathway (Robison et al., 1967, Barnes, 1995, Johnson, 2001, Delmotte et al., 2010). The use of β2-agonists as asthma treatments (discussed in section 1.1.5) takes advantage of this mechanism.

Additionally, prostanoids are physiologically relevant compounds consisting of prostaglandins and thromboxanes which regulate many processes in the airways, including airway inflammation and tone (Narumiya et al., 1999, Ricciotti and FitzGerald, 2011). Arachidonic acid, which is formed by phospholipid cleavage by phospholipase A enzymes (Dennis et al., 2011), is then synthesised into the prostanoids by either the constitutively expressed cyclooxygenase (COX)-1 enzyme, or the inducible COX-2 enzyme (Ricciotti and FitzGerald, 2011, Claar et al., 2015). Regarding airway tone, prostaglandins have been reported to have various effects. PGE_2, which is one of the more abundant prostaglandins produced in the airways, either by the ASM (Delamere et al., 1994) or the epithelium (Churchill et al., 1989), directly stimulates HASM relaxation (Sweatman and Collier, 1968, Mathé and Hedqvist, 1975, Pavord et al., 1993). COX pathway inhibitors such as indomethacin (Blanco et al., 1999) are useful tools in elucidating the functions of prostaglandins in the airways (Claar et al., 2015).

1.2.4.4 The regulation of smooth muscle contraction

As described in section 1.2.4.2, there must be an appropriate stimulus that results in an increase in $[Ca^{2+}]_i$ for myocytes to contract. This increase in $[Ca^{2+}]_i$ is referred to as the 'contractile Ca^{2+}'. The regulation of smooth muscle contraction is complex, but occurs via two main mechanisms. The first mechanism is Ca^{2+}-dependent,

whereby intracellular Ca^{2+} directly modulates the activity the contractile apparatus, and involves Ca^{2+}-binding proteins such as calmodulin (see section 1.2.4.4.1). The second mechanism, however, changes the sensitivity of the contractile apparatus to the contractile Ca^{2+}, so that the apparatus responds with greater force to the same concentration of contractile Ca^{2+}. This process is known as Ca^{2+} sensitization and the mechanism behind the regulation is Ca^{2+}-independent, not considering the contractile Ca^{2+} (see section 1.2.4.4.2).

1.2.4.4.1 Calcium-dependent regulation of smooth muscle contraction

The most important event in the initiation of smooth muscle contraction is the phosphorylation of MLC20 by MLCK, which is itself regulated by Ca^{2+} concentration via the Ca^{2+}-binding protein calmodulin (see figure 1.2.4) (Dabrowska et al., 1977, Blumenthal and Stull, 1980). An increase in $[Ca^{2+}]_i$ leads to the binding of Ca^{2+} to one of four Ca^{2+}-binding sites on calmodulin, and data suggests that at least three of these four sites must be occupied for calmodulin to positively regulate its downstream targets (Blumenthal and Stull, 1980, Crouch and Klee, 1980, Cox et al., 1982, Rasmussen and Barrett, 1984). Inactive MLCK contains an auto-inhibitory domain in its active site which prevents its binding to MLC20 and its subsequent activity (Kemp et al., 1987, Kemp and Pearson, 1991). However, binding of the Ca^{2+}-calmodulin complex to inactive MLCK induces a conformational change, removing the auto-inhibitory domain from the active site of MLCK and allowing the phosphorylation of MLC20 (Kemp and Pearson, 1991, Allen and Walsh, 1994). In this way, the $[Ca^{2+}]_i$ directly regulates smooth muscle contraction via MLCK activation.

There are also additional regulatory mechanisms of smooth muscle contraction, involving the thin-filament associated proteins, caldesmon and calponin. Caldesmon is arranged beside tropomyosin along the axis of the actin double helix (Moody et al., 1990) and has 4 distinct domains which can bind to actin, myosin, tropomyosin and calmodulin (Leszyk et al., 1989). Caldesmon is thought to stabilise the formation of actin filaments via its interaction with tropomyosin (Gusev, 2001). Through its ability to bind calmodulin, caldesmon is also regulated by Ca^{2+}. At low/resting Ca^{2+} concentrations, the actin-tropomyosin-caldesmon complex typically inhibits the ATPase activity of the myosin head, and calponin is bound to F-actin, negatively regulating contraction by disrupting the actin-myosin cross-bridges. At higher Ca^{2+} concentrations, there is a

conformational change in the actin-tropomyosin-caldesmon complex which favours the activation of the myosin-head ATPase catalytic site, supporting contraction (Pritchard and Moody, 1986, Roux et al., 2012). Furthermore, higher Ca^{2+} concentrations result in calponin phosphorylation, impairing its binding to F-actin. It follows that the reduction of Ca^{2+} concentration leads to the dephosphorylation of calponin and the restoration of its inhibition (Winder and Walsh, 1990, Winder et al., 1998).

It is therefore evident that smooth muscle contraction is heavily regulated by $[Ca^{2+}]_i$. Increases in cytosolic Ca^{2+} as a result of the described Ca^{2+} influx or Ca^{2+} release mechanisms result in MLCK activation and the elimination of the inhibitory effects of both calponin and caldesmon. This primes the contractile apparatus so that, when a contractile stimulus is next applied to the cell, it contracts with greater force. It is possible, therefore, that smooth muscle hyperresponsiveness in asthma is a result of dysregulated Ca^{2+} signalling mechanisms. However, the regulation of smooth muscle contraction is not wholly Ca^{2+}-dependent, particularly as sustained contraction has been shown to occur even if cytosolic Ca^{2+} levels decline (Mbikou et al., 2006). Therefore, Ca^{2+}-independent mechanisms must be considered.

1.2.4.4.2 Calcium-independent regulation of smooth muscle contraction

The use of Ca^{2+}-indicators enabled the observation in vascular smooth muscle that the application of different contractile stimuli results in different amounts of force generated, even if $[Ca^{2+}]_i$ are similar (Somlyo and Somlyo, 1968). Typically, agonist-induced contraction generates greater force than contraction induced by membrane depolarization (Bradley and Morgan, 1987, Himpens and Casteels, 1990). It is assumed that this could be due to downstream effects of receptor activation which change the sensitivity of the cell to Ca^{2+}, mainly by modulating the ratio of MLCK:MLCP (Somlyo and Somlyo, 2003). For example, as described above, the inhibition of MLCP will favour MLC20 phosphorylation and so greater force will be generated by the same levels of contractile Ca^{2+}. This is Ca^{2+} sensitization (Kitazawa et al., 1991a, Kitazawa et al., 1991b, Kubota et al., 1992), a mechanism that is known to take place in ASM. For example, the pre-treatment of bovine tracheal smooth muscle with guanosine 5'-O-(3-thiotriphosphate) (GTPγS) leads to Ca^{2+} sensitization (Kubota et al., 1992). Furthermore, the pre-treatment of canine ASM with ragweed pollen increases the MLCK content

activity (Jiang et al., 1992), potentially linking allergy with ASM sensitization. Additionally, a preliminary study using HASM has demonstrated that sensitized ASM has increased MLCK content (Ammit et al., 2000). Ca^{2+} sensitization can occur via two pathways, as summarised in figure 1.2.5 (Somlyo and Somlyo, 2003).

Figure 1.2.5 shows the generally accepted mechanisms which regulate Ca^{2+} sensitization. However, additional regulatory steps are still being elucidated, adding further complexity to the system. For example, CPI-17 phosphorylation is typically viewed as protein kinase C (PKC)-mediated. However, RhoK has been implicated in the direct phosphorylation of CPI-17 (Kitazawa et al., 2003, Niiro et al., 2003). Furthermore, the lipid-mediator arachidonic acid can activate Rho-kinase independently of Rho and has been implicated in Ca^{2+} sensitization (Gong et al., 1992, Gong et al., 1995, Fu et al., 1998, Feng et al., 1999). It must also be noted that activated RhoK has been shown to phosphorylate MLC20 directly (Amano et al., 1996, Van Eyk et al., 1998), bypassing a requirement for MLCK activation.

In the same way that Ca^{2+} sensitization increases the contractile response of a cell to the same contractile Ca^{2+} levels, Ca^{2+} desensitization can decrease the contractile response. An increase in MLCP activity can cause this. For example, the β-agonist isoprotenerol was shown to augment MLCP activity in porcine and bovine ASM, thereby reducing carbachol-mediated Ca^{2+} sensitization (Janssen et al., 2004). Furthermore, as contraction is dependent on the polymerisation of F-actin, inhibitors of this polymerisation and destabilisation of actin filaments have both been found to decrease contractile responses/induce relaxation in ASM without affecting $[Ca^{2+}]_i$ (Ito et al., 2002, Komalavilas et al., 2008).

Despite the complexity behind the processes, it is evident that Ca^{2+} sensitization and desensitization play a physiological role in ASM function and could be involved in its dysfunction in pathological states.

1.2.5 ASM in asthma

1.2.5.1 Possible mechanisms behind the hypercontractility of asthmatic ASM

As previously described, a hallmark feature of asthma is hyperresponsive ASM (Burrows et al., 1989, An et al., 2007, Holgate, 2008). Genetic and environmental factors could result in ASM cells that are fundamentally different in asthmatic patients

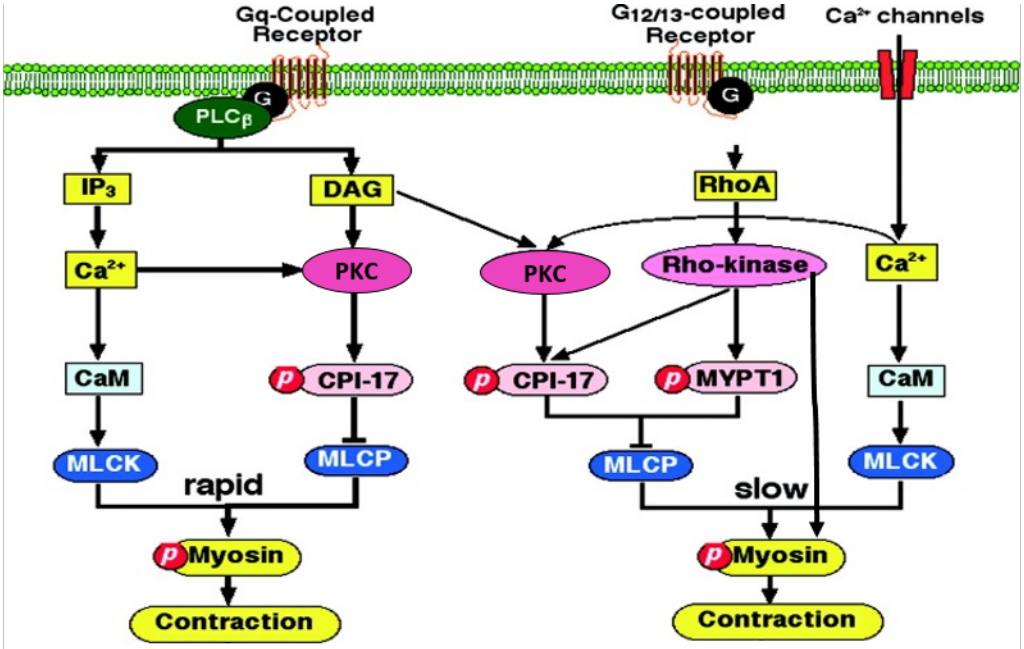

Figure 1.2.5. The mechanism of sensitization of contractile filaments to calcium.

Calcium sensitization, the mechanism by which contractile filaments are sensitized to contractile Ca^{2+}, occurs primarily via the Rho-mediated pathway. The activation of G12/13-, G_q- and G_i- coupled GPCRs activates the small, monomeric G protein Rho (Croxton et al., 1998, Katoh et al., 1998, Hirshman and Emala, 1999, Klages et al., 1999). Rho then activates Rho-kinase (Ishizaki et al., 1996, Leung et al., 1996, Matsui et al., 1996), which phosphorylates the targeting subunit of MLCP (MYPT1) (Kimura et al., 1996, Kitazawa et al., 2003). This phosphorylation then inhibits MLCP activity in smooth muscle (Ito et al., 2003, Niiro et al., 2003, Muranyi et al., 2005). Calcium sensitization also occurs via the protein kinase C (PKC)-mediated pathway. Ligand binding to G_q-coupled GPCRs leads to the activation of PKC via PLC (van Biesen et al., 1996, Blaukat et al., 2000, Kitazawa et al., 2000). PKC is then known to phosphorylate PKC-potentiated inhibitor protein (CPI-17) (Eto et al., 1995, Kitazawa et al., 1999, Kitazawa et al., 2000, Zemlickova et al., 2004), which then inhibits MLCP, favouring contraction (Eto et al., 1995, Eto et al., 2000).

Figure modified from Dimopoulos et al, 2007.

compared to healthy individuals, and these differences could contribute, in whole or in part, to the hypercontractile phenotype.

Due to the numerous cellular processes regulated by Ca^{2+}, including cell contraction, it is plausible to consider that Ca^{2+} handling (including mechanisms of Ca^{2+} release from intracellular stores and efflux) could be abnormal in ASM from asthmatic patients. However, data on this subject is contradictory. For instance, one study found no evidence for abnormal Ca^{2+} handing, including SERCA2 expression, in ASM from asthmatic individuals (Sweeney et al., 2015), whilst another study found decreased SERCA2 expression in asthmatic subjects (Mahn et al., 2009).

Other regulatory factors of ASM contraction could also be abnormal in ASM from asthmatic patients. For example, an increase in MLCK mRNA (Ma et al., 2002, Leguillette et al., 2009) and protein (Benayoun et al., 2003) has been observed in ASM from asthmatic individuals (Ma et al., 2002) compared to healthy controls, which could provide an explanation for the increased shortening capacity and velocity of asthmatic ASM. However, the literature regarding this idea becomes complicated as other groups found no differences in MLCK expression between asthmatic and non-asthmatic ASM (Woodruff et al., 2004, Matsumoto et al., 2007). These disparities could be explained, however, by the use of subjects with different severities of asthma, or by small sample sizes. Additionally, sensitization has been found to increase MLCK expression (Ammit et al., 2000). Furthermore, differences in the asthma subtypes of subjects could also explain contradictory results, as IgE antibodies have been shown to directly upregulate MLCK expression in human ASM cells (Balhara et al., 2014), which could obviously have implications for allergic asthma.

In addition to abnormalities resulting in hypercontraction, it could be that asthmatic ASM has impaired relaxation processes. For instance, cultured ASM cells from asthmatic donors produce less PGE_2 than those from healthy volunteers due to lower expression of the COX-2 enzyme (Chambers et al., 2003), which could lower the threshold of contractile agonists required to trigger contraction, in addition to impairing relaxation directly.

There is a possibility, therefore, that ASM from asthmatic patients is intrinsically different to that of healthy individuals. However, the degree to which they are different, and exactly how they are different, is currently unknown. Furthermore, perhaps an

asthmatic phenotype results in differences in the local environment surrounding the ASM which could explain the observed hyperresponsiveness. For example, the inflammatory state of asthmatic airways could increase resting intracellular ASM Ca^{2+} concentrations, particularly as treatment of cHASM cells with TNF-α and IL-13 results in increased NCX expression and increased $[Ca^{2+}]_i$, potentially due to an increase in the reverse mode of NCX function (Sathish et al., 2011).

1.2.5.2 Possible mechanisms behind the hyper-proliferation of asthmatic ASM

Although evidence points against ongoing ASM proliferation in asthma (Benayoun et al., 2003, Woodruff et al., 2004, Begueret et al., 2007, Ward et al., 2008), it has been noted that ASM cells from asthmatic individuals proliferate more than those from healthy donors (Johnson et al., 2001), which could suggest yet another intrinsic difference between healthy and asthmatic ASM.

The cell cycle and proliferation of ASM is regulated much like that of any other (smooth muscle) cell. Therefore, Ca^{2+} plays an important part in the regulation of cell proliferation (Berridge, 1995, Capiod, 2011), and a reduction in the extracellular Ca^{2+} concentration decreases/arrests cell proliferation (Hazelton et al., 1979, Whitfield et al., 1979, Durham and Walton, 1982, Wei et al., 1983), potentially via the inhibition of DNA synthesis (Boynton et al., 1976). Furthermore, increases in $[Ca^{2+}]_i$ are associated with cell cycle progression (Berridge, 1995, Kahl and Means, 2003). Consequently, dysregulated Ca^{2+} handling could explain these observations, as described above.

However, it seems more logical that changes in the local environment which occur throughout asthma pathogenesis could be the cause of any ASM hyper-proliferation (or even hyperresponsiveness) observed in asthmatic patients. For example, the inflammatory state associated with asthma pathogenesis could contribute to this phenomenon. Supporting this, direct contact between eosinophils and ASM cells has been shown to induce proliferation in the latter (Halwani et al., 2013). Furthermore, inflammatory mediators including histamine (Panettieri et al., 1990), tryptase (Brown et al., 1995) and transforming growth factor-β1 (Chen and Khalil, 2006) also induce proliferation of ASM cells. Even extracellular nucleotides have been implicated in the proliferation of rat tracheal ASM cells *in vitro* via the activation of their receptors, P2 receptors (Michoud et al., 1997, Michoud et al., 2002).

Despite evidence suggesting that ongoing ASM proliferation does not contribute to the increased ASM mass observed in asthma, the possibility that ASM proliferation occurs in discrete occasions, such as upon asthma exacerbations, instead of slowly but continuously over time cannot currently be ruled out.

1.2.6 ASM cells in culture

Cells can be studied *in vitro* typically after tissue dissection, digestion with proteolytic enzymes and then isolation of cells (Alberts et al., 2002c). However, even utilising this method, studying cells *in vitro* can be difficult. For example, the amount of starting material (e.g. strips of muscle) may be limited, resulting in few cells that may not be entirely pure populations. Furthermore, the isolation process may damage the cells, limiting the approaches that can be used to study them. These issues can be countered by culturing the cells after their isolation. This not only allows the cells to recover from the isolation, but results in larger numbers of cells and so increases the number of technical approaches that can be applied to their study. In addition, it is easier to obtain pure populations of cells, and the purity can even be determined by using cell-specific markers.

The most common contaminating cell types when culturing ASM cells are thought to be the epithelial and endothelial cells in the first instance, and also fibroblasts. However, these cells do not have the typical 'hill and valley' morphology of smooth muscle cells, and the epithelial and endothelial cells disappear from culture after a few days (Tom-Moy et al., 1987, Hirst, 1996). Furthermore, it is generally accepted that only smooth muscle cells express the smooth muscle specific markers, including α-smooth muscle actin and smooth muscle myosin isoforms (Chamley-Campbell et al., 1979, Campbell and Campbell, 1993), although fibroblasts can weakly express smooth muscle actin (Gown et al., 1985, Hirst, 1996). This allows the differentiation of smooth muscle cells from contaminating cells.

However, arguably the most troublesome contaminating cells are the myofibroblasts, which are fibroblasts that take on a smooth muscle-like phenotype and express α-smooth muscle actin (Hinz et al., 2007). A number of markers have been suggested to aid differentiation of smooth muscle cells from myofibroblasts. For example, whilst both cell types express smooth muscle actin, smooth muscle cells are

thought to express desmin, whilst myofibroblasts are not (Kalluri and Zeisberg, 2006). However, due to the heterogeneous nature and plasticity of both myofibroblasts (Hinz et al., 2007) and smooth muscle cells (Hirst, 1996), particularly in culture, it is extremely difficult to differentiate these two cell types and it has even been argued that it is not necessary to do so (Wenzel and Balzar, 2006). Furthermore, numerous studies which report using airway smooth muscle cells, specifically, use only α-smooth muscle actin and pan-cytokeratin antibodies as markers (Michoud et al., 1997, Michoud et al., 1999, Michoud et al., 2002, Liu et al., 2014) and so perhaps it is not necessary to differentiate between these two cells lines at this time.

Freshly isolated smooth muscle cells from a wide variety of sources have been used *in vitro*, from toad stomach (Singer and Walsh Jr, 1980) to mouse aorta (Serir et al., 2006). A number of groups have also made use of freshly isolated ASM cells, including those from bovine trachea (Welling et al., 1992) and even human bronchi (Snetkov et al., 1996).

It is important that cultured cells remain true to their *in vivo* state and so respond to physiologically relevant stimuli in ways that can still provide physiologically relevant data, despite their *in vitro* use. The development of an approach which allowed the culture of physiologically responsive human ASM cells was pivotal for the study of ASM (Panettieri et al., 1989) and numerous studies have since used cultured ASM cells (Panettieri, 2001), including for cell contraction assays using collagen gels (Matsumoto et al., 2007, Sutcliffe et al., 2012). These cells are typically cultured from explants following enzymatic digestion (Hall and Kotlikoff, 1995).

As discussed previously in section 1.2.1, ASM cells display a large capacity for phenotypic plasticity, and the effects of culture can sometimes augment this plasticity so that cells do not resemble those from their source. For example, the primary function of smooth muscle cells is considered to be contraction. However, when in culture, ASM cells switch from a contractile to a non-contractile phenotype, with a concomitant decrease in contractile marker expression including smooth muscle myosin heavy chain, α-smooth muscle actin and MLCK (Halayko et al., 1996). Still, smooth muscle cells can be returned to a contractile phenotype using treatments that reverse the decreased expression of contractile markers, including culturing the cells on type IV collagen (Hirose et al., 1999) or in the presence of retinoic acid (Gollasch et al., 1998). Typically,

the higher the passage number and the number of population doublings required to reach confluence (related to seeding density), the further from their natural state the cells will be (Hirst, 1996). Therefore, it is more desirable to utilise cells at lower passage numbers, whilst still ensuring a pure population.

Culturing smooth muscle cells also leads to other changes in protein expression, and particularly that of cell receptors, which can further affect the function of the cultured cell. For example, L-type VDCC expression is known to decrease in response to culture (Gollasch et al., 1998), as is that of certain Ca^{2+}-activated potassium channels such as $K_{Ca}1.1$ (Neylon et al., 1999) and even P2 receptors (Erlinge et al., 1998).

Culturing smooth muscle cells is a fantastic approach to facilitate their study *in vitro*, despite the effects that culture conditions can have on the cells. As long as these effects are considered when resulting data is contextualised, the data can be extremely meaningful and informative.

1.3 P2 receptors

While it is clear that Ca^{2+} signalling is important for ASM functions, further investigation is required to fully elucidate Ca^{2+} entry pathways into the cell, and those pathways that could be important to asthma pathogenesis particularly. To this end, a role for P2 receptors must be considered.

1.3.1 Nucleotide structures and functions

This section will discuss the known intracellular and extracellular functions of nucleotides. However, it must be acknowledged that early literature regarding nucleotide function is dominated by adenine-based nucleotides. This is demonstrated by the term 'purinergic signalling', which is now only appropriate to use in an historical context since the discovery of P2 receptors that respond to pyrimidine nucleotides (Communi and Boeynaems, 1997, Harden et al., 1997, Brunschweiger and Muller, 2006). Therefore, this section focuses mainly on adenine-based nucleotides only as a result of biased literature.

1.3.1.1 The structure of nucleotides and nucleosides

A nucleoside consists of nitrogenous base connected via an N-glycosidic bond to a 5-carbon sugar (either a ribose or a deoxyribose sugar, as present in ribonucleic or deoxyribonucleic acid, respectively). When a phosphate ester group is attached to the sugar in the 5' position, the nucleoside becomes a nucleotide (see figure 1.3.1). With one phosphate group, this is a nucleotide monophosphate, which then becomes a diphosphate and triphosphate with a total of two and three phosphate groups, respectively. Whether a nucleotide is a purine or a pyrimidine is determined by the base (Saenger, 1973, Bowater and Gates, 2015).

1.3.1.2 The historical intracellular roles of nucleotides

Nucleotides are mainly known for their historic intracellular roles, and primarily those in DNA coding and replication (Alberts et al., 2002a, Alberts et al., 2002b). ATP itself is arguable the most notable nucleotide owing to its additional role as an energy source. It was discovered independently by two groups in 1929, and was initially presumed to be a regulator of muscle contraction (Fiske and Subbarow, 1929, Lohmann, 1929). However, ATP and its 'high-energy' phosphate bonds were attributed to energy consumption in the cell in 1941 (Lipmann), and since then has become known as the

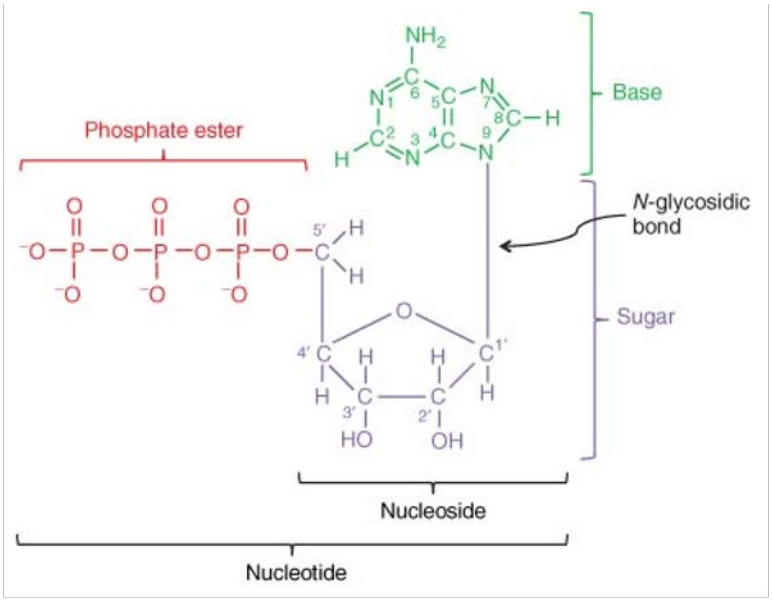

Figure 1.3.1. The structure of nucleosides and nucleotides.
Nucleosides such as adenosine are formed from a nitrogenous base (adenine here) joined to a ribose sugar via an N*-glycosidic bond. When phosphate groups are added to the sugar, nucleotides are formed. One, two and three phosphate groups results in the formation of nucleoside 5′ mono-, di- and tri-phosphates, respectively.*
Figure taken from Bowater and Gates, 2015.

energy source for all living cells in addition to a regulator of metabolism (Atkinson, 1971). Nucleotides also play a role in intracellular signalling pathways as second messengers, most notably as cyclic nucleotides such as cAMP (Rall et al., 1957, Sutherland and Rall, 1958, Reddix and Pacheco, 2007).

1.3.1.3 The discovery of adenine-based compounds as extracellular signalling molecules

Adenine-based compounds (such as ATP) were first reported to have extracellular signalling capabilities in 1929 when adenosine and adenosine 5'-monophosphate (AMP) were observed to have physiological effects on the body, including the ability to regulate cardiac and smooth muscle contraction. These actions also included the modulation of arterial dilation and, therefore, of blood pressure, and even the inhibition of intestinal contraction (Drury and Szent-Gyorgyi, 1929). Subsequent studies demonstrated that purines mediate countless functions in numerous tissue types throughout the body, including the dilation of coronary, renal and pulmonary vessels (Burnstock and Verkhratsky, 2012).

Adenine-based nucleotides were specifically implicated in signalling in nervous tissue when the stimulation of sensory nerves was shown to induce ATP release in quantities sufficient to induce vasodilation of rabbit ear arteries (Holton and Holton, 1953, Holton, 1959), demonstrating that ATP acts as a neurotransmitter. It was suggested in 1976 that ATP acts as a cotransmitter (Burnstock, 1976) and 'The Purinergic Nerve Hypothesis' was styled the following year, building on previous data to define purinergic nerves as neither cholinergic, nor adrenergic, but having purine nucleotide-based neurotransmitters (Burnstock, 1977). From this point, the discussion of purinergic signalling and the extracellular signalling capabilities of purine nucleotides became more commonplace. Today, ATP is widely known to act as a cotransmitter in neurones (Sneddon and Burnstock, 1984a, Sneddon and Burnstock, 1984b), and its release has long been linked to tissue depolarization (Abood et al., 1962).

ATP is now known to act as a cotransmitter with acetylcholine (Burnstock et al., 1978b, Zimmermann, 2008), and also with noradrenaline in ileum (Burnstock et al., 1978b), *vas deferens* (Westfall et al., 1978), rat tail artery (Sneddon and Burnstock, 1984a) and aorta (Sedaa et al., 1990).

Adenine-based nucleotides mediate their physiological effects in a number of ways. For example, when considering the regulation of vasodilation by purines, ATP and ADP were found to relax vascular smooth muscle in an endothelium-dependent manner, inducing the release of an inhibitory mediator of smooth muscle cells (endothelium-derived relaxing factor), whilst adenosine and AMP acted on the muscle itself to more directly induce its relaxation (De Mey and Vanhoutte, 1981). Furthermore, adenine-based nucleotides often have opposing functions in different tissues, and as such are implicated in both vasoconstriction and vasodilation (Burnstock and Kennedy, 1986).

The activity of adenine-based nucleotides has also been correlated with the length of their phosphate chains, and it has been noted that ATP is typically a more potent signalling molecule than adenosine 5'-diphosphate (ADP), which is itself often more potent than AMP (Drury, 1936, Green and Stoner, 1950, Moir and Downs, 1972). Why this was the case was largely unknown until the discovery of P2 receptors (see section 1.3.2), which then helped to dispel resistance to the purinergic signalling theory which existed due to the well-known intracellular roles of nucleotides.

1.3.1.4 Physiologically-relevant mechanisms of nucleotide release

For nucleotides to elicit extracellular responses, there must be physiological mechanisms of their release into extracellular spaces, particularly as ATP cannot pass through cell membranes by simple diffusion due to its size and charge (Glynn, 1968, Chaudry, 1982). To that end, this subchapter will discuss the known physiologically-relevant mechanisms of nucleotide release.

Again, recent literature primarily focuses on the release of ATP (and other adenine-based nucleotides including ADP) from cells. However, cellular mechanisms of nucleotide release are not selective for adenine-based nucleotides. For example, both adenine- and uridine-based nucleotides, including uridine-5'-diphosphate (UDP)-glucose, can be released from astrocytes (Lazarowski, 2006). Also, the release of UTP and ATP can be mechanically-induced from astrocytes (Lazarowski et al., 1997). Furthermore, airway epithelial cells have been observed to release both uridine-5'-triphosphate (UTP) and ATP (Watt et al., 1998). Therefore, all types of nucleotides can be released from cells.

1.3.1.4.1 Nucleotide release via tissue damage

Nucleotides can be released from cells following tissue damage and trauma. During the Second World War, it was noticed that crushed tissues (as a result of battle damage), and especially muscles, would release ATP and other adenine compounds, ultimately leading to an increase in ATP levels in the blood which resulted in vasodilatation (Biblschowsky and Green, 1943, Green, 1943, Stoner and Green, 1944). It was noted that after nucleotide release, the situation often worsened for the patient as high concentrations of ATP increase the permeability of erythrocyte cell membranes, allowing a huge efflux of intracellular ions and compounds, including ATP itself (Trams et al., 1980). A similar ATP-induced ATP release mechanism has also been suggested in smooth muscles (Katsuragi et al., 1991) and endothelial cells (Bodin and Burnstock, 1996).

1.3.1.4.2 Nucleotide release from excitatory/secretory tissues

As discussed, ATP is known to act as a cotransmitter with preliminary data suggesting its release from sensory nerves following antidromic stimulation (Holton and Holton, 1953, Holton and Holton, 1954), which was later confirmed (Holton, 1959). The presence of ATP in synaptic vesicles has been confirmed (Dowdall et al., 1974, Zimmermann, 2008), and it is assumed that its release from these and other types of vesicles is primarily by exocytosis (Aspinwall and Yeung, 2005).

ATP is also released from other excitatory/secretory tissues including chromaffin cells (Douglas and Poisner, 1966), Xenopus oocytes (in response to hypertonic stress) (Aleu et al., 2003), mast cells and pancreatic β-cells (Aspinwall and Yeung, 2005), osteoblasts (Romanello et al., 2005) and lung epithelial cells (in response to the air pollutant ozone) (Ahmad et al., 2005). ATP release is particularly well document from the platelet due to its important role in the regulation of haemostasis (Holmsen et al., 1969, Holmsen and Weiss, 1979, Ingerman et al., 1979, Kahner et al., 2006). These data are supported by the identification of vesicular nucleotide transporters (Sawada et al., 2008).

1.3.1.4.3 Nucleotide release from non-excitatory cells

Nucleotides can also be released from non-excitatory cells via mechanisms more dependent on channels and transporters than by exocytosis. For example, connexin

hemichannels are known to be involved in nucleotide release and have been identified in cells including astrocytes (Stout et al., 2002) and airway epithelia (Guyot and Hanrahan, 2002). Pannexon channels also exhibit ATP permeability, such as when heterologously expressed in Xenopus oocytes (Bao et al., 2004), and even in ciliary (Li et al., 2010) and lung (Ransford et al., 2009, Seminario-Vidal et al., 2009) epithelial cells. Furthermore, ATP can be released through activated P2X7 receptors in cells including astrocytes (Suadicani et al., 2006). ATP-binding cassette (ABC) transporters, including the cystic fibrosis transmembrane conductance regulator (CFTR) (Cant et al., 2014), have also been implicated in ATP release. However, their involvement is controversial, particularly as they are primary transporters that use the energy from ATP hydrolysis to power the transport of their target (Vasiliou et al., 2009, Wilkens, 2015). They were originally implicated in ATP transport as cell membrane patches containing the channels were observed to have ATP conductance (Reisin et al., 1994). This has since been disputed and the current hypothesis, particularly regarding the CFTR, is that their function modulates the activity of another channel that is actually involved in ATP transport (Grygorczyk et al., 1996, Reddy et al., 1996, Watt et al., 1998, Braunstein et al., 2001).

1.3.1.4.4 Nucleotide release induced by mechanical stimulation

Mechanical stimulation of ATP release was first documented in 1972 during sustained exercise of human forearm muscle (Forrester, 1972). There are several types of mechanical stimulation, including mechanical loading or stretching, hypotonic cell swelling (osmotic stress) and even cell surface poking (Lazarowski et al., 2003). Studying these mechanisms can be difficult as they must be specifically identified as non-lytic release mechanisms. However, there are a number of reported cases of nucleotide release by mechanical stimulation. For example, this has been reported in vascular endothelial and smooth muscle cells in culture (Pearson and Gordon, 1979), which was initiated via use of a cell scraper. It was considered to not be due to damaged/destroyed cells as trypan exclusion measurements were not affected and intracellular enzymes (mainly lactate dehydrogenase) were not released. Furthermore, mechanical stimulation by media replacement has been shown to release both ATP and UTP from astrocytes (Lazarowski et al., 1997).

Sheer stress, which can be encountered physiologically in response to an increase in flow rates, has also been shown to release endogenous ATP from a number of cell/tissue types, including endothelial cells from human umbilical vein (Milner et al., 1990b, Bodin and Burnstock, 1998, Yegutkin et al., 2009) and rabbit aorta (Milner et al., 1990a, Bodin et al., 1991). This mechanism has also been encountered in airway epithelial cells, which can evidently have implications on neighbouring cells including ASM cells, and has been demonstrated to be CFTR-independent (Grygorczyk and Hanrahan, 1997, Watt et al., 1998, Homolya et al., 2000, Guyot and Hanrahan, 2002).

Another mechanical stimulant for nucleotide release is osmotic stress, which has been observed in numerous cell types including Xenopus oocytes (Aleu et al., 2003), osteoblasts (Romanello et al., 2005), astrocytes (Darby et al., 2003) and airway epithelial cells (Taylor et al., 1998, Guyot and Hanrahan, 2002).

1.3.2 The discovery and classification of P2 receptors

Receptors for extracellular nucleotides were first conceptualised in 1965 when caffeine was found to inhibit adenosine-induced contraction in cardiac muscle, and the existence of the adenosine receptor (AR) was postulated (Degubareff and Sleator, 1965). 'Purinergic receptors' themselves were categorised by Burnstock in 1978 into P1 receptors for adenosine and P2 receptors for ATP and ADP. Like most early studies investigating receptor function before selective pharmacological tools for each subtype were developed, categorisation was based on agonist potencies. At P1 receptors, the typical agonist potency profile was adenosine>AMP>ADP>ATP, and their activation generally modified intracellular cAMP levels. At P2 receptors, the proposed general agonist potency profile was ATP>ADP>AMP>adenosine, and their activation not typically associated with cAMP changes, but was sometimes known to induce prostaglandin synthesis (Burnstock, 1978). As all discussion related to these receptors at the time involved purine-based nucleotides, the term purinergic signalling was appropriate. Furthermore, as investigations have continued, it is apparent that the stated agonist profiles are no longer accurate.

Receptors for adenosine, which at one time were synonymously called P1 receptors (Ralevic and Burnstock, 1998), are now mostly referred to as adenosine receptors. Now, there are four recognised subcategories of adenosine receptors, consisting of A1, A2A, A2B and A3 receptors (Daly, 1985, Fredholm et al., 2001,

Fredholm et al., 2011). They are all GPCRs and are typically Gi, Gs, Gs and Gi coupled, respectively. P2 receptors were also further subcategorised into 2 types of receptor, P2X and P2Y receptors, again based on agonist potencies and the action of some antagonists (Burnstock and Kennedy, 1985). However, at this time the P2 receptor subclasses were not fully here, but this was aided by the cloning of the receptors in subsequent years.

The first cloned ATP receptor was a P2Y receptor based on its pharmacological profile, now known as the GPCR P2Y1, and was cloned from embryonic whole chick brain (Webb et al., 1993). This was followed by P2Y2, a GPCR originally named P2U, which was cloned in the same year from mouse neuroblastoma cells (Lustig et al., 1993) and was known to be activated by both ATP and UTP. These were followed closely by the first two cloned P2X receptors a year later in 1994. P2X1 was cloned from rat *vas deferens* and was activated by ATP (Valera et al., 1994), and P2X2 cloned from rat pheochromocytoma (PC12) cells and was also activated by ATP (Brake et al., 1994). The cloning and characterisation of these receptors aided the formal identification of P2X and P2Y receptors (Abbracchio and Burnstock, 1994) and, since that moment, the discovery and classification of the purinergic receptors has progressed greatly. Indeed, the widespread expression of P2 receptors reaffirms the role of extracellular nucleotides in signalling.

In summary, the P2 receptor family now consists of 7 cloned P2X receptor subunits (P2X1-P2X7) (North, 2002, North, 2016) which make up ligand-gated non-selective cation channels, are activated by ATP and allow the passage of such ions as Na^{2+} and Ca^{2+} (Benham and Tsien, 1987, Dubyak, 1991, North, 2002, North, 2016), and 8 cloned P2Y receptors in mammals (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-P2Y14), which are GPCRs that are functionally coupled to several different G-proteins (Dubyak, 1991, von Kügelgen, 2006).

1.3.3 P2X receptors

Due to the relationship between receptor structure and function, the following subchapters will discuss the structure of P2 receptors.

1.3.3.1 The structure of P2X receptors

Functional P2X receptors are typically trimers (Nicke et al., 1998, Stoop et al., 1999, Bean, 1990, Barrera et al., 2005, Browne et al., 2011, North, 2002, North, 2016),

and experimental data regarding P2X receptor structure has been corroborated by the solving of the zebrafish P2X4 receptor crystal structure (Kawate et al., 2009) (see figure 1.3.2). Each P2X receptor trimer is thought to be activated by 3 molecules of ATP, where each molecule binds to (or between) each monomeric subunit (Bean, 1990, Coddou et al., 2011), as demonstrated in figure 1.3.2. Some data suggests that 2 molecules of ATP can fully activate P2X receptors, notably P2X2 receptors (Stelmashenko et al., 2012). P2X receptors do not always form homotrimers however, and functional heteromers have been observed.

Each P2X receptor monomer contains 2 hydrophobic transmembrane domains joined by a large intervening hydrophilic extracellular loop, and each has a pore-forming domain (Brake et al., 1994, Valera et al., 1994) (North, 1996, Ralevic and Burnstock, 1998). This P2X receptor monomeric structure is famously likened to the shape of a dolphin (see figure 1.3.2).

1.3.3.1.1 Homomeric P2X receptors

All P2X receptor subtypes form endogenous functional homotrimers except for P2X5 and P2X6 receptors. P2X5 occurs in humans predominantly as a non-functional (truncated) splice variant (Lê et al., 1997, Bo et al., 2003, Duckwitz et al., 2006), whilst data suggests that P2X6 subunits form tetramers and aggregates in the ER and so are not exported to the plasma membrane (Soto et al., 1996, Khakh et al., 1999b, Torres et al., 1999, King et al., 2000, Aschrafi et al., 2004, Barrera et al., 2005, Ormond et al., 2006). Further data using atomic force microscopy also suggests that P2X6 does not form functional homomers (Barrera et al., 2005).

When cloned, P2X5 and P2X6 receptors were expressed in human embryonic kidney (HEK) cells, and P2X5 homomers were observed to give extremely small currents (but with currents observed in 90-95% of transfected cells), whilst P2X6 homomers gave functional currents in an extremely small population of cells (0-15%) (Collo et al., 1996). Therefore, they are not thought to readily form functional homotrimers in native tissues unless they are N-linked glycosylated (see section 1.3.3.1.4) (Jones et al., 2004, Ormond et al., 2006).

Despite the conflicting evidence regarding their functional homomeric expression, P2X5 receptor transcripts are predominantly expressed in tissues related to immune system (Le et al., 1997) and it has been implicated in cell differentiation, for

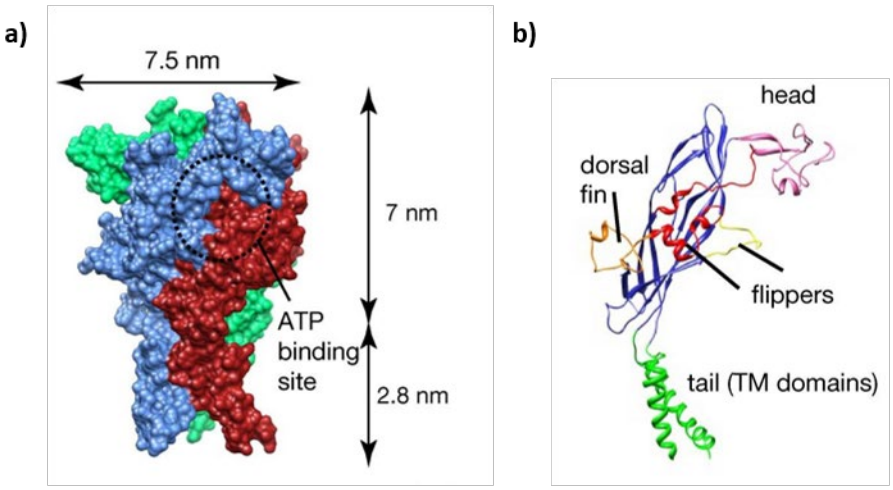

Figure 1.3.2. P2X receptor crystal structure.
The crystal structures of the P2X receptor a) trimer and b) monomer are shown. In a), the ATP binding site can be seen between two P2X receptor monomers. The monomeric form is likened to that of a dolphin, where the TM domains form a tail and the extracellular loop forms a dorsal fin, flippers and a head.
Figure modified from Young, 2010.

example in mouse neurogenesis (Guo et al., 2013). P2X6 receptors are predominantly expressed in skeletal muscle in humans, and have been implicated in oncogenesis (Urano et al., 1997).

It should also be noted that P2X1 can form hexamers in addition to trimers, but intermediate forms were not observed (Nicke et al., 1998). Also, despite the observation of P2X7 monomers in glial cells/astrocytes (Kim et al., 2001b), its native assembly is still assumed to be homotrimeric (Nicke, 2008).

1.3.3.1.2 Heteromeric P2X receptors

Native functional heteromers increase the diversity of, and add additional regulatory aspects to, receptor signalling. Some P2X receptor subunits appear to readily form heteromers, including P2X1 receptor monomers which form functional P2X1/P2X2 (Torres et al., 1999, Brown et al., 2002, Aschrafi et al., 2004, Marquez-Klaka et al., 2009), P2X1/P2X4 (Nicke et al., 2005) and P2X1/P2X5 (Torres et al., 1998b, Haines et al., 1999, Lê et al., 1999, Surprenant et al., 2000) heteromers. P2X1/P2X3 heteromers have also been observed (Nicke et al., 1998), although there appears to be a lack of functional evidence for this heteromer.

P2X2 receptor monomers are also known to form functional heteromers with P2X3 (Lewis et al., 1995, Werner et al., 1996, Radford et al., 1997, Jiang et al., 2003, Hausmann et al., 2012) and P2X6 (King et al., 2000, Barrera et al., 2005, Barrera et al., 2007, Hausmann et al., 2012) receptor subunits. Furthermore, functional P2X4/P2X6 receptor heteromers have been observed (Soto et al., 1996, Lê et al., 1998, Khakh et al., 1999b).

Data originally suggested that P2X7 receptor monomers did not co-assemble with any other P2X monomers (Torres et al., 1999, North, 2002), which could make sense considering its specific pharmacological properties, whilst P2X5 receptor monomers co-assembled with all but P2X7 subunits (North, 2002). Further data suggested that P2X7 receptor subunits have physical and functional interactions with P2X4 (Guo et al., 2007, Casas-Pruneda et al., 2009, Weinhold et al., 2010), although this is still controversial (Nicke, 2008) and even more recent data suggested that whilst P2X4 and P2X7 subunits do physically interact, they do not result in functionally distinct profiles. This could be due to the subunit stoichiometry where the more frequent receptor subtype dominates the functional profile (Schneider et al., 2017).

Data from recombinant expression studies must be considered carefully. Although different subunits may physically co-assemble in experimental systems, this does not mean that they readily co-assemble in native tissues. Furthermore, if they do co-assemble natively, this does not imply that they are functional, particularly as heteromer function is not always studied in co-expression studies. The best way to establish if heteromers function in native tissues is to determine if they're co-expressed, and then to perform functional investigations with the native tissues.

Investigations into P2 receptor expression in native tissues often show expression overlaps, which could suggest heteromer expression (Burnstock and Knight, 2004). For example, P2X1/P2X2 heteromers are expressed in a number of tissues, including vascular smooth muscle (Nori et al., 1998, Brown et al., 2002). Despite functional evidence when heterologously expressed in Xenopus oocytes (Brown et al., 2002) however, functional evidence of P2X1/P2X2 heteromers in native tissues is limited (Saul et al., 2013). Therefore, conclusions regarding functional heteromer expression in native tissues must be made conservatively.

There is a functional consequence to the presence of functional heteromers in native or heterologous expression systems as heteromers often have different functional and pharmacological profiles than homomers. For example, P2X1/P2X5 heterotrimers demonstrate pharmacology similar to that of P2X1 receptors [including sensitivity to α,β-methylene ATP (α,β-meATP)] but with modified kinetics (more slowly desensitizing than P2X1) (Lê et al., 1999). Another instance of this presents in P2X2/P2X3 heterotrimers, which functionally resemble P2X3 receptors overall (Jiang et al., 2003). However, P2X2 homomers are the only P2X homomers with Zn^{2+} sensitivity and where acidification potentiates ATP-induced currents. P2X2-containing heteromers, including P2X2/P2X3 receptors, adopt these characteristics (Li et al., 1996, Stoop et al., 1997, King et al., 2000). This is important to note, particularly when studying receptor expression in native tissues.

1.3.3.1.3 Alternative splicing of P2X receptors

The presence of splice variants can often add functional diversity to a population, and splice variants were originally reported for all P2X receptors but P2X7 (North, 2002). However, splice variants for P2X7 receptor have now been detected, with

serious implications for the use of P2X7 knock-out mice from Pfizer, as this functional splice variant was not targeted (Nicke et al., 2009). Furthermore, the P2X7 gene in particular is known to be extremely polymorphic in humans (Fuller et al., 2009), which could have implications for disease. For example, P2X7 plays a role in bone formation (Panupinthu et al., 2008) and loss-of-function mutations in P2X7 have been correlated with increased risk of fractures in post-menopausal women (Ohlendorff et al., 2007). Therefore, it is important to be aware of splice variants and polymorphisms, and their functional consequences.

1.3.3.1.4 Post-translational modifications of P2X receptors

Post-translational modifications, such as glycosylation and phosphorylation, can modify the function in addition to the molecular weights of receptors, and P2X receptors undergo these modifications. For example, P2X1 receptor has 4 conserved glycosylation sites which can all be glycosylated (Nicke et al., 1998). Furthermore, P2X2 receptor has 3 conserved glycosylation sites, all of which can be glycosylated (Newbolt et al., 1998, Torres et al., 1998a). P2X3-P2X7 receptors have 4, 6, 2, 3 and 3 glycosylation sites, respectively (North, 2002). It is accepted that glycosylation is actually necessary for the function of these receptors, particularly in the case of P2X2 where 2/3 sites glycosylated gives fully functional receptor at cell surface, whilst any less give small and non-existent currents respectively (Torres et al., 1998a). Indeed, it has been suggested that the lack of glycosylation of P2X6 receptors prevents their function (Jones et al., 2004, Ormond et al., 2006).

P2X receptor phosphorylation is sometimes integral to their function. For example, P2X1 receptors demonstrate basal phosphorylation (Vial et al., 2004) which, when disrupted, greatly impairs their function and the receptors desensitized ~10-fold faster than wild-type receptors (Ennion and Evans, 2002). However, the regulation of desensitization by phosphorylation is thought to be mediated by an accessory protein of the P2X1 complex, and not by the phosphorylation of P2X1 protein itself (Vial et al., 2004). Data also suggests that P2X7 receptor is basally phosphorylated and its activation leads to dephosphorylation (Kim et al., 2001a), which is thought to have functional consequences.

1.3.3.2 Endogenous ligands of P2X receptors

As described, P2X receptors are activated by their endogenous ligand, ATP, but not by ATP breakdown products or uracil-containing nucleotides (Abbracchio and Burnstock, 1994, Ralevic and Burnstock, 1998, Coddou et al., 2011, Jacobson and Muller, 2016). However, the various types of P2X receptor trimers display different affinities and EC_{50}s for ATP. The most notable subtype in this regard is P2X7 which has a low affinity for ATP and so must be activated by high concentrations in the milimolar range, as discussed in section 1.3.3.3. A summary of the endogenous agonist preferences and some important features of P2X receptor activation is shown in table 1.1.

1.3.3.3 P2X7 is functionally distinct from other P2X receptors

P2X7 receptors are often considered functionally distinct from the other P2X receptors. For example, they demonstrate a much lower affinity for ATP which makes them quite insensitive to ATP compared to other P2X receptors, and their activation typically requires millimolar concentrations of ATP (Surprenant et al., 1996, Rassendren et al., 1997, North, 2002).

Furthermore, whilst the initial activation of P2X7 receptors is similar to other P2X receptors in that it opens the non-selective cation channel (Surprenant et al., 1996, North, 2002, Coddou et al., 2011, Jiang et al., 2013), it has also been observed that repeated or continued application of agonist not only increases the affinity of the receptor to ATP but also increases the activity of the channel (Kim et al., 2001b, North, 2002, Roger et al., 2008, Roger et al., 2010). This then regulates a permeation pathway for large molecules, up to ~800-900 Da, including cellular metabolites and even fluorescent dyes (Gomperts, 1983, Steinberg et al., 1987, Rassendren et al., 1997, Wei et al., 2016). Therefore its activation can be described as biphasic (Coddou et al., 2011).

Two mechanisms for this biphasic response and the receptors large dilation have been proposed. There was initially assumed to be a dilatation of the P2X7 trimeric pore in the membrane (Surprenant et al., 1996, Coutinho-Silva et al., 1999, Chessell et al., 2009, Jiang et al., 2013). However, evidence has also suggested that P2X7 activation induces the recruitment of distinct pore-forming proteins, including the pannexin-1 channel, whose inhibition significantly reduces measurements of pore-function, such as dye-uptake assays (Pelegrin and Surprenant, 2006, Iglesias et al., 2008, Pelegrin and Surprenant, 2009, Sorge et al., 2012). Furthermore, whilst expression of native P2X7

Homomeric receptor subtypes	Agonist preference	Notable features
P2X1	ATP	-Rapid desensitization
P2X2	ATP	-Low pH -Zinc & copper sensitivity (potentiation) -Slow desensitization
P2X3	ATP	-Rapid desensitization
P2X4	ATP	-Ivermectin, cibacron blue and zinc potentiation -Slow desensitization - Insensitive to suramin/PPADS
P2X5	ATP	-Small currents compared to other P2X -Slow desensitization
P2X6	No functional homomer	-No functional homomer
P2X7	ATP (low affinity)	-Large pore -Prolonged activation -No desensitization -Inhibition by cations

Table 1.1. The endogenous agonists and notable features of P2X receptors.

receptors in Xenopus oocytes does not result in a pore-forming channel (only a non-selective cation channel), co-expression with pannexin-1 allows the formation of the pore (Locovei et al., 2007).

It must also be discussed that the other slowly desensitizing receptors (P2X2, P2X4 and the heteromeric P2X2/P2X3) have been shown to have additional permeation states/pore-forming capabilities (Khakh et al., 1999a, Virginio et al., 1999), perhaps lending support to the theory that it is the P2X7 trimeric pore itself which dilates.

1.3.3.4 Desensitisation of P2X receptors

Receptor desensitization can typically be described as the uncoupling of a receptor from its signalling and downstream effects after its activation, and holds physiological importance as it allows further modulation of receptor signalling beyond ligand presence and binding. For P2X receptors specifically, desensitization is the 'decline in the current elicited by ATP during the continued presence of ATP' (North, 2002), and has large implications for the function and the study of these receptors.

There are two groups of P2X receptors where desensitization is concerned. P2X1 and P2X3 receptors rapidly desensitize (within 100 - 300 ms) whilst P2X2, P2X4, P2X5 and P2X7 receptors slowly desensitize, with currents being maintained during continued activation for at least a few seconds (Ralevic and Burnstock, 1998, North, 2002).

The rapidly desensitizing P2X1 receptor gives smaller currents upon both repeated (Valera et al., 1994) and continuous (Coddou et al., 2011) activation. Data suggests that this could be due to receptor internalization after activation (Dutton et al., 2000, Ennion and Evans, 2001), and receptor phosphorylation is implicated in the regulation of its desensitization (Ennion and Evans, 2002). P2X3 receptor on the other hand, which also shows rapid desensitization (Zemkova et al., 2004), does not show complete desensitization to continued agonist application, unlike P2X1 (Coddou et al., 2011). There is even thought to be 2 types of desensitization in P2X3 receptors (which could be similar for P2X1 receptors), including a fast desensitization with a half time ($t_{1/2}$) of around 50 ms when activated with ATP 10 μM and a slower desensitization with $t_{1/2}$ of 35 s which can be triggered with even lower concentrations of ATP, such as 10 nM (Sokolova et al., 2006). However, such a low affinity for the slower desensitization could mean that this portion is consistently desensitized in physiological systems, raising

questions regarding its physiological relevance.

P2X receptor desensitization appears to be regulated by residues at both the N and the C termini (North, 2002, Coddou et al., 2011). Whilst exchange of either the N or C terminus of the P2X1 (rapidly desensitizing) receptor with that of the P2X2 (slowly desensitizing) receptor abolished the observed fast desensitization, both the N and C termini of the P2X2 receptor must be exchanged with that of P2X1 receptor in order to introduce rapid desensitization (Werner et al., 1996). Post-translational modification (glycosylation or phosphorylation) of these termini, or their interaction with intracellular proteins, could explain their involvement in receptor desensitization. Furthermore, external factors including extracellular Ca^{2+} can also affect receptor desensitization (North, 2002, Coddou et al., 2011).

1.3.4 P2Y receptors

The P2Y receptor structure and properties, including functional coupling to G proteins which can have implications for their function, will be explored here.

1.3.4.1 The structure of P2Y receptors

P2Y receptors are GPCRs and so have the general GPCR receptor structure consisting of seven transmembrane helices (von Kügelgen and Hoffmann, 2016), supported by the solving of the crystal structures for the P2Y1 and P2Y12 receptors (Zhang et al., 2014a, Zhang et al., 2014b, Zhang et al., 2015). P2Y receptors can also couple to different G proteins, which has implications for their functional diversity (von Kügelgen and Hoffmann, 2016).

1.3.4.1.1 Functional coupling of P2Y receptors

Broadly categorised, P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 receptor are all Gq-coupled receptors, whilst P2Y12, P2Y13 and P2Y14 receptors are Gi/o-coupled receptors (von Kügelgen, 2006, von Kügelgen and Harden, 2011, Erb and Weisman, 2012, Jacobson et al., 2012). In addition to being Gq-coupled, data suggests that P2Y11 is also Gs-coupled (Qi et al., 2001, Communi et al., 2009, von Kügelgen and Harden, 2011). There is also an indication that P2Y2 and P2Y4 receptors could display some Gi/o coupling in addition to Gq coupling (Jacobson and Boeynaems, 2010, Jacobson et al., 2012), but these differences in observed coupling could be due to where the receptor is

expressed (*i.e.* in native or recombinant systems). For a summary of P2Y receptor G protein coupling, see table 1.2.

1.3.4.1.2 Multimeric P2Y receptors

GPCRs are typically thought to function as monomers but are known to form homo- and hetero-dimers (Angers et al., 2002), and P2Y receptors are generally considered to follow this rule (Erb and Weisman, 2012). Functional P2Y4, P2Y6, P2Y12 and P2Y13 homodimers have been observed (Savi et al., 2006, D'Ambrosi et al., 2007). Additionally, P2Y1 receptors form functional heterodimers with P2Y2, P2Y4, P2Y11 and P2Y12 receptors (Ecke et al., 2008, Shrestha et al., 2010, Ribeiro-Filho et al., 2016). Functional P2Y4/P2Y6 heterodimers have also been observed (D'Ambrosi et al., 2007). Heterodimers between adenosine and P2Y receptors result in further signalling diversity. Notably, functional A1/P2Y1 and A1/P2Y2 heterodimers have been observed (Yoshioka et al., 2001, Suzuki et al., 2006).

As with P2X receptor heteromers, P2Y receptor heteromers also display functional differences to their monomeric or homomeric counterparts. For example, P2Y11 receptor does not typically internalise upon activation, yet activation of the P2Y1/P2Y11 heteromer results in its internalization (Ecke et al., 2008).

1.3.4.1.3 Alternative splicing of P2Y receptors

P2Y11 is the only P2Y receptor to contain an intron. Furthermore, there appears to be intergenic splicing between the *P2RY11* and adjacent *PPAN* genes in humans, creating a fusion P2Y11-PPAN protein. Despite acknowledgement of its widespread expression, the functional significance of this fusion protein remains unknown, although data suggests that the resulting protein is more unstable than P2Y11 receptor alone (Communi et al., 1997, Communi et al., 2001, Jacobson and Boeynaems, 2010, Dreisig and Kornum, 2016).

1.3.4.1.4 Post-translational modifications of P2Y receptors

P2Y receptors also undergo post-translational modifications, including glycosylation and phosphorylation. It is believed that all P2Y receptors undergo some glycosylation and that, as expected, this glycosylation can affect their function and can even be necessary for correct function and trafficking of the receptor (Nishimura et al., 2017). For example, P2Y12 receptor contains two potential glycosylation sites. Whilst

Homomeric receptor subtypes	Agonist preference	G protein coupling
P2Y1	ADP>ATP	-Gq
P2Y2	UTP=ATP>>ADP,UDP	-Gq -Possibly Gi
P2Y4	UTP	-Gq -Possibly Gi
P2Y6	UDP>UTP	-Gq
P2Y11	ATP	- Primarily Gs - Gq
P2Y12	ADP>>ATP	-Gi/o
P2Y13	ADP>>ATP	-Gi/o
P2Y14	UDP-glucose, UDP	-Gi/o

Table 1.2. The endogenous agonists and functional coupling of P2Y receptors.

impairment of its glycosylation does not appear to affect its ligand binding or expression at the cell surface, correct glycosylation appears to be necessary for its signalling (Zhong et al., 2004). P2Y2 receptor also contains two potential glycosylation sites and its glycosylation is required for its trafficking to the cell surface membrane (Nakagawa et al., 2017). P2Y receptors also undergo phosphorylation, which can regulate their activity. For example, P2Y2 receptor is regulated by agonist-induced phosphorylation, which is thought to affect its desensitization (Flores et al., 2005)

1.3.4.2 Endogenous ligands of P2Y receptors

P2Y receptors, unlike P2X receptors, are activated by a variety of nucleotides including ATP, ADP, UTP, UDP, and UDP-sugars such as UDP-glucose and UDP-galactose (von Kugelgen and Wetter, 2000, von Kügelgen, 2006, von Kügelgen and Harden, 2011, Jacobson et al., 2012, von Kügelgen and Hoffmann, 2016). For a summary of the reported endogenous nucleotide ligands for P2Y receptors, see table 1.2.

There are, however, some important individual features to note regarding P2Y receptor ligands. For example, P2Y2 receptor has historically been thought to be activated only by ATP and UTP, and not by ADP or UDP. However, data using canine and porcine P2Y2 receptors suggests that P2Y2 receptor can also respond to ADP and UDP (Zambon et al., 2000, Shen et al., 2004, von Kügelgen, 2006). Furthermore, P2Y14 is widely known to be activated by UDP-glucose. However, human P2Y14 receptor has also been reported to be activated by UDP itself (Carter et al., 2009, von Kügelgen and Harden, 2011, Jacobson et al., 2012, von Kügelgen and Hoffmann, 2016).

1.3.4.3 Desensitization of P2Y receptors

GPCRs also demonstrate receptor desensitization which is specifically defined here as a reduced ability to be stimulated due to the exhibition of a ‘memory’ of prior activation (Hausdorff et al., 1990, Gainetdinov et al., 2004). P2Y receptors, as GPCRs, also follow this rule and are known to desensitize.

One of the better studied P2Y receptors regarding its desensitization is the P2Y2 receptor (Garrad et al., 1998, Otero et al., 2000, Santiago-Pérez Laura et al., 2001, Flores et al., 2005). For P2Y2, agonist-induced receptor phosphorylation (Santiago-Pérez Laura et al., 2001, Flores et al., 2005), and phosphorylation of the C terminus of the receptor particularly (Otero et al., 2000), is involved in its desensitization. Furthermore,

(reversible) receptor internalisation and sequestration are thought to play a role too (Garrad et al., 1998, Sromek and Harden, 1998, Flores et al., 2005). Data suggests, however, that receptor desensitization and sequestration are discrete events and one is not necessary for the other to occur.

Studies involving other well-characterized GPCRs, and particularly the β2-adrenoreceptor, suggest that there are two levels of GPCR desensitization: short-term desensitization (with a 30 min exposure to agonist) and long-term desensitization (exposure over hours or even days). Short-term desensitization appears related to receptor modifications which regulate their function, such as phosphorylation or ubiquitination, or even arrestin-receptor binding and receptor internalisation, whilst long-term desensitization is related to the modulation of receptor expression (Lohse et al., 1996, Rajagopal and Shenoy, 2018). Data regarding P2Y2 receptor desensitization corroborates this idea, particularly as prolonged treatment with UTP (thought to activate a longer-stage desensitization) correlates with a decrease in P2Y2 transcript expression (Santiago-Pérez Laura et al., 2001), and suggests that the same phenomenon occurs in P2Y receptors.

It is interesting to note that different agonists can activate different desensitization pathways for the same receptor. For example, stimulation of P2Y2 receptors with ATP activates a different desensitization pathway than when activated with UTP, providing some explanation as to why desensitization with UTP occurs at lower concentrations than ATP, despite the ligands having the same potencies (Velázquez et al., 2000, Hoffmann et al., 2008). Furthermore, different P2Y receptors expressed in even the same cells can be desensitized in different ways. For example, it was originally thought that only P2Y1 receptors in platelets desensitized, and not P2Y12 receptors (Baurand et al., 2005). However, it was later established that P2Y12 receptors also desensitize and, for both receptors, this process is also regulated by their phosphorylation. However, the desensitization-inducing phosphorylation is mediated by GRKs in P2Y12, whilst that of P2Y1 is PKC-mediated (Hardy et al., 2005, Rodríguez-Rodríguez et al., 2009). This demonstrates the diversity of functional regulation even within the same receptor family.

1.3.5 The physiological roles of P2 receptors

When determining the functional expression and physiological roles of receptors in native tissues, it is often necessary to first establish expression and then function. Where there is low/restricted protein expression, it follows that there could be low/restricted function. For example, P2X2 and P2X3 are predominantly expressed in sensory neurones (Chen et al., 1995, Collo et al., 1996, Ralevic and Burnstock, 1998, Lewis et al., 1995). The absence of P2X3 is noted in tissues including the lung and other P2X receptor-rich smooth muscles, such as the *vas deferens* and bladder (Chen et al., 1995, Ralevic and Burnstock, 1998, North, 2002). Therefore, there has been a lack of functional P2X3 detected in these latter tissues.

However, P2 receptors are typically widely expressed and therefore are involved in a wide range of physiological functions in numerous tissues.

1.3.5.1 The regulation of immune cell functions by P2 receptors

P2 receptors are involved in the physiological functions of a wide range of immune cells. For example, P2X1 regulates neutrophil chemotaxis (Lecut et al., 2009), whilst P2Y11 regulates neutrophil apoptosis (Vaughan et al., 2007). Furthermore, functional P2X1, P2X4 and P2X7 receptors are found in mast cells, corroborating their roles in mast cell biology including chemotaxis, degranulation and apoptosis (Wareham et al., 2009, Bulanova and Bulfone-Paus, 2010). P2Y14 has even been implicated in regulating mast cell degranulation (Gao et al., 2010). In addition, P2 receptors, and specifically P2X1 receptors, regulate eosinophil migration and adhesion (Wright et al, 2016)(Alberto et al., 2016).

Additionally, functional P2X4 receptors have been observed in microglia and alveolar macrophages from rat, with data also indicating functional P2Y1 and P2Y2 receptors in the latter (Bowler et al., 2003). Currents for both P2X4 and P2X7 receptors have also been detected in murine peritoneal macrophages (Brône et al., 2007), correlating well with other studies that implicate P2X4 receptors in PGE_2 and cytokine production in macrophages (Ulmann et al., 2010, Layhadi et al., 2017). The role of P2X7 receptors in the immune system, and particularly in the regulation of IL-1β maturation and release in macrophages, is well documented (Solle et al., 2001, Labasi et al., 2002, Ferrari et al., 2006, Riteau et al., 2010).

Further complexity regarding P2 receptor signalling and the immune system is also present as PGE_2 has been observed to impair P2Y receptor-mediated macrophage migration (and so aiding the resolution of inflammation) (Traves et al., 2013).

1.3.5.2 P2 receptors and platelet functions

P2 receptors are also integral for platelet cell functions, which are involved in thrombosis (Holinstat, 2017) and are also effectors of the immune system (Trzeciak-Ryczek et al., 2013). The typical 'platelet P2 receptors' are P2X1, P2Y1 and P2Y12 receptors, which regulate platelet shape change and aggregation (Daniel et al., 1998, Jin and Kunapuli, 1998, Dorsam and Kunapuli, 2004). Indeed, evidence suggests that both P2Y1 and P2Y12 are essential for platelet aggregation (Jin and Kunapuli, 1998). Platelets also express functional P2X1 receptors which contribute to their aggregation and granule secretion (Oury et al., 2003, Mahaut-Smith et al., 2011). Therefore, P2 receptor dysfunction in platelets is linked to defective secretion and aggregation (Cattaneo et al., 2000, Foster et al., 2001), and so congenital bleeding (Nurden et al., 1995, Cattaneo et al., 2003).

1.3.5.3 The regulation of smooth muscle contraction and relaxation by P2 receptors

P2 receptors are also known for their roles in the regulation of smooth muscle tone. The study of purinergic innervation demonstrated a role for P2 receptors in the contraction of smooth muscles from a number of sources, including guinea-pig urinary bladder and *vas deferens* (Burnstock et al., 1978a, Westfall et al., 1978). Since then, P2 receptors have been widely explored in the context of smooth muscle contraction. Indeed, P2X1 receptor expressed on smooth muscle cells plays a dominant role in the regulation of smooth muscle contraction (Mulryan et al., 2000, Vial and Evans, 2000b, Vial and Evans, 2002), whilst P2X4 receptors expressed on endothelial cells are implicated in vascular tone regulation in response to flow (Yamamoto et al., 2006). P2Y receptor activation, including that of P2Y12, has also been shown to induce the contraction of mouse urinary bladder (Yu et al., 2014).

P2 receptors have also been implicated specifically in the regulation of ASM contraction. For example, both ATP and UTP were shown to contract isolated guinea-pig trachea (Fedan et al., 1993) and further pharmacological data implicated P2X1 receptor in this (Candenas et al., 1992). P2X receptors have been associated with rat

ASM contraction (Mounkaila et al., 2005, Gui et al., 2011), whilst P2X4 and P2Y2/4 receptors have specifically been linked with the contraction of porcine and murine ASM, respectively (Bergner and Sanderson, 2002, Nagaoka et al., 2009). P2 receptors have also been specifically associated with human ASM contraction. Inhalation of ATP induced bronchoconstriction in both healthy and asthmatic subjects (Pellegrino et al., 1996). Furthermore, P2X and P2Y1 receptors have been implicated in the contraction of human intra-pulmonary bronchi and cHASM cells, respectively (Govindaraju et al., 2005, Mounkaila et al., 2005).

P2 receptor activation not only induces smooth muscle contraction, but plays a role in the regulation of smooth muscle contractility (*i.e.* in its sensitization). For example, nucleotides including the non-hydrolysable nucleotide analogue GTPγS increase the phosphorylation of MLC20 in rabbit vascular smooth muscle (Kitazawa et al., 1991a), potentially via decreased MLCP activity (Kitazawa et al., 2003), and data also suggests that this mechanism exists in bovine ASM (Kubota et al., 1992). Specific P2 receptors have been implicated in this sensitization process, including P2Y6 receptor whose activation enhanced the P2X1-induced contraction of mouse bladder smooth muscle (Yu et al., 2013). Furthermore, the P2X1-selective agonist α,β-meATP induced airway hyperresponsiveness in isolated, epithelium-free guinea-pig trachea, with sensitization of methacholine-induced contraction potentially mediated by RhoK (Oguma et al., 2007).

However, P2 receptor activation also induces smooth muscle relaxation, in addition to its contraction. For instance, purinergic nerves are involved in the relaxation of guinea-pig intestinal smooth muscle (Burnstock et al., 1978b) and the endothelium-dependent relaxation of porcine aorta (Martin et al., 1985). Also, the activation of P2Y2 receptors on airway epithelial cells induces PGE_2 production, leading to ASM relaxation (Marcet et al., 2007). Therefore, it is important to appreciate that P2 receptors can have opposing actions regarding smooth muscle from different tissues, and particularly from different species.

Therefore, these data not only implicate P2 receptors in the direct stimulation of ASM contraction and relaxation, but also in its sensitization.

1.3.5.4 The regulation of cell proliferation and apoptosis by P2 receptors

P2 receptors also play a role in core cellular processes including cell proliferation and apoptosis. For example, P2X7 receptor activation is strongly associated with the induction of cell apoptosis and even necrosis, which is regulated by several mediators including reactive oxygen species (ROS) and caspases (Ferrari et al., 1999, Kong et al., 2005, Bartlett et al., 2013, Chen et al., 2013, Massicot et al., 2013). Furthermore, P2Y1 receptor activation has also been implicated in the induction of cell apoptosis (Coutinho-Silva et al., 2005), whilst that of P2Y6 receptors has been shown to protect cells from apoptosis (Kim et al., 2003). Other P2 receptors, including P2Y2/4 receptors, directly induce cell proliferation (Coutinho-Silva et al., 2005).

Some P2 receptors have even been specifically implicated in the regulation of ASM cell proliferation. For instance, extracellular ATP and UTP induce the proliferation of rat ASM cells (Michoud et al., 1997, Michoud et al., 1999, Michoud et al., 2002), potentially mediated via P2Y2 receptor, whilst P2Y2 and P2Y6 receptors expressed on cultured ASM cells from lung have been shown to regulate their proliferation (Abbracchio et al., 2006).

1.3.5.5 The roles of extracellular nucleotides and P2 receptors in the airways

Due to the numerous different mechanisms of nucleotide release into the extracellular space, especially by mechanical stimulation (as described in section 1.3.1.4.4), and to the regulation of nucleotide concentrations by ectonucleotidases (to be discussed in section 1.3.6), it is extremely difficult to accurately measure the resting extracellular nucleotide concentrations in the airways. However, one group has estimated the concentration of extracellular ATP in the nasal ASL of humans at ~470 nM ± 131 nM (Donaldson et al., 2000). This estimated resting concentration, in addition to potential further induction of nucleotide release during inflammation (as described in section 1.3.1.4.2) and the presence of microdomains which would lead to even higher concentrations, makes it feasible to consider that all P2 receptor subtypes could be activated in physiological and pathophysiological conditions.

Therefore, it is not surprising that extracellular nucleotides have numerous roles in the airways, including regulating airway inflammation (as described in section 1.3.5.1) and inducing ASM cell contraction and proliferation (as outlined in sections 1.3.5.3 and 1.3.5.4, respectively). P2 receptors also regulate chloride secretion in the airway

epithelium (Knowles et al., 1991), which therefore regulates mucous viscosity by osmosis and has implications for airways diseases including cystic fibrosis (CF). This function has been attributed to P2Y2 receptor and the mechanism, which is still functional in epithelia from CF patients, has been shown to be independent of the CFTR (Clarke and Boucher, 1992, Stutts et al., 1992). However, CF epithelia fail to release ATP under basal conditions due to dysfunctional CFTR (Taylor et al., 1998), which could explain the defective chloride secretion observed in CF patients.

1.3.5.6 Pharmacological tools for studying P2 receptors

The elucidation of P2 receptor functions, and particularly the assignment of these functions to a specific P2 receptor subtype, has been greatly hindered by the lack of selective agonists and antagonists. There is high receptor homology between the P2 receptors and so there can be cross-reactivity of activators and inhibitors. However, there are now a number of commercially available selective pharmacological tools which have aided the clarification of P2 receptor functions, some of which have even been used in clinical trials due to their promise in treating diseases. This section will explore the different tools currently available.

The wide array of pharmacological tools used in the laboratory have been extensively reviewed (Coddou et al., 2011, Jacobson and Muller, 2016), and those used in this report have been summarised in tables 1.3 and 1.4. However, there are some important points to make regarding selective targeting of P2 receptors using pharmacological tools.

Cultured cells are often used to study the functions of both receptors and cells. A common component of growth media is phenol red, which has been shown to antagonise rat P2X1 receptors and therefore could give false negatives when they are studied (King et al., 2009). Furthermore, compounds are often dissolved in solvents such as dimethyl sulfoxide (DMSO). However, DMSO itself showed some activity at rat P2X1 and P2X4 (King et al., 2009), and this demonstrates the importance of performing experimental controls. 3'-O-(4-Benzoyl)benzoyladenosine 5'-triphosphate (BzATP) has historically been used as a selective agonist for P2X7 receptor, particularly as it is a more potent activator of the receptor than ATP (Surprenant et al., 1996, Rassendren et al., 1997, Donnelly-Roberts Diana et al., 2009). However, it has now been shown to be active at other P2X receptor subtypes, and so is not suitable for use as a selective agonist

Target receptor	Drug	EC50 (μM)	Concentration used in this report (μM)	Use in literature				
				Species	Cell/tissue type	Concentration used (μM)	Assay type (observed function)	Reference
P2X1, P2X3	α,β-methylene ATP	1-3, 1	10	Murine	*Vas deferens*	10	Myography (induced contraction)	Mulryan *et al.,* 2000
					Bladder	10		Yu *et al.,* 2013
				Human	LAD2 lung mast cell line	10	Electrophysiology (induced P2X1-like currents)	Wareham *et al.,* 2009
					Primary eosinophils	10		Wright et al., 2016
P2Y4	MRS4062	0.023	3	Rat	Cardiac fibroblasts	10	Calcium imaging (induced oscillations), cell proliferation (no effect)	Certal *et al.,* 2015
					Glomeruli podocytes	1, 10 and 50	Calcium imaging (no responses observed)	Ilatovskaya and Staruschenko, 2013
				Murine	Cardiac adipose-derived stem cells	100	Immunocytochemistry (negative regulation of cell differentiation)	Lemaire *et al.,* 2017
				Human	MCF-7 breast cancer cell line	1 and 10	Cell migration (no effect with 1 but induced migration with 10)	Chadet *et al.,* 2014
P2Y6	MRS2693	0.015	10	Murine	C2C12 skeletal muscle cell line	≤1	Flow cytometry (anti-apoptotic effect observed)	Mamedova *et al.,* 2008
					Bladder	1.5	Myography (induced contraction alone and sensitized P2X1-mediated contraction)	Yu *et al.,* 2013
				Human	Primary monocytes	10 and 30	Flow cytometry (induced differentiation)	Obba *et al.,* 2015

Table 1.3. List of selective P2 receptor agonists used in this study.

Target receptor	Drug	IC50 (μM)	Concentration used in this report (μM)	Use in literature				
				Species	Cell/tissue type	Concentration used (μM)	Assay type (observed effect)	Reference
P2X4	5-BDBD (5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one)	0.5 - Hamster	20	Guinea-pig	Isolated cochlear endothelial cells, *in vivo* spiral ligament capillaries	30	Electrophysiology (decreased current amplitudes induced by ATP 1mM), *in vivo* imaging (blocked ATP-induced capillary dilation)	Wu *et al.*, 2011
				Murine	*In vivo* ovalbumin-induced allergic model	30	Reduced several signs of airway inflammation	Chen *et al.*, 2016
				Human	HK-2 kidney epithelial cell line	2	Calcium imaging (decreased high-glucose induced calcium rises)	Chen *et al.*, 2013
					Embryonic stem cell-derived microglia	1 and 20	Phagocytosis and migration (reversed suppression of phagocytosis and migration induced by ethanol), calcium imaging (decreased ethanol-induced calcium rises)	Gofman *et al.*, 2014
	PSB12054	0.2 - Human, 1.8 - Mouse	10	x	x	x	x	x
P2X7	AZ10606120 dihydrochloride	0.2 - Panda	1	Murine	J774 macrophage cell line	10	ELISA (inhibited ATP-induced IL-1β release)	Sluyter and Vine, 2016 and Bhaskaracharya *et al.*, 2014
				Murine	Pancreatic stellate cells	10	Calcium imaging (inhibited BzATP-induced calcium rises), BrdU-uptake assays (inhibited ATP-induced proliferation)	Haanes *et al.*, 2012
				Murine, Human	Primary B cells	10, 0.1	Flow cytometry (inhibited ATP-induced receptor shedding in both cell types at respective concentrations)	Pupovac *et al.*, 2015
P2Y1	MRS2179	0.3 - Turkey	10	Guinea-pig	Ileal smooth muscle cells	10	Calcium imaging and electrophysiology (blocked ATP-mediated inhibition of IP3-induced calcium responses)	MacMillan *et al.*, 2012
				Murine	Leukocytes	0.3	Adhesion (reduced 2-MeSATP-induced adhesion)	Oliveira *et al.*, 2016
				Murine	Macrophages and vascular smooth muscle cells	100	Proliferation (inhibited unstimulated smooth muscle cell proliferation by cell counting and MTT assay), migration (inhibited stimulated migraiton of both cell types)	Liu *et al.*, 2015
				Rat, human	Platelets	3 and 10	Calcium imaging (inhibited ADP-induced calcium rises at 3 μM), platelet aggregation (inhibited ADP-induced platelet aggregation)	Baurand *et al.*, 2001
P2Y2	AR-C118925XX	x	10	Human	Bronchial epithelial cells	0.1-10	Enzyme-linked lectin assay (maximal and almost complete inhibition of ATPγ-S induced mucous secretion at 10 μM)	Kemp *et al.*, 2004
P2Y11	NF-340	x	10	Rat	Cardiac fibroblasts	10	Calcium imaging (small reduction in UTP-induced calcium rises), proliferation (augmented UTP-induced proliferation)	Certal *et al.*, 2015
				Human	hCMEC/D3 endothelial cell line (of the blood-brain barrier)	1	Calcium imaging (failed to inhibit BzATP-induced calcium rises)	Bintig *et al.*, 2012
					Monocyte-derived dendritic cells	10	Calcium imaging (inhibited ATP-induced calcium rises), ELISA (decreased ATP-mediated inhibition of IL8 and IL12 secretion)	Meis *et al.*, 2009
						10	Calcium imaging (reduced ATP-induced calcium rises), flow cytometry (significantly reduced CD83 receptor expression indiced by hypoxia), ELISA (decreased ATP-mediated inhibition of IL12 secretion)	Chadet *et al.*, 2015

Table 1.4. List of selective P2 receptor antagonists used in this study.
'x' represents no available data found in current literature.

(Evans et al., 1995, Bianchi et al., 1999, North, 2002).

In addition to selective agonists and antagonists, there are also commercially available modulators of receptor function which can indicate the functional presence of a receptor subtype. A well-known modulator for P2 receptor function is ivermectin, which positively modulates P2X4 receptors in a completely reversible fashion (with a 10 minute wash-off time in Xenopus oocytes (Khakh et al., 1999b). However, it has been demonstrated that whilst ivermectin potentiates the currents of human P2X4 receptors, it does not do the same at murine P2X4 receptors (Nörenberg et al., 2012).

Several pharmacological tools for P2 receptor study have shown promise at treating disease *in vivo*. For example, the general P2 receptor antagonist suramin reduced immune cell-induced inflammation and airway hyperresponsiveness in a murine model of asthma (Idzko et al., 2007). Some P2 receptor binding molecules have previously been patented for use in treating diseases, including diaminopyridines which are used as P2X3 and P2X2/X3 heterodimer modulators (Dillon et al., 2007). Furthermore, a carboxylic acid-based pyridine compound has been patented for use as a P2X1 and P2X3 antagonist and as a possible treatment for neurological pain and inflammatory diseases (Kim et al., 2013). Some of these compounds have even entered clinical trials, including Pfizer's CE-224535 (Stock et al., 2012) and AstraZeneca's AZD9056 (Keystone et al., 2012), which entered Phase IIb clinical trials as P2X7 receptor antagonists. However, both of these compounds were found to be ineffective at treating their target disease, rheumatoid arthritis, and therefore did not pass the trials. Denufosol, a P2Y2 receptor agonist, underwent Phase III clinical trials, but proved unsuccessful at maintaining an improved diseased state for sufferers of CF, despite early promise (Ratjen et al., 2012).

Often, the aim with any study investigating receptor contribution to pathologies is the development of treatments to ameliorate the conditions for patients. However, particularly regarding P2 receptors, the high homology between receptors leads to a lack of specificity of drugs, and the ubiquitous nature of the receptors can also lead to unpleasant side-effects as certain physiological functions can be unwittingly targeted. For example, a recent study used the P2X3 receptor selective antagonist AF-219 as a potential treatment for chronic cough. However, although cough frequency was reduced, several patients opted out of the trial due to taste disturbances (Abdulqawi et

al., 2015). Despite this, it is possible to develop pharmacological tools for P2 receptors suitable for *in vivo* use as treatments. For example, the active metabolite of the P2Y12 antagonist, clopidogrel, is currently in use as an antithrombotic drug (CAPRIE, 1996), and functions by breaking up P2Y12 oligomers in addition to partitioning its subunits out of their typically associated lipid rafts (Savi et al., 2006).

1.3.6 The regulation of nucleotide signalling by ectonucleotidases

Ectonucleotidases are membrane-bound enzymes that are present on the extracellular surface of cells, and are involved in the metabolism of external nucleotides. They were first reported on pig aortic smooth muscle cells to degrade ATP to ADP and AMP, and then further to adenosine (Cooper et al., 1979, Pearson et al., 1980). However, it is now known that there are a number of different classes of ectonucleotidases. This section will give a brief summary of these enzymes and their physiological importance.

There are four main families of ectonucleotidases: ectonucleotide triphosphate diphosphohydrolases (E-NTDPases), ectonucleotide pyrophosphatase/ phosphodiesterases (E-NPPs), alkaline phosphatases (APs) and ecto-5'-nucleotidase. These enzymes can be cleaved from the membrane in a number of ways, including by sheer stress, and soluble isoforms also occur naturally. When released, these enzymes are referred to as exonucleotidases (Zimmermann, 2000, Zimmermann, 2001, Yegutkin et al., 2009).

They each mediate different steps in nucleotide degradation. For example, the E-NTDPases, which include CD39, preferentially catalyse nucleotide 5'-triphosphates and nucleotide 5'-diphosphates (Zimmermann, 2000, Zimmermann, 2001, Robson et al., 2006), whilst the E-NPPs are capable of cleaving ATP to ADP, whilst also cleaving ATP and ADP directly to AMP. E-NPPs are also capable of cleaving AMP to adenosine (Zimmermann, 2000, Zimmermann, 2001, Goding et al., 2003). Alkaline phosphatases are non-specific compared to other ectonucleotidases, catalysing each step of nucleotide metabolism and so are singularly able to degrade a nucleotide triphosphate to its nucleoside analogue (Zimmermann, 2000, Zimmermann, 2001, Sharma et al., 2014). Lastly is the ecto-5'-nucleotidase, also known as CD73, which is a sole enzyme despite being in a category of its own. CD73 catalyses only the final step in nucleotide degradation, breaking nucleotide monophosphates down into nucleosides (and inorganic phosphates) (Zimmermann, 2000, Zimmermann, 2001, Sträter, 2006). These

pathways are summarised in figure 1.3.3.

There is a ubiquitous and often overlapping expression of ectonucleotidases, demonstrating the importance of signalling mechanisms induced by extracellular nucleotides. As such, ectonucleotidases are heavily involved in the regulation of both adenosine and P2 receptor-mediated signalling, with an important example being their roles in the regulation of thrombosis, immunity and inflammation (Zimmermann, 2000, Zimmermann, 2001, Robson et al., 2005, Burch and Picher, 2006, Deaglio and Robson, 2011, Antonioli et al., 2013).

Ectonucleotidases play several roles in the airways, specifically, and in the pathogenesis of airway diseases (Picher, 2011). For example, CD39 expression is upregulated in the airways of smokers after acute cigarette smoke exposure, and so is thought to have a protective role against cigarette smoke-induced lung inflammation (Lazar et al., 2016). The expression and activity of ectonucleotidases is also increased in patients with COPD and CF (Picher et al., 2004, Lazar et al., 2016).

The inhibition of ectonucleotidases in the airways can be used to elucidate their functions. For example, their inhibition/deficiency results in enhanced emphysema in mouse models (Lazar et al., 2016), and has been shown to can worsen ovalbumin-induced bronchospasm in guinea-pig lungs (Chavez et al., 2013). Conversely, the administration of the commercial ectonucleotidase apyrase ameliorated immune cell-induced inflammation and airway hyperresponsiveness in mice (Idzko et al., 2007).

It can be concluded, therefore, that ectonucleotidases are extremely important in the regulation of extracellular nucleotide-mediated signalling, and this strengthens the opinion that extracellular nucleotides play central roles in the function of many tissues, whilst dysfunctional P2 receptor signalling could play a role in numerous pathologies.

1.3.7 The implication of P2 receptors in asthma pathogenesis

As described, asthma is a heterogeneous inflammatory disease characterized by airways inflammation and obstruction, and ASM hyperresponsiveness (section 1.1.3). Due to their key roles in inflammation (section 1.3.5.1) and smooth muscle contraction (section 1.3.5.3), P2 receptors could play a role in asthma pathogenesis, particularly as they are implicated in the pathogenesis of several other airway diseases (section 1.3.6). This is supported by the fact that ATP concentrations are increased in the airways of

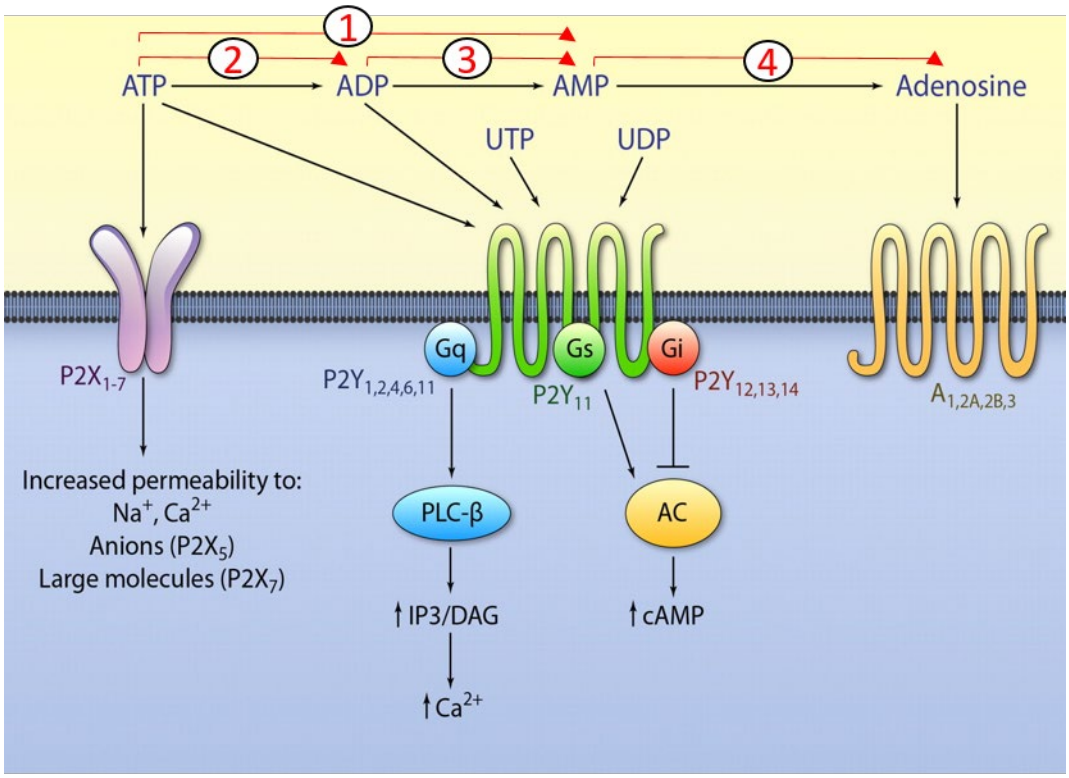

Figure 1.3.3. The nucleotide signalling cascade.

The nucleotide signalling cascade, summarised above, outlines the receptors activated by each nucleotides and also gives the steps of nucleotide metabolism. 'A' refers to adenosine receptors. Each metabolic step is carried out by the following enzymes: step 1 by E-NPPs/APs, steps 2 and 3 by E-NPPs/E-NTDPases/APs, and step 4 by E-NPPs/CD73/APs where E-NPPs are ectonucleotide pyrophosphatase/ phosphodiesterases, APs are alkaline phosphatases and E-NTDPases are ectonucleotide triphosphate diphosphohydrolases.

Figure modified from Yang and Liang, 2012.

asthmatic patients compared to those of healthy individuals (Idzko et al., 2007). It follows that the additional ATP observed in asthmatic airways could activate P2 receptors on ASM cells and contribute to ASM dysfunction in asthma, potentially by sensitizing the ASM to contraction, particularly as P2 receptors have been implicated in the sensitization of smooth muscle and, specifically, ASM contraction (section 1.2.4.5).

1.4 Hypotheses

P2 receptors play pivotal roles in the regulation of ASM functions including contraction and proliferation, and contribute to the dysfunction of ASM observed in asthma.

1.5 Study aims

This study aims to:

- Determine and compare the expression of P2 receptors in ASM cells from wild-type mice, and from healthy and asthmatic humans.
- Characterise the functional P2 receptors in ASM cells from wild-type mice, and from healthy and asthmatic humans.
- Identify the physiological roles of P2 receptors in ASM cells from wild-type mice, and from healthy and asthmatic humans, particularly in regards to ASM contraction and proliferation.

2 Materials and Methods

2.1 Drugs and reagents

All drugs and reagents were purchased from Sigma-Aldrich, Gillingham, UK unless otherwise stated.

2.2 Tissue and cell origins

2.2.1 Animals

Both male and female C57BL/6J mice between 3-6 months old were culled by Schedule 1: sedation with isofluorane followed by increased concentration of CO_2. This approach was chosen as cervical dislocation very often damages the trachea. Death was pronounced after femoral artery rupture with a scalpel blade. Organs were then rapidly dissected according to the experimental approach used.

2.2.2 Primary cultured mouse tracheal airway smooth muscle cells

Mouse tracheal ASM (mT-ASM) strips were dissected from the trachea along its length and were successively enzymatically digested with papain (15 units) /1,4-dithioerythritol (3 μM) for 30 min at 35°C, and collagenase (3 FALGPA units) /hyaluronidase (~700 units) for 5 min at 35°C. The digested tissue mix was then diluted ~1:3 in Hanks Buffered Saline Solution (HBSS, Gibco, Paisley, UK) containing 1.26 mM $CaCl_2$, pre-heated to 35°C, to stop the digestion reaction. The digested tissue mix was centrifuged at 500 g for 6 min before re-suspension and culture in mT-ASM cell media [Dulbecco's Modified Eagle Medium (DMEM) Glutamax media with sodium pyruvate (1 mM) (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco), 50 units/ml penicillin (Gibco), 50 μg/ml streptomycin (Gibco), 1X Modified Eagle Medium non-essential amino acids (NEAA) (Gibco)] and cultured in 25 cm^3 culture flasks until they reached confluence (after ~9 days). Media was changed every 3-4 days. Cultured mT-ASM cells (cmT-ASM cells) were always harvested using 0.25% trypsin-ethylenediamine tetra-acetic acid (EDTA) solution (Gibco) after washing in commercial phosphate buffered saline (PBSc). Cells, passaged no more than twice, were kept at 37°C in a humidified atmosphere of 5% CO_2/95% O_2.

Characterization of several of these samples using quantitative polymerase chain reactions (qPCR) and immunohistochemistry (IHC) showed that these cells strongly express α-smooth muscle actin transcript and protein (a smooth muscle

marker) whilst negligibly expressing pan-cytokeratin (a marker for epithelial tissue) transcript and protein (see results section 3.2.1.2.2). These cells also retain a typical 'hill and valley' morphology that is characteristic of smooth muscle cell cultures (data not shown).

2.2.3 Primary cultured human airway smooth muscle cells

2.2.3.1 Origin and culture of primary cultured human airway smooth muscle cells

Both healthy control and asthmatic subjects were recruited from respiratory clinics via Glenfield Hospital (Leicester, UK). All patients gave written consent to donate bronchial biopsies from fibreoptic bronchoscopies, which were performed as outlined in a previously published study (Brightling et al., 2005) and according to British Thoracic Society guidelines (Du Rand et al., 2013), and this procedure was approved by the Leicestershire Ethics Committee. The presence of asthma was defined using the current Global Initiative for Asthma Guidelines (GINA, 2015, GINA, 2017).

Explants obtained from bronchoscopies were further dissected to remove non-muscle contaminating tissues and were cut into sizes of ~2 mm^3. Using 6-well plates, ASM cells were then cultured from these explants in cHASM cell media (DMEM Glutamax media (Gibco) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, 1X Modified Eagle Medium NEAA and 1 mM sodium pyruvate) and were kept at 37°C in a humidified atmosphere of 5% CO_2/95% O_2. When confluent, cells were harvested as described using 0.25% trypsin-EDTA solution, which was neutralised with cHASM cell media, before centrifugation at 368 x g for 8 min at room temperature (RT). Cells were then resuspended and cultured in 75 cm^3 flasks until use. Cells up to and including passage 5 were used for experiments if ≥70% of the population stained positively for the smooth muscle marker, α–smooth muscle actin (see section 2.2.3.2).

2.2.3.2 Characterisation of primary cultured human airway smooth muscle cells

Before characterisation, cHASM cells were serum-starved in insulin-transferrin-selenium (ITS) media [DMEM Glutamax media containing 1% ITS (Gibco), 166 μM oleic acid, 166 μM lineoic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, 1X Modified Eagle Medium NEAA and 1 mM sodium pyruvate] for 24 hours at 37°C in a humidified atmosphere of 5% CO_2/95% O_2. Cells were then harvested

as described previously using 0.25% trypsin-EDTA solution before centrifugation at 368 x g for 8 min at 4°C. Cells were counted using a haemocytometer and, in duplicate, 10,000 cells were taken and centrifuged at 368 x g for 8 min at 4°C before being fixed on ice for 15 min using 4% paraformaldehyde solution containing 0.1% saponin (a cell membrane permeabilising agent) in PBSc. PBSc containing 0.5% bovine serum albumin (BSA) and 0.1% saponin was then added to the cells before centrifugation at 368 x g for 8 min at 4°C. Cells were then resuspended in PBSc containing 0.5% BSA and 0.1% saponin before incubation for 60 min on ice with either the mouse anti-human α-smooth muscle actin (Clone 1A4; Agilent Technologies, Cheadle, UK) or the isotype control (IC) (mouse IgG2a; Agilent Technologies) primary antibodies, both at 4.2 µg/ml. Following centrifugation at 368 x g for 8 min at 4°C, the cells were resuspended in PBSc containing 0.5% BSA, 0.1% saponin and the fluorescent secondary antibody (goat, anti-mouse APC; Life technologies, Warrington, UK) at 10 µg/ml and incubated for 60 min on ice in the dark. From this point, cells were kept in the dark. Following the incubation, cells were centrifuged at 368 x g for 8 min at 4°C before resuspension in PBSc containing 0.5% BSA. Cells were then analysed using a FACS Canto A flow cytometer (Becton Dickinson, Oxford, UK). ASM cells were gated using the forward and side scatter. Data were analysed using the FlowJo software (version 10, FlowJo, LLC) and comparisons of the two conditions enable to detection of α-smooth muscle actin positive cells. Donors were used if ≥70% of the cell population stained positively for α–smooth muscle actin.

2.3 Quantitative PCR in Airway Smooth Muscle

2.3.1 RNA isolation

For the quantification of P2 receptor transcripts in ASM, RNA was obtained from strips of mT-ASM, cmT-ASM cells (passage 1) or from cHASM cells (Glenfield Hospital, Leicester, UK). RNA was also isolated from different sections of mouse lung (upper, middle and peripheral lung) according to figure 2.1.

For strips of mT-ASM, tracheas were dissected and cleaned of contaminating connective tissues in phosphate buffered saline (PBS) (in mM: 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, 0.44 Na_2HPO_4, 0.4 $NaH_2PO_4.H_2O$, 4.2 $NaHCO_3$, pH 7.4). The strip of muscle was dissected along the length of the trachea and was then crushed on ice in

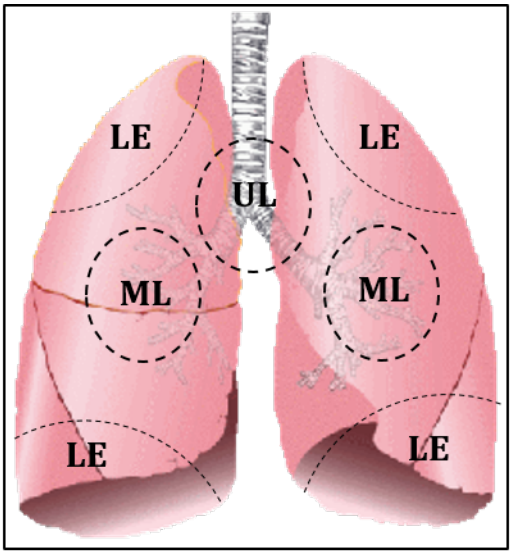

Figure 2.1. Sections of mouse lung used for qPCR and western blotting. *Mouse lung samples were dissected as shown and are named as follows: Upper Lung (UL), Middle Lung (ML) and Lung Extremities or Peripheries (LE).*
Figure modified from http://drugdiscoveryopinion.com/.

700 μl RLT lysis buffer from the RNeasy Mini Kit (Qiagen, Manchester, UK) containing 1% β-mercaptoethanol with a clean, fine scalpel blade. Total RNA was then extracted using the QIAshredder and the RNeasy Mini Kit (Qiagen). For RNA isolation from primary cultured ASM cells, cmT-ASM and cHASM cells were grown in 25 and 75 cm^3 flasks, respectively, until confluence when media was removed and the cells were washed in PBSc. 700 μl RLT lysis buffer containing 1% β-mercaptoethanol was then applied directly to the cells. A cell scraper was used, on ice, to mechanically detach and crush the cells. Total RNA was extracted using the RNeasy Mini Kit. RNA was quantified using a Tecan Infinite M200 NanoQuant (Männedorf, Switzerland).

2.3.2 Reverse transcription

When the amount of starting tissue, and therefore RNA, was limiting (such as for mT-ASM strips), total RNA (50 ng) was reverse transcribed using the Sensiscript Reverse Transcription Kit (Qiagen), which allows the reverse transcription of low quantities of RNA (≤50 ng).

When RNA concentrations were not limiting (*e.g.* for cHASM cells) the Superscript III Reverse Transcriptase Kit (Invitrogen, Life Technologies, Renfrew, UK) was used for reverse transcription.

2.3.3 Primer design

The cDNA sequences for mouse P2 receptor and reference genes were obtained from NCBI Nucleotide Search (NCBI, 2015) and ENSEMBL Genome Browser (ENSEMBL, 2015). Primers were then designed using the Primer 3 primer design software (Rozen and Skaletsky, 1998) and parameters, such as product size and primer melting temperatures (Tm), were imposed upon the primer design. The primers were purchased from Sigma and their sequences are given in tables 3.1 and 3.2 for P2 receptors and reference genes, respectively.

Primers for human P2 receptors and reference genes were designed and characterised in the same manner as above by previous team members, and their sequences are given in tables 2.1 and table 2.2, respectively. The primers for human P2X receptors and several of the reference genes have also been used in published work (Wright et al., 2016).

Target gene	Primer sequence (Forward, Reverse)	Amplicon size (bp)	Amplification efficiency (%)
P2X1	5′-CTGGTGGAGGAGGTGAATG-3′ 5′-AAGTTGAAGCCTGGGGAGAG-3′	260	98
P2X2	5′-CATCGGGGTCATTATCAAC-3′ 5′-CAGTCGCACAGGAAGGAG-3′	300	97
P2X3	5′-GACCCTTTCTGCCCCATC-3′ 5′-CACTGCCATTTTCCATTTTG-3′	250	95
P2X4	5′-GGAGAACGCAGGACACAG-3′ 5′-CCTTCCCAAACACAATGATG-3′	272	95
P2X5	5′-TGGTCGTATGGGTGTTCCTG-3′ 5′-TGCCTTCATTCTCAGCACAG-3′	240	99
P2X6	5′-CCCAAGTTCAGGGCAGATG-3′ 5′-GAAGGTGACTGTGTTTTTGATG-3′	257	96
P2X7	5′-TACATCGGCTCAACCCTCTC-3′ 5′-GCAGGTCTTGGGACTTCTTG-3′	293	95
P2Y1	5′-GTCCCCTTGGTGCTGATTC-3′ 5′-TGGCATAAACCCTGTCATTG-3′	232	93
P2Y2	5′-GTAACCTGCCACGACACCTC-3′ 5′-GAAGACAGCCAGCACCAC-3′	276	91
P2Y4	5′-TAACGCCCCAACCCTATG-3′ 5′-GCAGGTGAGGAAAAGGACAC-3′	229	90
P2Y6	5′-AACCGCACTGTCTGCTATGAC-3′ 5′-CTGTCTTGGTGATGTGAAAAGG-3′	271	91
P2Y11	5′-GGTGGTTGAGTTCCTGGTG-3′ 5′-GTAGCGGTTGAGGCTGATG-3′	280	94
P2Y12	5′-CTGTTGTCATCTGGGCATTC-3′ 5′-CCTACACCCCTCGTTCTTACG-3′	250	91
P2Y13	5′-GAGCAACAAGGAAGCAACAC-3′ 5′-ACAAAGAAGACAGCCACGAC-3′	255	93
P2Y14	5′-TCTGCCGTGCTCTTCTACG-3′ 5′-CACTTCCGTCCCAGTTCAC-3′	260	91

Table 2.1. List of human P2 receptor primers used for qPCR experiments. *Primers were designed and validated using Primer3 by previous lab group members. This table also includes the amplicon size (base pairs) and the amplification efficiency (%) for each pair of primers tested. Primers were used for final qPCR experiments only when the amplification efficiency was ~90-105%.*

Target gene	Primer sequence (Forward, Reverse)	Amplicon size (bp)	Amplification efficiency (%)
ACTβ	5′-TCCTATGTGGGCGACGAG-3′ 5′-ATGGCTGGGGTGTTGAAG-3′	242	97
GAPDH	5′-ATCATCTCTGCCCCCTCTG-3′ 5′-GCCATCCACAGTCTTCTGG-3′	213	97
HPRT1	5′-CATTGTAGCCCTCTGTGTGC-3′ 5′-ACTTTTATGTCCCCTGTTGACTG-3′	165	95
PPIB	5′-CGTCTTCTTCCTGCTGCTG-3′ 5′-AGCCAAATCCTTTCTCTCCTG-3′	200	100
GUSβ	5′-TCAGAGCGAGTATGGAGCAG-3′ 5′-CCCTTTTTATTCCCCAGCAC-3′	210	95
YWHAZ	5′-CCGTTACTTGGCTGAGGTTG-3′ 5′-AGGCTTTCTCTGGGGAGTTC-3′	188	97
RPL13A	5′-CTGCCCCACAAAACCAAG-3′ 5′-TCTCTTTCCTCTTCTCCTCCAG-3′	226	97
SDHA	5′-TGGAGACCTAAAGCACCTGAAG-3′ 5′-CTCATCAATCCGCACCTTG-3′	181	99
POLR2A	5′-TTGACCTCCTGCTTGATGC-3′ 5′-TCATTCCACTCCCAACACTG-3′	207	97

Table 2.2. List of human gene primers tested as potential reference genes for qPCR experiments.

Primers were designed and validated using Primer3 by previous lab group members. Primers were designed and validated using Primer3 by previous lab group members. This table also includes the amplicon size (base pairs) and the amplification efficiency (%) for each pair of primers tested. Primers were used for final qPCR experiments only when the amplification efficiency was ~90-105%.

2.3.4 Quantitative PCR

qPCR were carried out using the iQ SYBR Green Supermix qPCR kit (Bio-Rad Laboratories Ltd., Hertfordshire, UK) on a Roche LightCycler 480 (Roche LifeScience, Mannheim, Germany) using the LightCycler 480 software release 1.5.0 and the 384-well plate block type. Primers were used at 2 μM and reactions were performed in triplicate in a final volume of 10 μl. The cDNA concentrations were kept constant for the same sample type across all qPCR experiments. The qPCR thermal profile comprised 3 min at 95°C followed by 38 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°. The 3 most stably expressed reference genes used in experiments were determined for each cell or tissue sample type, and the final combinations are shown in table 3.3.

2.3.5 Primer validation and product sequencing

The amplification efficiencies of each primer pair were calculated by performing qPCR using cDNA synthesised from a commercial mouse reference total RNA (Agilent Technologies, Texas, USA), which ensured broad gene expression. The cDNA was diluted by 1:2 over 5 concentrations. Each primer pair was used at each cDNA concentration, and the $\log_{10}$[cDNA] was plotted against the average cycle threshold value for each condition. The slope of the line was inputted into Thermo Fisher qPCR primer efficiency calculator (ThermoScientific, 2016). Primer pairs were used for experiments if their efficiencies were ~90-105%. The amplification efficiencies of the primer pairs used are given in table 3.1 and table 3.2.

The qPCR products of each primer pair were mixed with 6x blue/orange loading dye (Promega, Southampton, UK) and loaded onto a 1.8% agarose (Bioline, London, UK) gel made up in tris[hydroxymethyl]aminomethane (tris)-acetate-EDTA 1x buffer (Invitrogen, Life Technologies, Renfrew, UK) with 0.005% ethidium bromide and separated by electrophoresis to ensure that each primer pair amplified only one product (see figures 3.1 and 3.6). The qPCR product bands were then cut from the gel and extracted using the QIAquick Gel Extraction Kit (Qiagen) before sequencing by DNA Sanger Sequencing at The Protein Nucleic Acid Chemistry Laboratory (The University of Leicester, Leicester, UK). The sequences were compared to their target gene sequences using the NCBI Blast search (NCBI, 2015) to confirm the specificity of the primers.

The primer pairs of all putative reference genes were tested against several

samples of each cell/tissue type in order to determine the most stably expressed genes. qPCR were performed on the appropriate samples using each primer pair. The $2^{-\Delta CT}$ values were extracted and analysed using the Microsoft Excel VBA applet geNorm (version 3.5, Ghent University, Belgium) to give the gene expression stability measure (M) for each gene. Stepwise exclusion of the gene with the highest M value (and therefore the lowest stability) enabled the determination of the 3 most stably expressed reference genes for each sample type (see results section 3.2.1.2.2). A summary of the 3 different reference genes used for each cell/tissue type is given in table 3.3.

2.4 Western blotting

Western blotting was carried out using a Mini-Protean 3 Western Blotting kit (Biorad, Hertfordshire, UK). When samples from mouse were used, nitrocellulose membranes were probed with the following 3 rabbit primary antibodies (Alomone Labs, Jerusalem, Israel); anti-P2X1 (APR-001) and anti-P2X7 (APR-004) at a final concentration of 0.4 μg/ml, anti-P2X4 (APR 002) at 0.43 μg/ml final concentration (optimisation shown in section 3.2.2.1.2). When samples from cHASM cells were used, the same anti-P2X1, anti-P2X4 and anti-P2X7 antibodies were used at 0.8 μg/ml, 0.43 μg/ml and 0.4 μg/ml, respectively (see optimisation in section 3.2.2.1.2). In each case a goat anti-rabbit HRP-conjugated secondary antibody (A6154, Sigma) was used at 1:2000 dilution from the stock.

2.4.1 Protein sample preparation

Tissues/organs were collected from either native mice or Xenopus laevis oocytes expressing recombinant P2X receptors and were immediately mashed using a clean scalpel blade and lysed on ice for 30 min in lysis buffer [in mM: 20 Tris-base, 250 NaCl, 3 EDTA (ethylenediaminetetraacetic acid), 3 EGTA (ethylene glycol tetra acetic acid) with 0.5% Triton X-100 and 1% Protease Inhibitor Cocktail (PIC), pH 8]. Proteins from mouse lung were isolated in different sections (upper, middle and peripheral lung) according to figure 2.1. When lysing primary cultured ASM cells (from both mouse and human), cells were washed in PBSc and lysis buffer was applied directly to the cells on ice for around 10 min. During this time, a cell scraper was used to mechanically disrupt the attached cells. The lysates were then collected, regularly vortexed and kept

on ice for a further 20 min. Lysates were centrifuged at 4°C /368 x g for 10 min before supernatants containing proteins were collected and stored at -20°C until used. Protein concentrations were assessed using a CB-Protein assay kit (Calbiochem, Nottingham, UK). Before use, samples were slowly thawed on ice and diluted in lysis buffer to give the required concentrations. Protein samples were then mixed in equal parts with 2x SDS-PAGE sample buffer [0.18M Tris-base, 5.7% SDS, 29% glycerol, 0.003% bromophenol blue (pH 6.8), 5% β-mercaptoethanol]. The protein samples were denatured at 95-100°C for 3-5 min before loading onto the gel.

2.4.2 Electrophoresis

For P2X1, P2X4 and P2X7 receptor detection, a 5% SDS-stacking gel [0.05% ProtoFLOWGel (Flowgen Bioscience, Scientific Laboratory Supplies, Wakefield, UK), 125mM Tris pH 6.8, 0.1% SDS, 0.1% ammonium persulfate (APS), 0.2% N,N,N',N'-tetramethylethylenediamine (TEMED)] and a 10% SDS-resolving gel [9.9% ProtoFLOWGel, 375 mM Tris pH 8.8 (Biorad), 0.1% SDS, 0.1% APS, 0.07% TEMED in distilled water (dH_20)] were used. The gels were inserted into the electrophoresis tank system, which was filled with Tris-glycine electrophoresis buffer [in mM: 25 Tris-base, 250 glycine (pH 8.3), 0.1% SDS]. Precision Plus Protein All Blue Standards (10-250 kD) (Biorad) protein markers were used. 7.5 μg and 10 μg of protein were loaded per lane for human and mouse samples, respectively, apart from positive controls for which a maximum volume (not a quantity) was loaded. The proteins were then separated by electrophoresis under 120 volts for around 95 min.

2.4.3 Membrane transfer

Nitrocellulose membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) were cut to the size of the gel and soaked along with the gel and extra thick blot paper (Biorad) in transfer buffer 1x (in mM: 25 Tris-base, 192 glycine, 20% methanol). The electroblot system was assembled as shown (figure 2.2), and care was taken to ensure that no bubbles were present in the system. The proteins were then transferred to the membrane by electroblotting at 100 V for 1 hour.

2.4.4 Membrane probing and exposure

The membranes were removed from the electroblot system and blocked overnight at 4°C. Optimisation was carried out to determine if milk powder or Animal

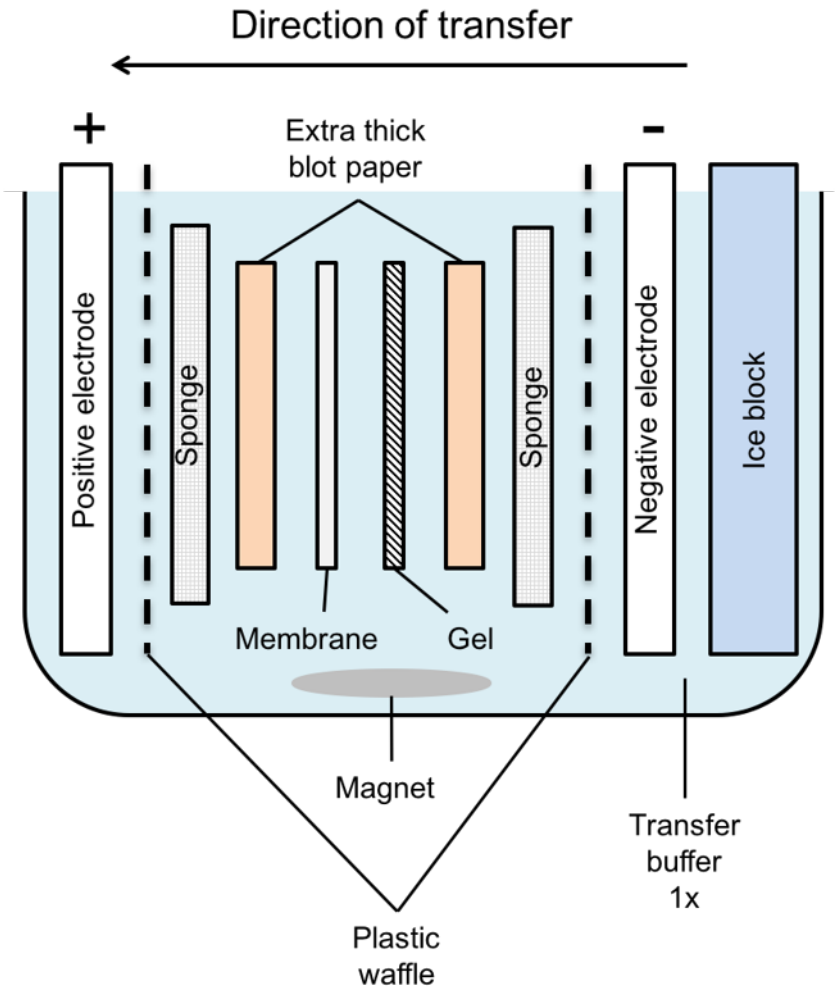

Figure 2.2. The setup of the electroblot system.

Free Blocker (AFB, Vector Labs, Peterborough, UK) blocking solution (AFB-W) [AFB 1x in dH_2O] was the optimal blocking solution (see section 3.2.2.1.1). Following optimisation, the blocking solution for western blotting (BS-W) contained 10% milk powder in Tris-buffered saline/Tween solution (TTBS) (in mM: 50 Tris-base, 150 NaCl, 0.1% Tween-20, pH 8).

The following morning, membranes were washed 3 times in TTBS for 5 min each before being incubated in the presence of the primary antibodies (made up in BS-W) for 2 hours at RT. The primary antibody concentrations used were also optimised (see results section 3.2.2.1.2). Membranes were washed again in TTBS 3 times for 10 min each before probing with the secondary antibody (made up in BS-W) for 2 hours at RT. Afterwards, membranes were washed in TTBS 5 times for 5 min each. To reveal the presence of the target protein on the membrane, the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used according to the manufacturer's recommendations. The membrane was then wrapped in clean saran-wrap in a liquid-proof manner (care was taken to avoid air bubbles between the membrane and the wrap), taped into an Amersham Hypercassette (GE Healthcare Life Sciences) and exposed onto ECL Hyperfilm (GE Healthcare Life Sciences) for a minimum period of 30 seconds or more if required. The film was processed using a Hyperprocessor (GE Healthcare Life Sciences).

2.5 Immunohistochemistry

2.5.1 Tissue collection

Bladder, *vas deferens*, trachea and lungs were dissected in PBS. Each sample was embedded in Optimal Cutting Temperature Compound (O.C.T, VWR International, Belgium) in a small, soft plastic tube and were immediately snap-frozen on a mixture of dry ice and hexane, and then stored at -20°C for a minimum of 24 hours before sectioning.

2.5.2 Tissue sectioning

Sections were cut to a thickness ranging from 10-15 μm (typically 12 μM) at -12°C using a thermo cryostat (Model CryoStar NX50, Thermo Scientific, Runcorn, UK). Section thickness was kept constant for each tissue used within an experiment. Sections were mounted onto positively charged microscope slides (Thermo Scientific, Cheshire,

UK) and allowed to dry. They were then fixed in 2% paraformaldehyde solution containing 80 mM Na_2HPO_4 and 20 mM NaH_2PO_4 for 10-15 min at RT.

2.5.3 Immunofluorescence

For immunofluorescence experiments, the rabbit anti-P2X1 antibody and its antigen peptide (the peptide sequence against which the antibody was raised) were purchased from Alomone Labs. The secondary antibody was a donkey anti-rabbit fluorescein isothiocyanate-conjugated antibody (Jackson Immunoresearch, Suffolk, UK). Antibodies were made up in blocking solution for immunofluorescence (BS-IF) containing 0.5% Triton X-100 and 10% donkey serum (Jackson Immunoresearch) in PBS immediately before use and were used at 4 μg/ml final concentration.

Following fixation, slides were washed once in PBS for 15 min. Excess buffer was removed and BS-IF was pipetted carefully onto the sections. The slides were placed in a humid chamber for 30 min at RT. All subsequent incubations were kept in these conditions unless otherwise stated. Slides were washed twice in PBS for 15 min each and the sections were incubated overnight at 4°C with either of the following conditions; BS-IF alone; the secondary antibody alone; both the primary and the secondary antibodies; the primary and secondary antibodies, but where the primary antibody was pre-incubated with its antigen peptide (for 1 hour before application to the section).

The next day, the slides were washed 6 times in PBS for 10 min each before incubation with the secondary antibody for 2 hours in the dark at RT. Slides were then washed 3 times in PBS for 10 min each, the excess of buffer removed, and a drop of Citifluor-AF1 mounting fluid (Citifluor Ltd., London, UK) was applied to each section before a coverslip was mounted onto the sections. The samples were protected from light before being viewed using an epifluorescence microscope (Nikon, model EFD-3, Tokyo, Japan) and images were taken using a DeltaPix camera (DeltaPix, model DP450, Nibe, Denmark) using DeltaPix InSight software. FITC was detected on channel FL1 and, if necessary, a filter was used to decrease the intensity of the signal for all samples within the same experiment.

2.5.4 DAB staining

To perform 3,3'-diaminobenzidine (DAB) staining, a DAB substrate kit (Cell Signalling Technology, Hertfordshire, UK) was used in conjunction with the following

primary rabbit antibodies: anti-P2X1 (final concentration 1.3 μg/ml), anti-P2X4 (final concentration 0.85 μg/ml), anti-P2X7 (final concentration 1.3 μg/ml), anti-pan cytokeratin (final concentration 3 μg/ml, Novus Biologicals, Cambridge, UK) and anti-α-smooth muscle actin (final concentration 2.5 μg/ml, Novus Biologicals). The DAB IC antibody was a rabbit polyclonal IgG antibody (Immunostep, Spain) and was adjusted to the highest required concentration for each experiment. Antibody concentrations were optimised before final experiments were performed with the above antibody concentrations (see section 3.2.3.2.1.4).

2.5.4.1 DAB staining

After fixation in 2% paraformaldehyde solution, slides were washed once in PBS for 5 min, and then twice in Tris-buffered saline and Tween 20 solution (TBST) (in mM: 137 NaCl, 20 Tris-base, 0.1% Tween-20, pH 8) for 5 min each.

Technical optimisation was performed to investigate the effect of antigen retrieval on DAB staining with the different primary antibodies (see section 3.2.3.2.1.5). During this process, after washing in TBST, samples were either treated with a 1% sodium dodecyl sulphate (SDS) in PBS for 10 min before washing twice in TBST for 5 min each, or were left in TBST. The optimisation demonstrated that antigen retrieval was not necessary for DAB staining for these proteins. Therefore, all subsequent experiments were carried out without antigen retrieval.

Samples were then incubated for 10 min at RT in a peroxidase solution, consisting of 10% peroxide in methanol (Fisher Scientific, Loughborough, UK), to irreversibly block endogenous cellular peroxide activity. Sections were then washed twice in TBST for 5 min each before being blocked.

Technical optimisation was also performed to determine the appropriate blocking conditions for DAB staining (see section 3.2.3.2.1.3). Following optimisation, sections were blocked with AFB-BS [AFB 1x containing 0.3% Triton X-100 in dH_2O] overnight at 4°C. The blocking solution was then removed and sections were incubated with the appropriate primary antibodies, or the isotype control antibody, for 5 hours at RT.

Following washing in TBST (3 times for 5 min each), the slides were incubated for 30 min in SignalStain Boost Detection Reagent (Cell Signalling Technology) containing

a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (30 µl/section directly from the kit). Slides were then washed in TBST (3 times for 5 min each) and a SignalStain DAB solution [3% SignalStain DAB Chromogen Concentrate (Cell Signalling Technology) in SignalStain DAB Diluent (Cell Signalling Technology)] was added to each section. Following this, the times for incubation varied between experiments (from 1-10 min as recommended by the supplier) depending on the tissue, the primary antibody and its concentration. Within each experiment, the incubation time was the same for each condition. The reaction was stopped by immersing the slides in dH_2O.

2.5.4.2 Counterstaining with haematoxylin and coverslip mounting

Sections were washed in tap water for 3 min before counterstaining with haematoxylin (to stain basophilic substances, including nuclei) until optimal staining was obtained. The slides were then washed gently in running tap water and immersed twice in 70% ethanol and twice in 100% ethanol. Afterwards, the slides were placed in xylene (Fisher Scientific) twice before sections were mounted in DPX mounting agent (Fisher Scientific). All immersions were for 3 min except for the last xylene immersion, which was for 5 min. The slides were allowed to dry in a fume cupboard overnight before viewing under a brightfield microscope. Images were then captured using a Zeiss AxioCam HRc (Zeiss, Göttingen, Germany) and the Zen lite software (version 1.1.2.0, Zeiss, 2012).

2.6 Measurement of $[Ca^{2+}]_i$

2.6.1 Widefield epifluorescence microscopy

cmT-ASM and cHASM cells were sparsely seeded onto glass coverslips (Thermo Scientific, Loughborough, UK) the afternoon before use. The cells were washed once in epifluorescence solution (ES, containing in mM: 118.4 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgCl_2$, 11.1 D-glucose, 10 HEPES, pH 7.4). Cells were then loaded with 2 µM fura-2-AM (Molecular Probes, Invitrogen, Fisher Scientific) in ES containing 2.5 mM probenecid and 0.04% w/v pluronic acid-F127 for 50 min in the dark at RT. The cells were visualised using a 20x objective lens on an inverted epifluorescence microscope (Roper Scientific Cascade, 512B, Germany) and were alternately excited by monochromatic UV light at 340 nm and 380 nm to give 1 ratio image every ~3 seconds. Fluorescence emissions at

525 nm were then captured and digitized using a charge-coupled device (CCD) camera (Roper Cascade Photometrics, Arizona, USA). This setup is summarised in figure 2.3.

Each coverslip was superfused with ES at 37°C (perfusion rate ~2 ml/min) and around 10 cells/coverslip were monitored simultaneously. A fluorescence region of interest (ROI) was defined around all cells [and so responses were measured as a population, similar to Flexstation experiments (see section 2.6.2)]. On each coverslip, a positive control response to bradykinin 5 μM was performed 5 min after recording started, followed by the test response after a further 15 min. Therefore, responses were compared between coverslips in a similar way that wells are compared between other wells in Flexstation experiments. Data are expressed as a % change in ratio F_{340}/F_{380} (Grynkiewicz et al., 1985) compared to the positive control response, bradykinin 5 μM. Cells were pre-treated with antagonists for 5 min before use where appropriate. Experiments performed in the absence of extracellular calcium (Ca^{2+}_e) used Ca^{2+}-free epifluorescence solution (0Ca-ES, in mM: 118.4 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 11.1 D-glucose, 10 HEPES, pH 7.4), which was applied 2 min before agonist application (in 0Ca-ES) to ensure that no Ca^{2+}_e remained in the bath. For desensitization experiments, the agonist was applied immediately after any $[Ca^{2+}]_i$-response induced by the desensitizing agent returned to baseline. The data were extracted from the WinFluor software (Version 3.7.2, University of Strathclyde, Glasgow, Scotland, UK) and analysed in Microsoft Excel (Microsoft Office, 2013) and GraphPad Prism 7 (Version 7.00, GraphPad Software, Inc.).

2.6.2 $[Ca^{2+}]_i$ measurements using the Flexstation™

2.6.2.1 Flexstation™

cmT-ASM and cHASM cells were seeded into black-walled/clear-bottomed 96-well plates (Costar, Corning, NY, USA) and grown to confluence. Cells were washed once in Flexstation saline (FS, in mM: 118.4 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgCl_2$, 11.1 D-glucose, 10 HEPES, pH 7.4) and loaded (50 min at RT in the dark) with fura-2 AM 2 μM in FS containing 0.02% pluronic acid-F127 (PA) and 0.16 IU/ml apyrase (Grade VII). Cells were briefly washed once in FS after the loading dye was removed and 200 μl FS was then added to each well. Where appropriate, cells were incubated in the presence of antagonists for ~5 min before the experiment was run. Measurements were made

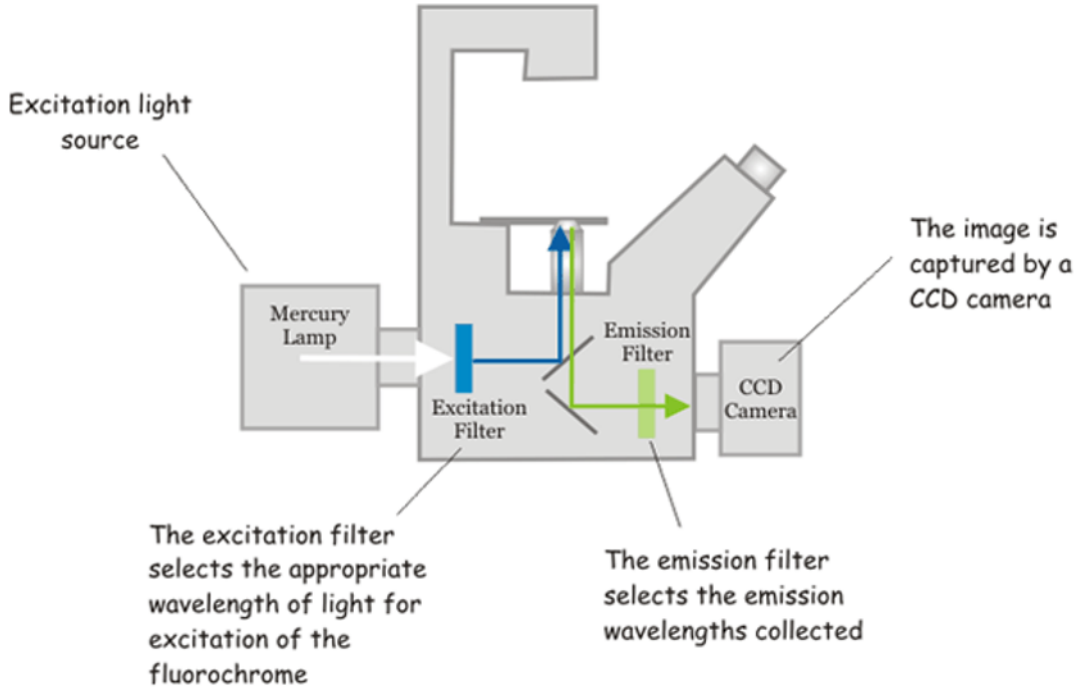

Figure 2.3. The setup of the widefield epifluorescence microscopy system.
Image taken from http://www.robarts.ca/confocal/techniques.html#widefield.

using a Flexstation™ II fluorimeter (Molecular Devices, Wokingham, UK) and SoftMax® Pro software (Version 4.7, Molecular Devices). Loaded cells were excited at 340 nm and 380 nm and emission was collected above 510 nm (495 nm cut-off). 50 μl of agonists (containing antagonists where appropriate) were then added via automated injection at a pipette height of 215 μl. Data were extracted and analysed using Microsoft Excel and GraphPad Prism 7. Based on the fact that injections of saline buffer induced peak $[Ca^{2+}]_i$ rises of 18±3 nM and 16±2 nM in cHASM cells from healthy and asthmatic donors, respectively (see section 4.2.2.1), experiments where antagonists are used but where there is a peak $[Ca^{2+}]_i$ rise <20 nM for the controls were excluded.

2.6.2.2 Calculating $\Delta[Ca^{2+}]_i$ using Flexstation™

340 nm/380 nm ratio (R) measurements indicate the relative $[Ca^{2+}]$ levels in the cell and can show the effect of a compound/condition on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ itself can be quantified using the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R) \times (F_{380max} / F_{380min})$$

Here, K_d is the dissociation constant for fura-2, taken as 257 nM (Mason and Mahaut-Smith, 2001). R_{min} and R_{max} were determined for each Flexstation plate in Ca^{2+}-free (containing 15 mM EGTA) and Ca^{2+}-saturated (containing 10 mM Ca^{2+}) conditions, respectively, following treatment with ionomycin (10 μM). R_{min} and R_{max} were corrected by applying a viscosity correction factor of 0.85 (Poenie, 1990). F_{380max}/F_{380min} is the maximum/minimum fluorescence intensity at 380nm. Any background signal was determined by treating cells with Mn (25 mM), and subtracting the value obtained from R before use in the equation.

2.7 Myography

2.7.1 Tissue collection

Mouse tracheas were dissected in physiological saline solution (PSS, in mM: 5 KCl, 136 NaCl, 10 HEPES, 11.1 D-glucose, 0.5 $NaH_2PO_4.2H_2O$, 0.5 Na_2HPO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, pH 7.4) and connective tissue was removed. The tracheas were then cut into cylindrical segments of around 2.5 mm long (just slightly smaller than the size of the jaws of the myograph, as shown in figure 2.4).

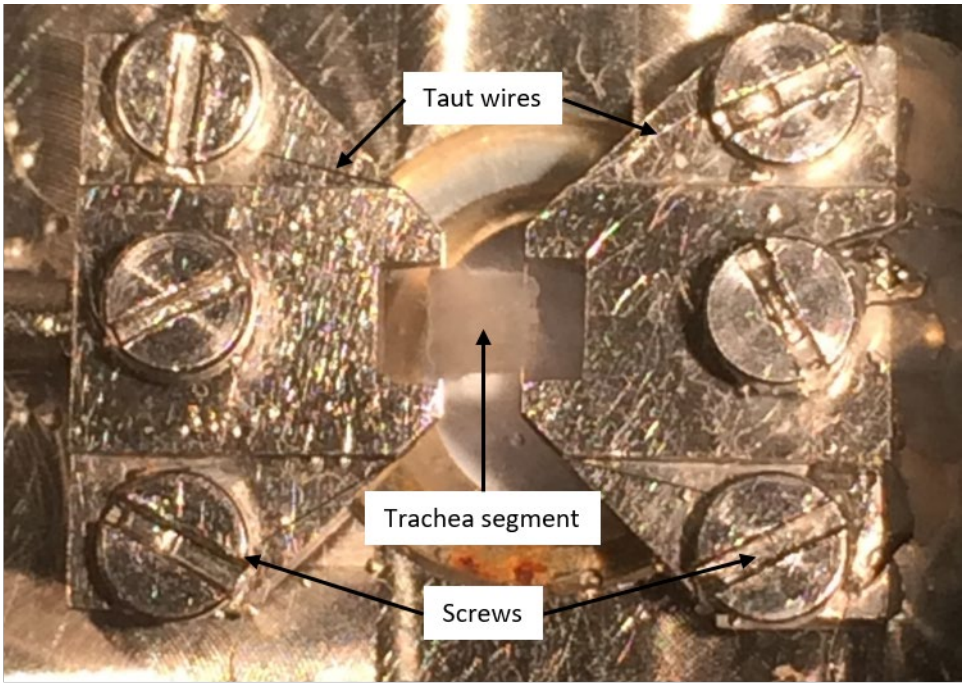

Figure 2.4. The myograph setup.

In the myography setup, the trachea segment (~2.5 mm long) is shown in the middle of the myograph jaws. Wires are threaded through the trachea segment and are then screwed into place over the ridge the jaws so that they are taut.

2.7.2 Mounting of the trachea in the myograph

Myography was performed using a Myo-Interface (Model 500A, JP Trading, Aarhus, Denmark), which contains two chambers, allowing two segments of trachea to be mounted and studied simultaneously. The organ bath was filled with ice-cold PBS, and an appropriate length of 40 μm thick wire was cut and threaded through the segment, which was then transferred to the middle of the myograph chamber and the wire was screwed into place on the jaws of the myograph. One of the jaws is stationary and attached to a sensitive isometric transducer and the other is attached to a micropositioner. The wire was attached so that it fell over a ridge on the jaws, remaining taut and allowing the force along the whole length of the trachea to be measured accurately. The jaws were then moved closer to enable a second wire to be threaded through the trachea and screwed into place on the opposite side of the jaws. Afterwards, the jaws were moved slightly apart so that normalization could be performed. This setup is shown in figure 2.4.

2.7.3 Normalization of trachea segments

Organs and tissues are subjected to a resting physiological tension in *vivo*. When these tissues are used in *in vitro* experiments, these conditions are replicated as much as possible as it has been found that the resting tension applied to vessels (and in this case trachea segments) can affect the extent of contraction or relaxation (McPherson, 1992). Therefore, it is crucial that initial conditions are standardized to enable the comparison of physiological responses between different trachea segments. This procedure is known as normalization (Mulvany and Halpern, 1977). Here, normalization and all subsequent myography were performed at 37°C. When the true physiological tension that a tissue is subjected to *in vivo* is unknown, a common method of normalization is to apply a tension that gives the optimal contraction (Adner et al., 2002, Zhang et al., 2005, Zhang et al., 2007). In this preparation, the trachea segments rested for 30 min in 0Ca-PSS (in mM: 5 KCl, 136 NaCl, 10 HEPES, 11.1 D-glucose, 0.5 $NaH_2PO_4.2H_2O$, 0.5 Na_2HPO_4, 1.2 $MgCl_2$, pH 7.4). Tension, in 0.2 mN increments, was manually applied to the trachea and a 5 minute application of 60KPSS (in mM: 60 KCl, 81 NaCl, 10 HEPES, 11.1 D-glucose, 0.5 NaH_2PO_4, 0.5 Na_2HPO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, pH 7.4) was performed between each increment to contract the ASM following membrane

depolarisation. The optimal resting tension which gave the biggest contractions to 60KPSS was found to be 0.8 mN (see section 5.2.1.1.1), which agrees with current literature (Adner et al., 2002, Zhang et al., 2005, Zhang et al., 2007). Therefore, all experiments were performed under a resting tension of 0.8 mN.

2.7.4 Measurement of trachea contraction responses to extracellular nucleotides

Following normalisation, three 5 minute applications of 60KPSS were performed with a 5-minute wash in PSS in between each application. This was used to assess the integrity and viability of the tissue (to ensure that the dissection and mounting procedure had not impaired its physiological responses). The trachea segments were then equilibrated for 1 hour in PSS. The organ bath is set up with a perfusion system using a peristaltic pump which allows drugs to be rapidly and readily applied into or washed out from the myograph chambers where the tissue is located (flow rate ~2 ml/min). Carbachol (0.3 μM), a known bronchoconstrictor, was applied to ensure that the segments also contracted in a physiologically relevant manner. All drugs and nucleotides were applied in PSS via the perfusion system. 45 min were left between each application unless otherwise stated. All data were normalised to the peak amplitude of the 3rd 60KPSS-induced contraction.

2.8 Collagen-gel contraction assays

cHASM cells were treated with ITS media ~12 hours before use for collagen-gel contraction assays. A 24-well plate was pre-coated with 2% BSA in PBSc for at least 60 min before use. A collagen gel (total volume of 500 μl) containing DMEM was made by combining liquid collagen (PureCol, Advanced Biomatrix, Cell Systems, Germany) with 0.125x10^6 cHASM cells [harvested using accutase cell dissociation solution] in ITS media before setting with 4% sodium bicarbonate (Gibco) for 90 min at 37°C in a humidified atmosphere of 5% CO_2/95% O_2. A rounded, metal tool was used to gently detach the gels from the sides of the wells and 500 μl ITS media was added per well. To investigate the effect of nucleotides on bradykinin-induced contraction of cHASM cells, cells were treated with appropriate nucleotides in ITS media for 10 min before bradykinin application. Plates were scanned using a table-top scanner (Epson Perfection, V37, Epson, Leicester, UK) at RT for 10 and 5 min before drug addition, then every 5 min for the first 30 min, and finally every 10 min for the following 30 min (total recording time

spanning 1 hour). Between scans, plates were kept at 37°C in a humidified atmosphere of 5% CO_2/95% O_2. ImageJ (Version 1.49) was used to measure the surface area of the gels and the data were expressed as % gel area compared to H_2O diluent control, which was averaged from the -10 and -5 min time-points. Area under the curve (AUC) analyses were performed using GraphPad Prism 7.

2.9 Cell proliferation assays

The following protocol was modified from Michoud *et al*. (2002). cmT-ASM (passage 2) and cHASM cells (up to passage 5) were harvested using 0.25% trypsin-EDTA solution and seeded into 24-well plates at 10,000 cells/well. Cells were cultured for 48-72 hours in their appropriate media (cmT-ASM or cHASM cell media) containing 10% FBS before being growth-arrested for 48 hours by serum-starving in the same media containing only 0.5% FBS media. Treatment conditions were then added either in 0.5% FBS media or 10% FBS media for 48 hours before the cells were counted using a haemocytometer. For counting, the cells were washed in PBSc and harvested with 0.25% trypsin-EDTA solution. Once all cells had detached, a sample of cells was taken directly from the well in order to minimise cell loss and counted. Statistical analyses were performed using a one-way ANOVA followed by Dunnett's multiple comparisons test (compared to diluent control).

2.10 Statistics

Unless stated otherwise, significance was determined using Student's paired *t*-tests for experiments using cmT-ASM and cHASM cells from the same mouse or donor, and in experiments run on the same day, without and with treatment. Student's unpaired *t*-tests were used when comparing healthy and asthmatic responses in cHASM cells. When several conditions were compared at the same time, one-way ANOVA tests were performed followed by either Dunnett's test when comparing column means to that of a control or by Sidak's test when comparing the means of specific conditions against each other. Significance was accepted when $P<0.05$ (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

3 P2 receptor expression in the airways

3.1 Introduction

Only a small number of studies have previously directly investigated the expression of P2 receptors in ASM, including Govindaraju *et al.* who demonstrated using RT-PCR and western blotting that P2Y1, P2Y2, P2Y4 and P2Y6 receptors are expressed in cHASM cells (Govindaraju et al., 2005). However, they did not investigate the full range of P2 receptors and often only utilised a single tissue or cell type. Therefore, the aim of this chapter was to determine the expression of P2 receptors in a range of tissues/cells relating to the airways, including: mouse lung, strips of mT-ASM and cmT-ASM cells, in addition to cHASM cells from healthy and asthmatic volunteers, using several experimental approaches. First, the P2 receptor transcript profile was determined in the above samples by qPCR. Then, western blotting was used to determine the presence/absence of select P2 receptor proteins in the same samples before immunohistochemistry (immunofluorescence and DAB staining) was used to examine whether these P2 receptor proteins were present in the ASM, specifically. It is known that there is a lack of good, specific antibodies for many of the P2 receptor, and especially for P2Y receptor, subtypes [*e.g.* antibodies against P2Y6 receptor (Yu et al., 2013)]. However, P2X2 and P2X3 receptor proteins are typically only found in cells of nervous tissues (Chen et al., 1995, Lewis et al., 1995, Collo et al., 1996, Ralevic and Burnstock, 1998) and there is limited data regarding functional P2X5 and P2X6 receptors in native tissues (Collo et al., 1996, Lê et al., 1997, Bo et al., 2003, Jones et al., 2004, Duckwitz et al., 2006, Ormond et al., 2006). In this way, the direct investigation of P2 receptor proteins was narrowed to the P2X1, P2X4 and P2X7 receptor subtypes, particularly as antibodies against these receptors have been shown to work well in western blotting and immunohistochemistry (Vial and Evans, 2000b) experiments.

3.2 Results

3.2.1 Measurement of P2 receptor transcript expression levels in airways by qPCR

3.2.1.1 Optimisation of qPCR conditions

3.2.1.1.1 The design and validation of primers for putative mouse reference genes

Primers were designed for putative mouse reference gene transcripts as shown in table 3.1. To validate the specificity of these primers, qPCR were performed on cDNA

Target gene	Primer sequence (Forward, Reverse)	Amplicon size (bp)	Amplification efficiency (%)
18srRNA	5'-CGAACGTCTGCCCTATCAAC-3' 5'-GCCTCGAAAGAGTCCTGTATTG-3'	203	85
B2M	5'-CCTGGTCTTTCTGGTGCTTG-3' 5'-TTCCCGTTCTTCAGCATTTG-3'	174	93
GUSβ	5'-CCGACATGAGAGTGGTGTTG-3' 5'-GGGTATGAGGGGTCAGTGTG-3'	195	96
HPRT1	5'-GTCAACGGGGGACATAAAAG-3' 5'-GGCCTGTATCCAACACTTCG-3'	201	96
LDHAL6B	5'-AATGAAGGGTGAGACGATGG-3' 5'-GCTTGATAATTTCCGCAACC-3'	209	90
NONO	5'-AGCATCATCAGCATCACCAC-3' 5'-GGAAGATTGCCCACAAAGAG-3'	199	97
PPIA	5'-TGCCAGGGTGGTGACTTTAC-3' 5'-ATCCAGCCATTCAGTCTTGG-3'	186	92
RPL13A	5'-TCCCTCCACCCTATGACAAG-3' 5'-TGCTTCTTCTTCCGATAGTGC-3'	196	95
TBP	5'-GCCTTCCACCTTATGCTCAG-3' 5'-CTACTGCCTGCTGTTGTTGC-3'	201	99
TFRC	5'-TAAATTCCCCGTTGTTGAGG-3' 5'-ACAGCTTCCTTCCATTTTTCC-3'	202	95

Table 3.1. List of mouse gene primers tested as potential reference genes for qPCR experiments.

Primers were designed using Primer3. This table also includes the amplicon size (base pairs) and the amplification efficiency (%) for each pair of primers tested. Primers were used for final qPCR experiments only when the amplification efficiency was ~90-105%.

synthesised from a commercial mouse reference total RNA (Agilent Technologies LDA UK Limited, Cheshire, UK) derived from 11 different cell lines. Analysis of the melting curves for each primer pair suggested that each pair amplified single products (data not shown). The products were then run on an agarose gel. A single PCR product band was obtained for each gene (n=1, figure 3.1). The PCR product was extracted from the gel and sent for sequencing. BLAST searching these sequenced products demonstrated that primers amplified their target genes, indicating primer specificity. The amplification efficiencies of each primer pair were then determined (table 3.1).

3.2.1.1.2 The determination of the optimal combination of reference genes

All putative reference gene primer pairs were tested against each cell/tissue type of our airway samples.

In mouse lung samples, the 3 most stably expressed genes were β2M, GUSβ and TBP (n=5, figure 3.2). However, in fresh strips of mT-ASM the 3 most stably expressed genes were TBP, NONO and PPIA (n=5, figure 3.3). In cmT-ASM cells (see results section 3.2.1.2.2) the most stable combination of reference genes was RPL13A, PPIA and HPRT1 (n=4, figure 3.4).

For cHASM cells from both healthy (n=3) and asthmatic (n=4) donors, the 3 most stably expressed reference genes across both phenotypes were YWHAZ, HMBS and HPRT1 (figure 3.5). The different combinations of reference genes used in each sample type are summarised in table 3.2.

3.2.1.1.3 The design and validation of primers for mouse P2 receptor

Primers were designed for mouse P2 receptor transcripts as shown in table 3.3. To validate the specificity of these primers, qPCR were performed either on tissues where the P2 receptor expression will be determined or those known to express the appropriate P2 receptor subtypes. The tissues used for each primer pair are summarised in table 3.4. The products were then run on an agarose gel. A single PCR product band was obtained for each receptor (figure 3.6). The PCR product was extracted from the gel and sent for sequencing. Each set of primers amplified their target gene, demonstrating primer specificity. The amplification efficiencies of each primer pair were also determined and are shown in table 3.3.

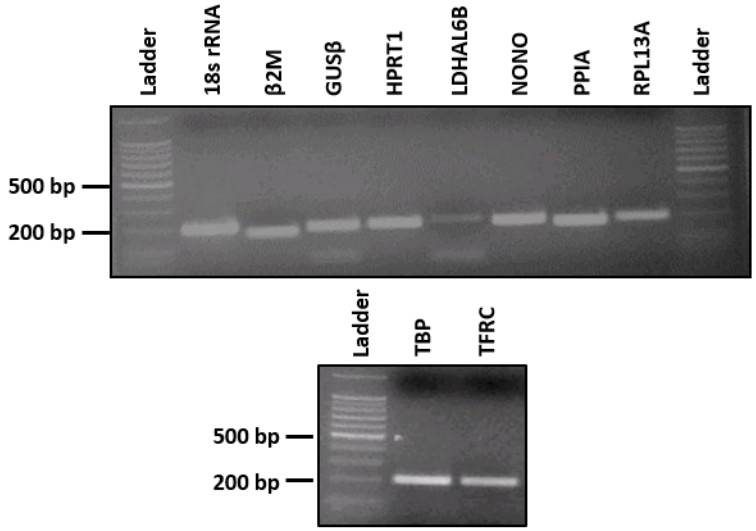

Figure 3.1. Primers for potential mouse reference genes amplify single DNA products.
qPCR were performed with primer pairs for the named transcripts and products were run on a 1.8% agarose gel (n=1). Single products of the expected size (~200 base pairs) were amplified for each primer pair. These PCR products were then extracted and sent for sequencing to confirm primer specificity.

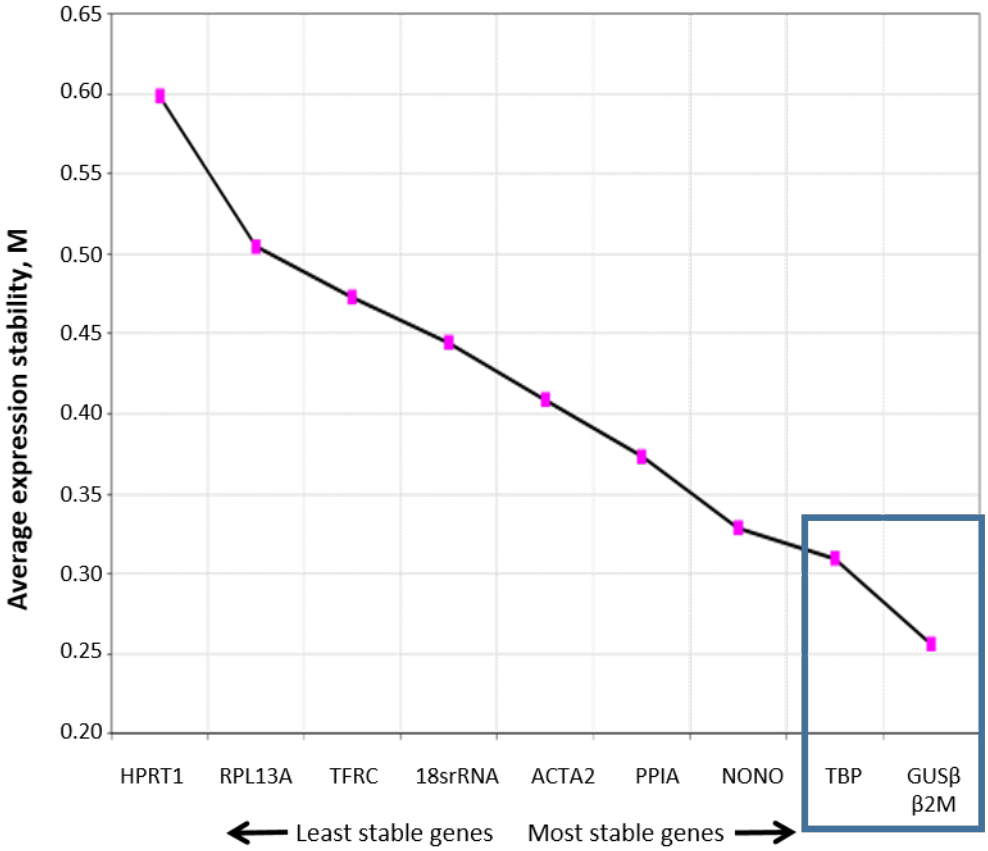

Figure 3.2. Determination of the most stable reference genes to be used for qPCR experiments on mouse lung.

qPCR was performed using a panel of 10 genes and material extracted from mouse lungs (n=5). GeNorm analysis was then performed to determine the 3 most stable genes to be used for gene normalisation (blue box).

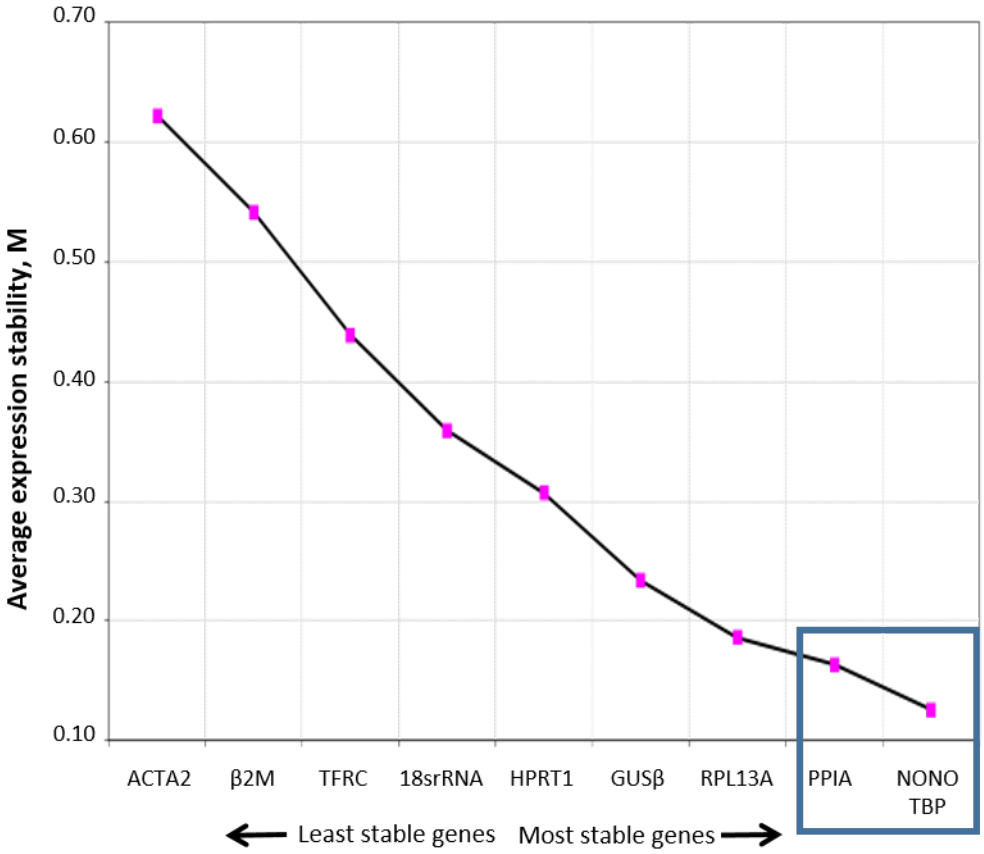

Figure 3.3. Determination of the most stable reference genes to be used for qPCR experiments on fresh mT-ASM strips.

qPCR was performed using a panel of 10 genes and material extracted from strips of mT-ASM (n=5). GeNorm analysis was then performed to determine the 3 most stable genes to be used for gene normalisation (blue box).

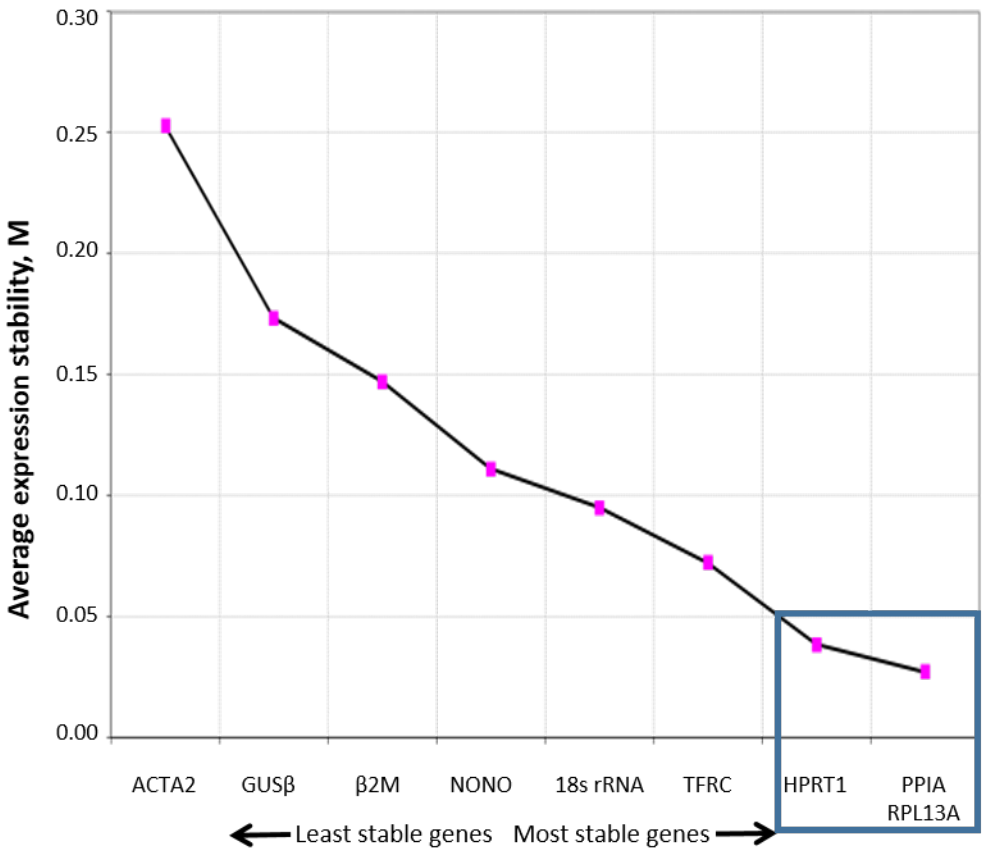

Figure 3.4. Determination of the most stable reference genes to be used for qPCR experiments on cmT-ASM cells.

qPCR was performed using a panel of 9 genes and material extracted from cmT-ASM cells (n=4). GeNorm analysis was then performed to determine the 3 most stable genes to be used for gene normalisation (blue box).

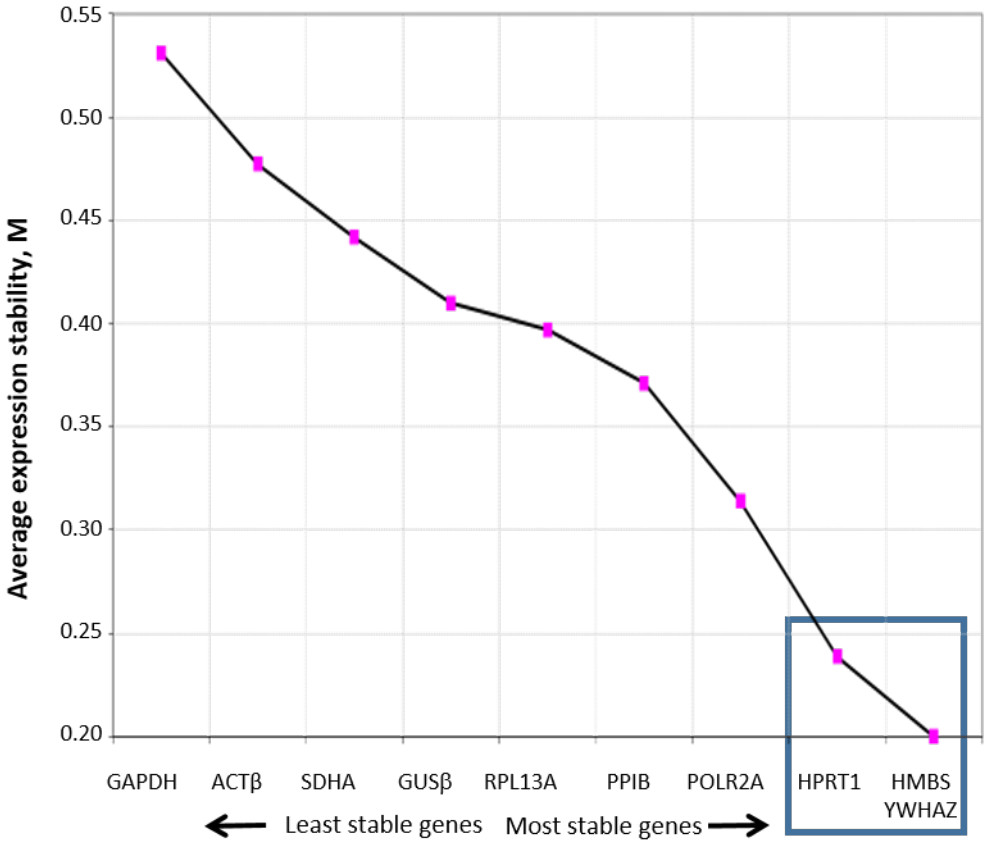

Figure 3.5. Determination of the most stable reference genes to be used for qPCR experiments on cHASM cells from healthy and asthmatic donors.

qPCR was performed using a panel of 10 genes and material extracted from cHASM cells from healthy (n=3) and asthmatic (n=4) donors. GeNorm analysis was then performed to determine the 3 most stable genes to be used for gene normalisation (blue box).

Tissue	Genes tested	Selected reference genes
Mouse lungs	HPRT1, RPL13A, TFRC, 18s rRNA, ACTA2, PPIA, NONO, TBP, GUSβ, β2M	GUSβ, β2M, TBP
Mouse T-ASM (fresh)	HPRT1, RPL13A, TFRC, 18s rRNA, ACTA2, PPIA, NONO, TBP, GUSβ, β2M	PPIA, NONO, TBP
cmT-ASM cells	HPRT1, RPL13A, TFRC, 18s rRNA, ACTA2, PPIA, NONO, GUSβ, β2M	HPRT1, PPIA, RPL13A
cHASM cells	GAPDH, ACTβ, HPRT1, PPIB, SDHA, RPL13A, GUSβ, POLR2A, HMBS, YWHAZ	HPRT1, HMBS, YWHAZ

Table 3.2. Summary of reference genes used in qPCR experiments.
List of the reference genes tested for each tissue/cell type (middle column) and the 3 most stable genes used for normalisation in qPCR experiments (right column).

Target gene	Primer sequence (Forward, Reverse)	Amplicon size (bp)	Amplification efficiency (%)
P2X1	5′-CCTCAAGTGGCCTTATCAGC-3′ 5′-TCTGGGTTCTCTGCACAATG-3′	196	100
P2X2	5′-CGAGGTTACTCCTTCCCAGAC-3′ 5′-TCAGAAGTCCCATCCTCCAC-3′	210	90
P2X3	5′-TCCCCTGGCTACAACTTCAG-3′ 5′-CAGAGAACAGTCCCCACTCC-3′	194	98
P2X4	5′-ATCCCTTCTGCCCCATATTC-3′ 5′-TAGCCAGGAGACACGTTGTG-3′	205	91
P2X5	5′-GATAAAGCTGCCTCCCACTG-3′ 5′-CCGCCTTGCCATTAACTATC-3′	190	92
P2X6	5′-TGACACCAGCTCAAGTCCAG-3′ 5′-CACAGCACCACTCTCCACTG-3′	200	91
P2X7	5′-TGGGGTGACGAAGTTAGGAC-3′ 5′-CTTTGCTCTGTGGGTCCATC-3′	203	95
P2Y1	5′-TCCCTTTGGTGCTGATCTTG-3′ 5′-TCACACATTTCTGGGGTCTG-3′	208	100
P2Y2	5′-CTTTTTGCTGTGCCCTTTTC-3′ 5′-GGTCAAGTGATCGGAAGGAG-3′	211	99
P2Y4	5′-TGACCAGTGCAGACTCCTTG-3′ 5′-TGTAAGTGGCTGTTGCATCC-3′	207	91
P2Y6	5′-TGCTTGGGTAGTGTGTGGAG-3′ 5′-TATGAAGGGCAGCAAGAAGC-3′	190	95
P2Y12	5′-GTCAGAACAAGGGGTTCAGC-3′ 5′-GTCAGCCATAGGGTGCTCTC-3′	200	89
P2Y13	5′-ATGCAGGGCTTCAACAAGTC-3′ 5′-TCAAGTCTGCCACCAGAGTG-3′	193	92
P2Y14	5′-GGCAGGTGAATGTGTTTGTG-3′ 5′-ATGTTTGGGACAGCAAGGAG-3′	211	95

Table 3.3. List of mouse P2 receptor primers used for qPCR experiments. *Primers were designed using Primer3. This table also includes the amplicon size (base pairs) and the amplification efficiency (%) for each pair of primers tested. Primers were used for final qPCR experiments only when the amplification efficiency was ~90-105%.*

Target gene	Tissue used
P2X1	Mouse bladder
P2X2	mT-ASM strips
P2X3	
P2X4	
P2X5	
P2X6	
P2X7	
P2Y1	Mouse bone marrow
P2Y2	mT-ASM strips
P2Y4	Mouse liver
P2Y6	mT-ASM strips
P2Y12	Mouse bone marrow
P2Y13	Mouse liver
P2Y14	mT-ASM strips

Table 3.4. Summary of tissues used to test mouse P2 receptor primer pairs for qPCR. *A summary of the tissues used in order to test the specificity of mouse P2 receptor primer pairs for qPCR. After qPCR were performed, products were run on a gel, excised and sent for sequencing. Resulting sequences were BLAST searched, where they were all found to amplify only their target genes.*

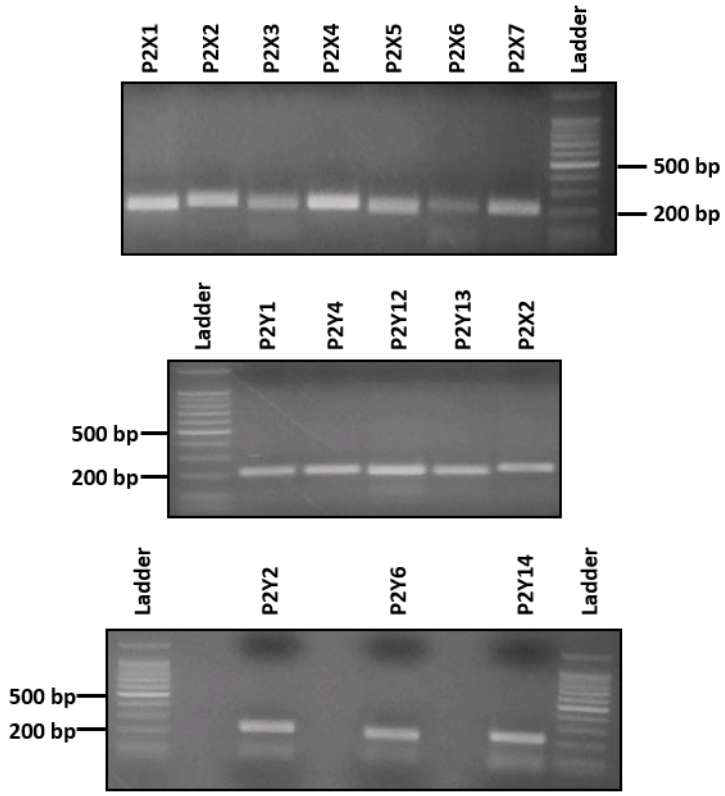

Figure 3.6. Mouse P2 receptor primers (all subtypes) amplified single DNA products.
qPCR were performed with primer pairs for the named transcripts and products were run on a 1.8% agarose gel (n=1). Single products of the expected size (~200 base pairs) were amplified for each primer pair. These PCR products were then extracted and sent for sequencing to confirm primer specificity.

3.2.1.2 P2 receptor transcript expression levels in mouse airways

RNA was isolated from different sections of mouse lung (upper lung, middle lung and peripheral lung, according to figure 2.1), strips of mT-ASM and cmT-ASM cells. The RNA was reverse transcribed and the cDNA used to investigate the expression profiles of P2 receptors in these different tissues by qPCR. The obtained expression profiles are summarised in table 3.5.

3.2.1.2.1 P2 receptor transcript expression levels in mouse lungs

In mouse upper lung, middle lung and the peripheral lung, the P2X receptor transcript expression profile was P2X4>P2X6≈P2X7>P2X1≈P2X5>P2X2≈P2X3 (n=4 for all), while that of P2Y receptors in the same samples was P2Y2≈P2Y6≈P2Y14>P2Y13≈P2Y12>P2Y1 (n=3, n=4 and n=4 for upper, middle and peripheral lung samples, respectively). P2Y4 receptor transcripts could not be confidently detected in these samples (figure 3.7).

3.2.1.2.2 P2 receptor transcript expression levels in mT-ASM

In mTASM strips, the P2X receptor transcript expression profile was P2X4>P2X6≈P2X7>P2X5>P2X3≈P2X2≈P2X1 (n=4), while that of P2Y receptors was P2Y1>P2Y14>P2Y6≈P2Y2≈P2Y12>P2Y13>P2Y4 (n=3) (figure 3.8).

RNA was isolated from the cells cultured from mT-ASM at passage 1 (around 10 days of culture) and qPCR were performed to determine the expression of *ACTA2* (a smooth muscle cell marker) and *PCK* (an epithelial cell marker) transcripts (n=4, figure 3.9). These cells were found to express *ACTA2* transcripts at more than 3 times the abundance of the reference genes, whilst expressing *PCK* transcripts around 100,000 times less than the reference genes. DAB staining was also performed on these cells (passage 1). Anti-α-smooth muscle actin antibodies labelled the majority of the cells in fibre-like intracellular structures whilst anti-pancytokeratin antibodies labelled only one cell in the field shown, despite similar cell densities for each condition. Staining with the isotype control antibody showed negligible background immunoreactivity (n=3 different mice, figure 3.9). These data suggest that mT-ASM cells can be cultured and are still a good model for studying ASM cells at passage 1 as they retain ASM features, such as α-smooth muscle actin expression and negligible amounts of pancytokeratin expression.

Sample type	P2X receptor expression profile	P2Y receptor expression profile
mT-ASM strips	P2X4>P2X6≈P2X7>P2X5>P2X3≈P2X2≈P2X1	P2Y1>P2Y14>P2Y6≈P2Y2≈P2Y12>P2Y13>P2Y4
Mouse upper lung	P2X4>P2X6≈P2X7>P2X1≈P2X5>P2X2≈P2X3	P2Y2≈P2Y6≈P2Y14>P2Y13≈P2Y12>P2Y1
Mouse middle lung	P2X4>P2X6≈P2X7>P2X1≈P2X5>P2X2≈P2X3	P2Y2≈P2Y6≈P2Y14>P2Y13≈P2Y12>P2Y1
Mouse peripheral lung	P2X4>P2X6≈P2X7>P2X1≈P2X5>P2X2≈P2X3	P2Y2≈P2Y6≈P2Y14>P2Y13≈P2Y12>P2Y1
cmT-ASM cells	P2X4≈P2X7>P2X2>P2X5>P2X3≈P2X6>P2X1	P2Y6≈P2Y2≈P2Y14>P2Y12≈P2Y1>P2Y13
cHASM cells from healthy individuals	P2X4>P2X6≈P2X7>P2X5>P2X1≈P2X2≈P2X3	P2Y1>P2Y11>P2Y2≈P2Y6≈P2Y4≈P2Y12>P2Y13
cHASM cells from asthmatic individuals	P2X4>P2X6≈P2X7>P2X5>P2X1≈P2X2≈P2X3	P2Y11>P2Y1≈P2Y4>P2Y6≈P2Y12≈P2Y13≈P2Y2

Table 3.5. A summary of the P2 receptor transcript expression profiles obtained in this study.

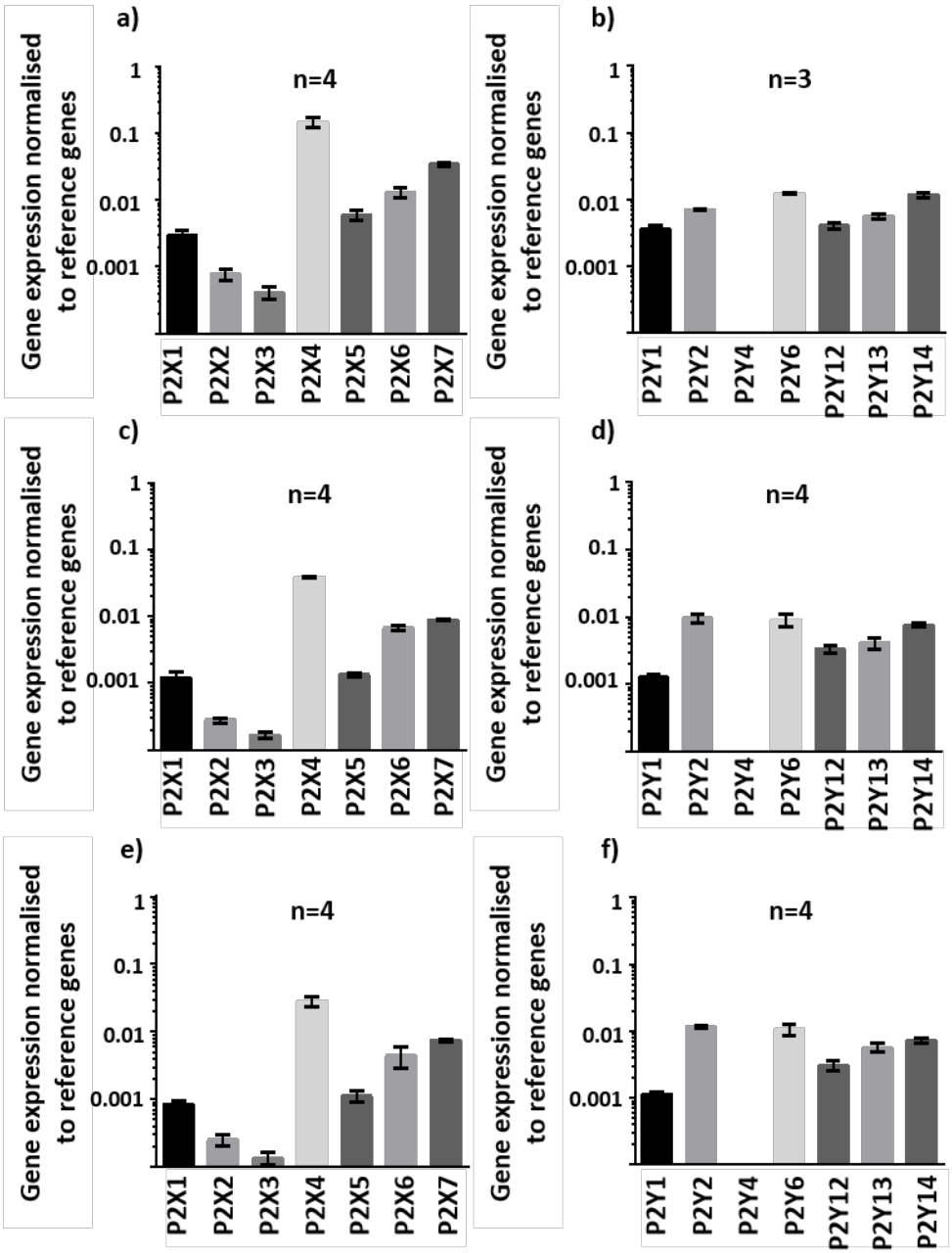

Figure 3.7. Relative expression of P2 receptor transcripts in mouse lungs.
qPCR were performed using material extracted from mouse upper, middle and peripheral lungs. The P2X receptor transcript expression profiles are shown in a) for upper lung, c) for middle lung and e) for peripheral lung. The P2Y receptor transcript expression profiles are shown in b) for upper lung, d) for middle lung and f) for peripheral lungs. Gene expression was normalised to GUSβ, β2M and TBP. Reactions were run as triplicates and n represents the number of mice tested. Data are expressed as mean +/- SEM.

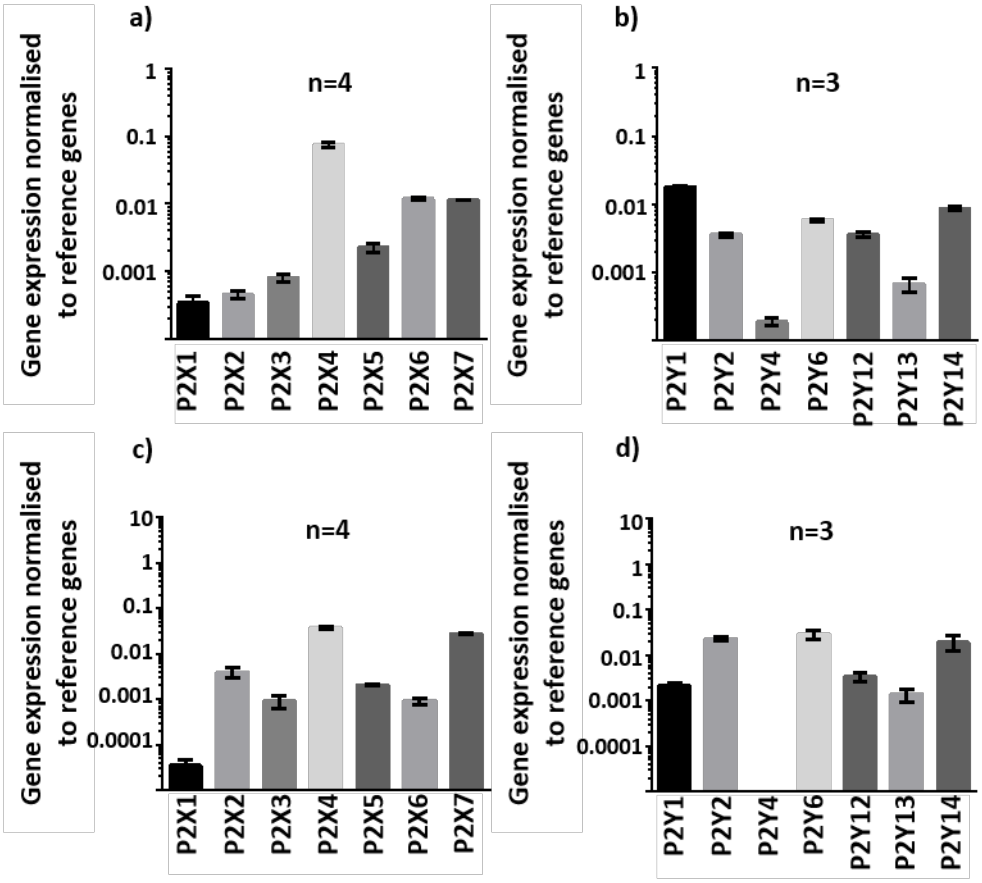

Figure 3.8. Relative expression of P2 receptor transcripts in mT-ASM.
qPCR were performed using material extracted from fresh mT-ASM muscle strips and cmT-ASM cells. The P2X receptor transcript expression profiles are shown in a) for fresh mT-ASM strips and c) for cmT-ASM cells. The P2Y receptor expression profiles are shown in b) for fresh mT-ASM strips and d) for cmT-ASM cells. Gene expression in fresh mT-ASM strips was normalised to PPIA, NONO and TBP, and to PPIA, HPRT1 and RPL13A in cmT-ASM cells. Reactions were run as triplicates and n represents the number of mice tested. Data are expressed as mean +/- SEM.

a)

Target gene	Primer sequence (Forward, Reverse)	Amplicon size (bp)	Amplification efficiency (%)	Tissue used
ACTA2	5'-ACTGGGACGACATGGAAAAG-3' 5'-CAGAGGCATAGAGGGACAGC-3'	204	101	mT-ASM strips
PCK	5'-AACTTCAGCTCAGGCTCTGC-3' 5'-AGCCACACTCTTGGAGATGC-3'	192	102	Mouse lungs

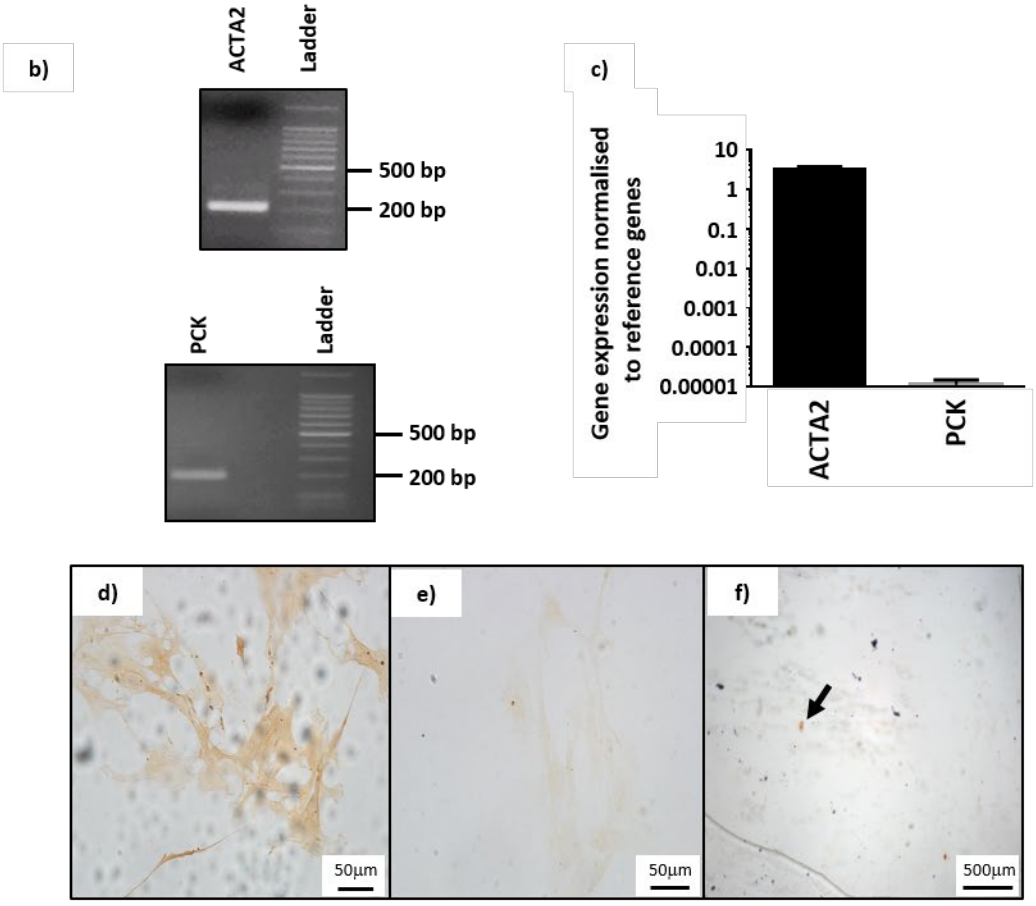

Figure 3.9. mT-ASM cells can be cultured and retain native ASM features.
a) ACTA2 *(α-smooth muscle actin) and* PCK (pan-cytokeratin) *primers were designed using Primer3. The amplicon size (base pairs) and the amplification efficiency (%) for each pair of primers are shown. Primers were used for final qPCR experiments only when the amplification efficiency was ~90-105%. b) qPCR were performed with primer pairs for the named transcripts and products were run on a 1.8% agarose gel (n=1). Single products of the expected size (~200 base pairs) were amplified for each primer pair. These PCR products were then extracted and sent for sequencing to confirm primer specificity. c) qPCR were performed using material extracted from cmT-ASM cells (passage 1). The relative expression of* ACTA2 *and* PCK *transcripts were determined. Gene expression was normalised to PPIA, HPRT1 and RPL13A. Reactions were run as triplicates and data are expressed as mean +/- SEM (n=4). DAB staining was performed on cmT-ASM cells (passage 1) with similar cell densities in each condition. Cells were incubated with d) an anti-α-smooth muscle actin antibody (2.5 µg/ml), e) a polyclonal rabbit IgG isotype control antibody (3 µg/ml) and f) an anti-pancytokeratin antibody (3 µg/ml) (n=3 mice for each condition).*

In cmT-ASM cells (passage 1), the P2X and P2Y receptor transcript expression profiles were P2X4≈P2X7>P2X2>P2X5>P2X3≈P2X6>P2X1 (n=4) and P2Y6≈P2Y2≈P2Y14> P2Y12≈P2Y1>P2Y13 (n=3). P2Y4 receptor transcripts could not be confidently detected in these samples (figure 3.8).

3.2.1.3 P2 receptor transcript expression levels in cHASM cells from healthy and asthmatic individuals

cDNA was produced from cHASM cells from both healthy and asthmatic donors to investigate the expression profiles of P2 receptors in these cells. The obtained expression profiles are summarised in table 3.5.

3.2.1.3.1 P2X receptors

In cHASM cells from both healthy and asthmatic donors, the P2X receptor transcript expression profile was P2X4>P2X6≈P2X7>P2X5>P2X1≈P2X2≈P2X3, respectively (n=3 and 4, figure 3.10). No difference in P2X receptor transcript expression levels between healthy and asthmatic donors was observed.

3.2.1.3.2 P2Y receptors

In cHASM cells from healthy and asthmatic individuals, the P2Y receptor transcript expression profiles were P2Y1>P2Y11>P2Y2≈P2Y6≈P2Y4≈P2Y12>P2Y13 and P2Y11>P2Y1≈P2Y4>P2Y6≈P2Y12≈P2Y13≈P2Y2, respectively (n=4 for all). There were no significant differences in expression levels for any P2 receptor transcripts between cells isolated from healthy or asthmatic individuals using unpaired *t*-tests (P=0.16 for relative gene expression of P2Y1 receptor for healthy versus asthmatic cHASM cells) (figure 3.10).

3.2.2 Study of P2 receptor protein expression in the airways by western blotting

Proteins were extracted from cHASM cells (up to passage 5) from both healthy and asthmatic donors in addition to the following mouse samples: bladder, *vas deferens*, spleen, thymus, lung (in sections of upper, middle and peripheral lung according to figure 2.1), mT-ASM strips and cmT-ASM cells (passage 1).

3.2.2.1 Optimisation of western blotting conditions

Technical optimisation of both the membrane blocking agent and the primary antibody concentrations used for western blotting experiments was performed to determine the optimal conditions.

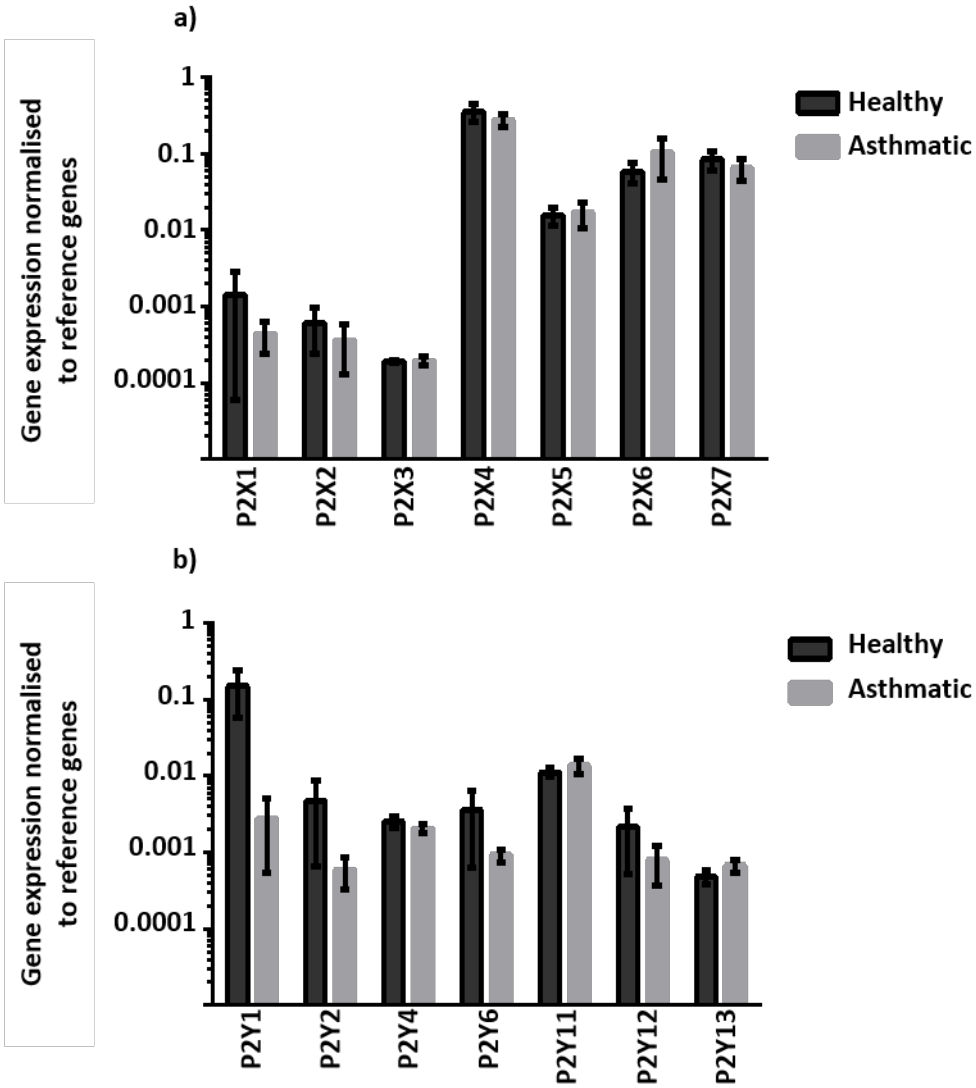

Figure 3.10. Relative expression of P2 receptor transcripts in cHASM cells from healthy and asthmatic donors.

qPCR were performed using material extracted from cHASM cells from healthy (dark bars) and asthmatic donors (light bars). The a) P2X (n=4 for all transcripts except P2X1 and P2X3 in cHASM cells from healthy donors and P2X2 in cHASM cells from asthmatic donors where n=3) and b) P2Y (n=4) receptor transcript expression profiles are shown. Gene expression was normalised to HPRT1, HMBS and YWHAZ. Reactions were run as triplicates and data are expressed as mean +/- SEM.

3.2.2.1.1 The determination of the optimal membrane blocking agent for western blotting

Gels were ran using protein extracted from samples of cHASM cells isolated from healthy and asthmatic donors. Proteins isolated from Xenopus laevis oocytes injected with rat P2X7 mRNA were used as the positive control. Membranes were blocked overnight at 4°C with either AFB-W (AFB 1x in dH_2O) or BS-W (10% milk powder in TTBS) before incubation with anti-P2X7 antibody at 0.4 μg/ml (n=1, figure 3.11). Blocking with AFB-W resulted in strong background noise while blocking with BS-W gave almost no background noise. Therefore, BS-W was used as the blocking agent for all future western blotting experiments.

3.2.2.1.2 The determination of the optimal primary antibody concentrations for western blotting

Proteins were extracted from samples of cHASM cells isolated from healthy and asthmatic donors and were separated by electrophoresis before transfer to nitrocellulose membranes and blocking with BS-W overnight at 4°C. Membranes were incubated with anti-P2X1, anti-P2X4 and anti-P2X7 antibodies at the following concentrations: 0.8 or 0.4 μg/ml, 0.86 or 0.43 μg/ml and 0.8 or 0.4 μg/ml, respectively (n=1, figure 3.12). The positive controls used for P2X1, P2X4 and P2X7 receptor protein expressed were mouse bladder, Xenopus laevis oocytes injected with rat P2X4 mRNA and Xenopus laevis oocytes injected with rat P2X7 mRNA, respectively. No signal was observed with the lower concentration of anti-P2X1 antibody whilst the higher concentration of 0.8 μg/ml revealed bands of the expected MW. On the other hand, the use of the higher concentrations of anti-P2X4 and anti-P2X7 antibodies resulted in too much background staining which completely masked the visualisation of potential bands. However, the lower concentrations tested (0.43 and 0.4 μg/ml for anti-P2X4 and anti-P2X7 antibodies, respectively) allowed the observation of bands at the expected MW without strong background staining. Therefore, for all subsequent experiments, anti-P2X1, anti-P2X4 and anti-P2X7 antibodies were used at 0.8 μg/ml, 0.43 μg/ml and 0.4 μg/ml, respectively.

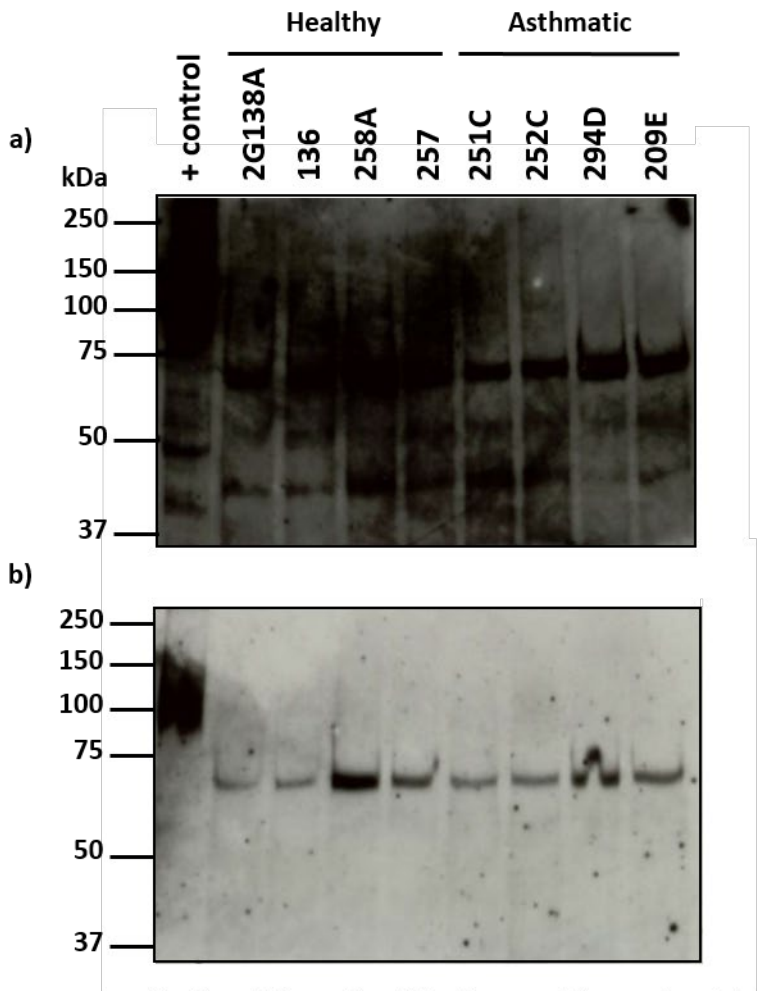

Figure 3.11. Determination of the optimal blocking agent for western blotting.
Western blots showing the presence of P2X7 receptor protein in cHASM cells where membranes were blocked with either a) AFB-W or b) BS-W overnight at 4°C. 7.5μg of protein lysates from cHASM cells (passages 2-5) isolated from different healthy and asthmatic donors (as illustrated by their identifier codes) were loaded per lane and membranes were incubated with an anti-P2X7 antibody at 0.4 μg/ml (n=1). The positive control used was Xenopus laevis oocytes injected with rat P2X7 mRNA.

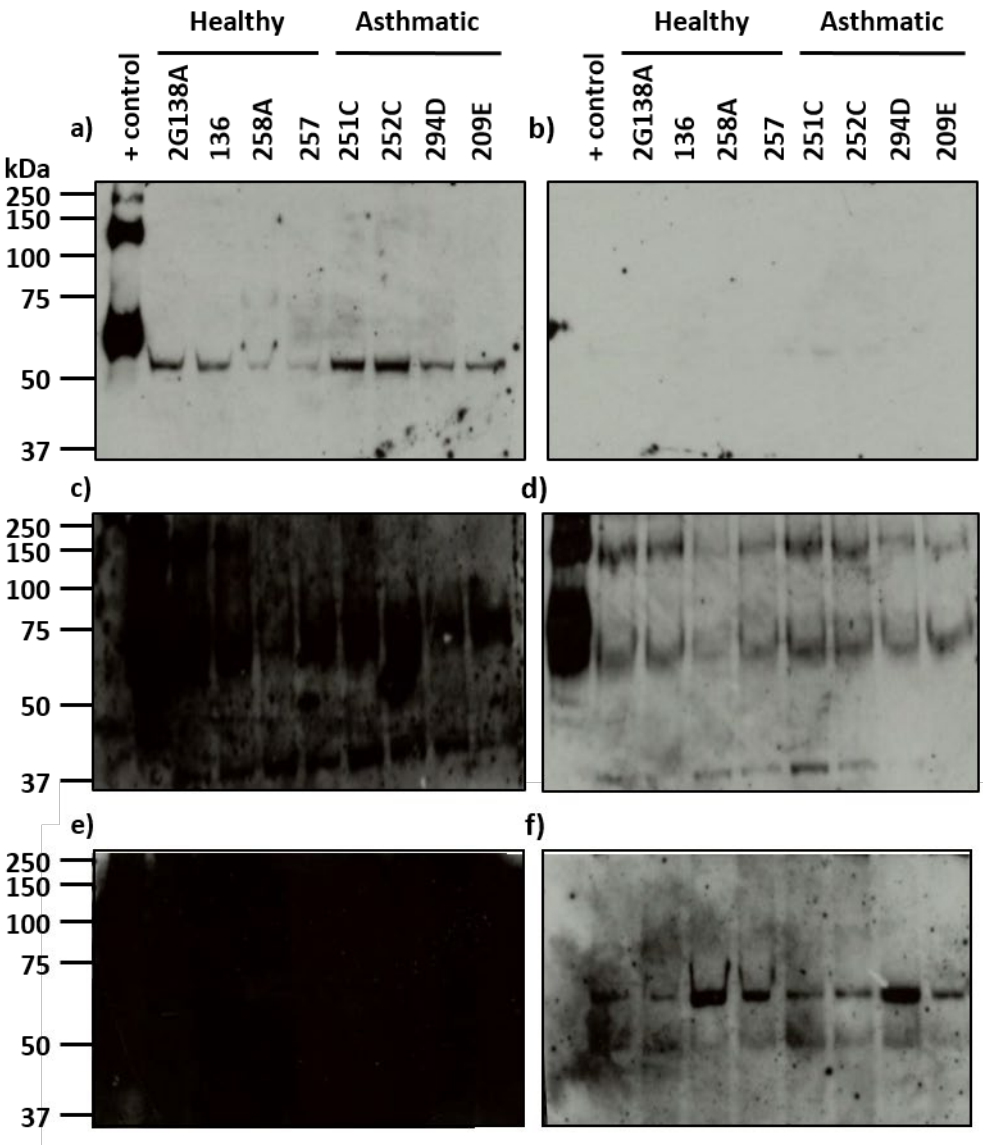

Figure 3.12. Optimisation of anti-P2X1, anti-P2X4 and anti-P2X7 antibody incubation concentrations for western blotting.

cHASM cells were isolated from several healthy and asthmatic donors as indicated by their identifier codes. 7.5μg/lane of protein lysates from these donors were loaded for all conditions tested. After SDS-PAGE, transfer of the proteins onto nitrocellulose membranes and blocking overnight at 4°C with BS-W, the membranes were incubated with anti-P2X1 antibody at either a) 0.8 μg/ml or b) 0.4 μg/ml, anti-P2X4 antibody at either c) 0.86 μg/ml or d) 0.43 μg/ml and anti-P2X7 antibody at either e) 0.8 μg/ml or f) 0.4 μg/ml (n=1). The positive controls used were: a) and b) mouse bladder, c) and d) Xenopus laevis oocytes injected with rat P2X4 mRNA and e) and f) Xenopus laevis oocytes injected with rat P2X7 mRNA.

3.2.2.2 P2X receptor protein expression in mouse tissues by western blotting

To detect the presence of P2X1, P2X4 and P2X7 receptor proteins in mouse trachea, proteins were extracted from whole tracheas and strips of mT-ASM to perform western blotting.

The monomeric (~55kDa) and trimeric (~150kDa) forms of the P2X1 protein were detected in mouse urinary bladder and *vas deferens*, which acted as positive controls for P2X1 expression (Mulryan et al., 2000, Vial and Evans, 2000b). Both the monomeric and trimeric forms were also present in the spleen, thymus, all lung samples and cmT-ASM cells. Only the trimeric form was present in mT-ASM strips (n=4, figure 3.13).

P2X4 receptor transcripts have been detected in the spleen of C57BL/6 mice (de Baaij et al., 2014) whilst the presence of both transcript and protein have been observed in rat thymus (Bo et al., 1995, Glass et al., 2000). Therefore, mouse spleen and thymus were used as positive controls for P2X4 receptor expression, and indeed both the monomeric (~70kDa) and the dimeric (~150kDa) forms of the P2X4 receptor were detected. In all tested samples from mouse airways including the upper, middle and peripheral lungs, and both strips of mT-ASM and cmT-ASM cells, both the monomeric and dimeric forms were detected (n=4, figure 3.13).

P2X7 receptor transcripts are expressed in crude rat thymus extracts (Glass *et al.*, 2000), 2BH4 cells (a C57BL/6 thymic epithelial cell line) (Bisaggio et al., 2001) and crude extracts from mouse spleen (Masin et al., 2012). Therefore, mouse spleen and thymus were used as positive controls for P2X7 receptor expression, in which the monomeric (~85kDa) form of the P2X7 receptor was found. This band was also present in all other tested samples, including samples from mouse upper, middle and peripheral lungs, and both strips of mT-ASM and cmT-ASM cells. An additional band (~65kDa) observed in the lung samples could be due to truncated isoforms of the receptor (n=4, figure 3.13), which have been described for P2X7 receptors (Sluyter and Stokes, 2011).

3.2.2.3 P2X receptor protein expression in cHASM cells by western blotting

For western blotting experiments using cHASM cells, the positive controls for P2X1, P2X4 and P2X7 receptor expression were mouse bladder and *Xenopus laevis* oocytes injected with rat P2X4 and P2X7 mRNA, respectively.

The monomeric form (~55kDa) of the P2X1 receptor protein was present in cHASM cells, with no observable difference between healthy and asthmatic donors. A

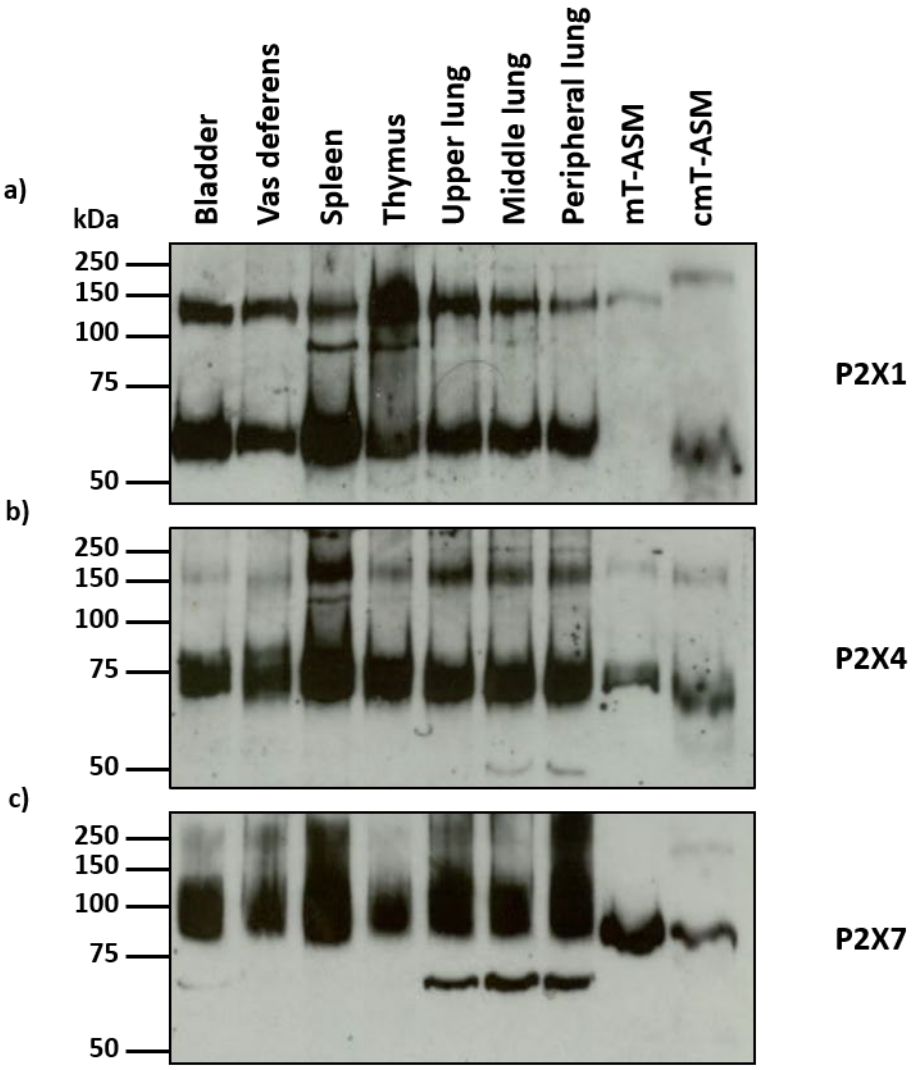

Figure 3.13. Expression of P2X1, P2X4 and P2X7 receptor proteins in various mouse tissues, including lungs, strips of mT-ASM and cmT-ASM cells.

10μg of protein lysates from various tissues were loaded per lane. After SDS-PAGE and transfer of the proteins onto nitrocellulose membranes, the membranes were incubated with a) 0.4 μg/ml of anti-P2X1 antibody, b) 0.43 μg/ml of anti-P2X4 antibody and c) 0.4 μg/ml of anti-P2X7 antibody. Bladder, vas deferens, spleen and thymus were used as positive control tissues for the expression of the 3 different subtypes of P2X receptors. mT-ASM and cmT-ASM refer to strips of mouse T-ASM and cultured mT-ASM cells, respectively. Figures are representative of 4 different mice.

band which could be attributed to the monomeric form (~65kDa) was present in the positive control of mouse bladder. The difference in MW between the cHASM cells and the positive control could be attributed to post-translational modifications, such as glycosylation, which are present in the mouse tissue that have been lost in cHASM cells, potentially due to the effect of cell culture. Additionally, the trimeric form (~145kDa) of P2X1 receptor was present in the positive control.

Both the monomeric (~70kDa) and dimeric (~150kDa) forms of the P2X4 receptor protein were found in the positive control as well as all tested human samples, including all healthy and asthmatic donors. There was no apparent difference in P2X4 expression between healthy and asthmatic donors.

Single bands correlating to the monomeric form of P2X7 receptor protein were observed in all human samples (~69kDa) and the positive control (~80kDa). Again, the difference in the observed MW could be due to the loss of post-translational modifications from cHASM cells due to the effect of cell culture which are present in the positive control of Xenopus laevix oocytes injected with rat P2X7 mRNA. No apparent difference in P2X7 receptor protein expression was observed between healthy and asthmatic donors (n=4 for each receptor in both healthy and asthmatic donors, figure 3.14).

3.2.3 Investigation of P2X receptor protein expression in the airways using immunohistochemistry

Western blotting can determine if a protein is expressed in an organ or tissue, but does not allow discrimination between the cell types in those tissues. Therefore, to further investigate the presence and tissue localisation of the P2X receptors (P2X1, P2X4 and P2X7 receptors) in mouse airways, samples of mouse airways were subjected to immunofluorescence- and DAB-based immunohistochemistry. These samples consisted of mouse trachea and mouse lung. The lungs were not split into upper/middle/peripheral samples as in previous experiments in order to maintain the structural integrity of the samples, and as qPCR data suggested that there was no difference in P2 receptor transcript expression along the airway tree.

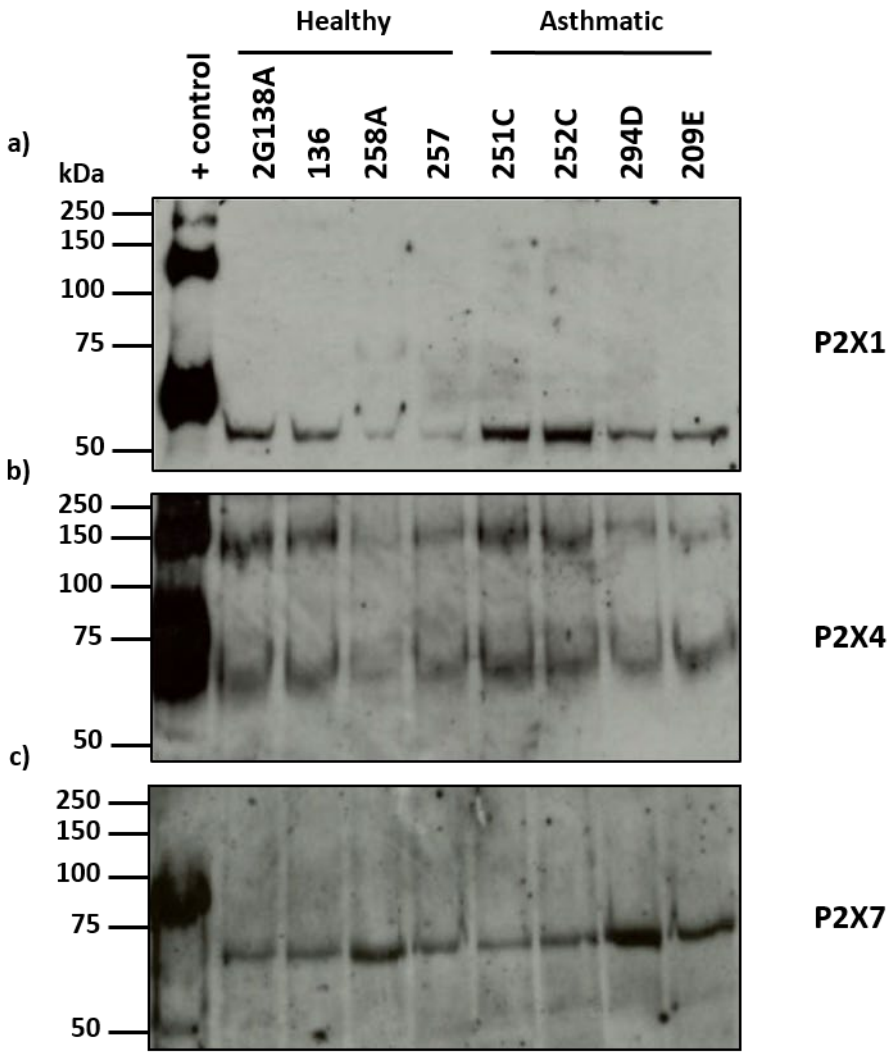

Figure 3.14. Expression of P2X1, P2X4 and P2X7 receptor proteins in cHASM cells from healthy and asthmatic donors.

7.5μg of protein lysates from cHASM cell samples were loaded per lane. After SDS-PAGE and transfer of the proteins onto nitrocellulose membranes, the membranes were incubated with 0.8 μg/ml, 0.43 μg/ml and 0.4 μg/ml of a) anti-P2X1, b) anti-P2X4 and c) anti-P2X7 antibodies respectively. The positive controls were: a) mouse bladder, b) Xenopus laevis oocytes injected with rat P2X4 mRNA and c) Xenopus laevis oocytes injected with rat P2X7 mRNA. Sample names relate to donor identifier codes from healthy and asthmatic donors (n=4 donors for each condition).

3.2.3.1 Immunofluorescence

3.2.3.1.1 Technical optimisation of sample preparation

To maintain lung structure during sectioning, lungs were inflated with a viscous substance before flash freezing on dry ice and hexane. Two viscous substances were tested: 50% sucrose and O.C.T. O.C.T-perfused lungs had better structural preservation than 50% sucrose-perfused lungs, but the level of background autofluorescence was very high (n=2, figure 3.15). Therefore, lungs were perfused with 50% sucrose in subsequent immunofluorescence experiments.

3.2.3.1.2 P2X1 receptor expression in mouse lungs and trachea by immunofluorescence

Due to high background fluorescence in the lungs, no positive staining in the anti-P2X1 antibody treated sections could be discerned when compared to the negative controls (sections treated with non-immune serum, with the secondary antibody only and with anti-P2X1 antibody pre-incubated with its antigen peptide) (n=3, figure 3.16). However, in mouse trachea, P2X1 immunoreactivity was detected in the smooth muscle layer of the trachea and was absent in the negative controls (n=3, figure 3.17).

As the high background fluorescence observed in the lungs was present in all conditions, including the negative controls, and was present with either viscous perfusion substance, immunofluorescence is not an appropriate approach to use to investigate P2 receptor localisation. Therefore, a DAB-based immunohistochemistry approach was optimised next to test its suitability for the same purpose.

3.2.3.2 DAB staining

3.2.3.2.1 Technical optimisation

3.2.3.2.1.1 Method of fixation

To ensure that the fixation method used does not damage tissue morphology, fixation of mouse lungs and trachea sections was performed with either 2% paraformaldehyde or 10% neutral buffered formalin for 10 min before counterstaining with haematoxylin and eosin. While there was no observable difference in tracheal sample structure and integrity when fixed with either formalin or paraformaldehyde, lung fixation with paraformaldehyde maintained the integrity of the connective tissues and epithelium more than fixation with formalin (n=1, figure 3.18).

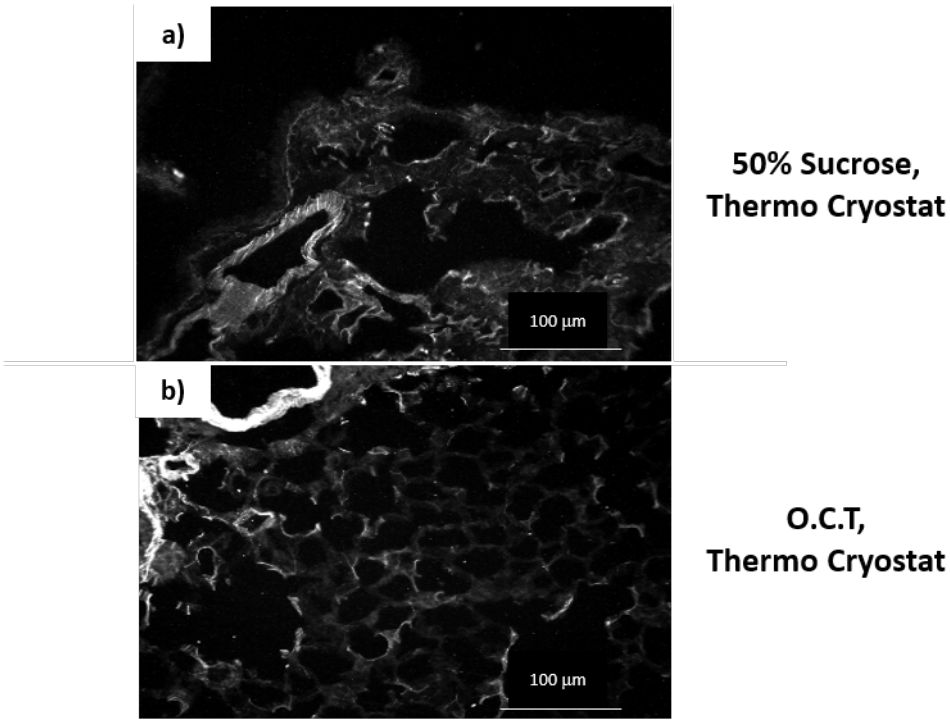

Figure 3.15. Technical optimisation of mouse lung section sample preparation to maintain tissue integrity for use with immunofluorescence.

Sections of mouse lung, all treated with non-immune serum from donkey only and sectioned at 12µm, were imaged to show the structure of the lung. a) Lung samples were perfused with 50% sucrose solution and sectioned with the Thermo Cryostat. b) Lung samples were perfused with O.C.T and sectioned with the Thermo Cryostat (n=2).

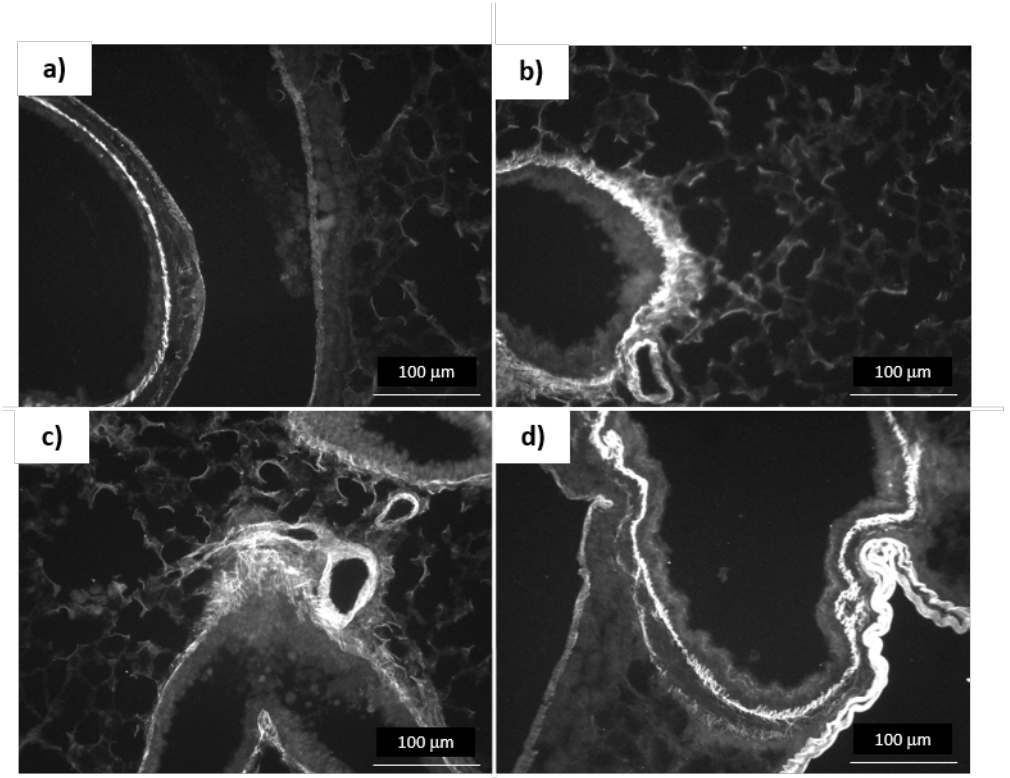

Figure 3.16. Determination of P2X1 receptor expression in mouse lungs using immunofluorescence.

Sections of mouse lung, all perfused with 50% sucrose and sectioned at 12 µm, were incubated either with a) non-immune serum from donkey, b) the secondary antibody, c) anti-P2X1 antibody at 4 µg/ml or d) anti-P2X1 antibody pre-incubated with its antigen peptide, both at 4 µg/ml (n=3).

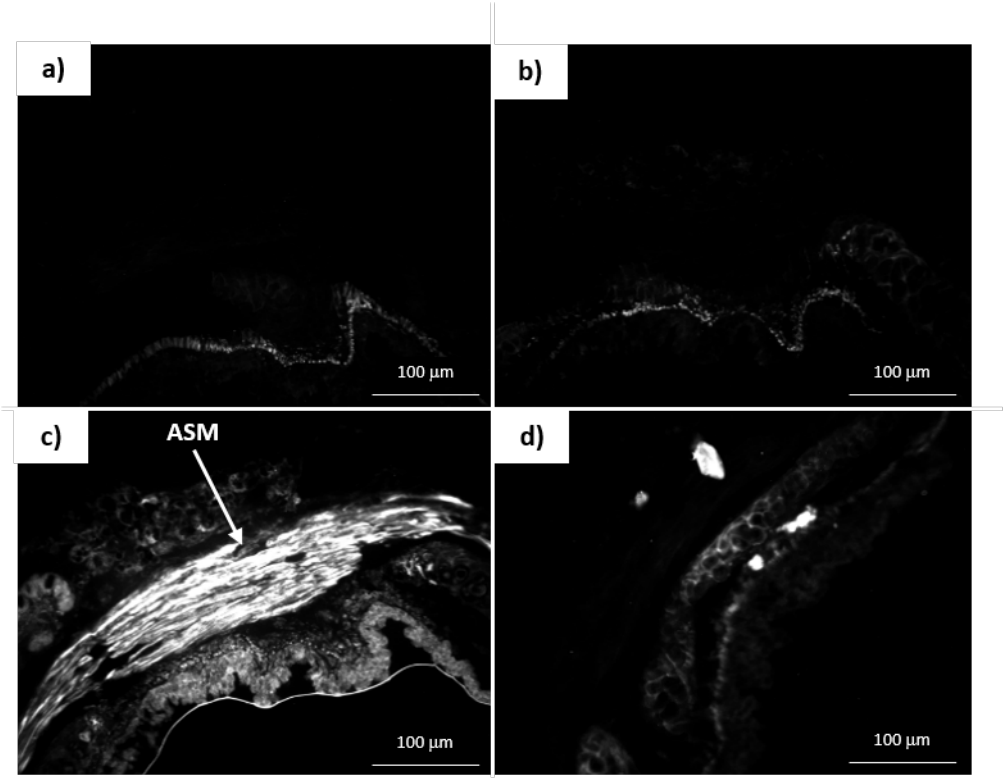

Figure 3.17. Determination of P2X1 receptor expression in mouse trachea using immunofluorescence.

Sections of mouse trachea, sectioned at 12µm, were incubated either with a) non-immune serum from donkey, b) the secondary antibody, c) anti-P2X1 antibody at 4 µg/ml or d) anti-P2X1 antibody pre-incubated with its antigen peptide, both at 4 µg/ml (n=3). The arrow indicates the ASM layer present on the dorsal side of the trachea.

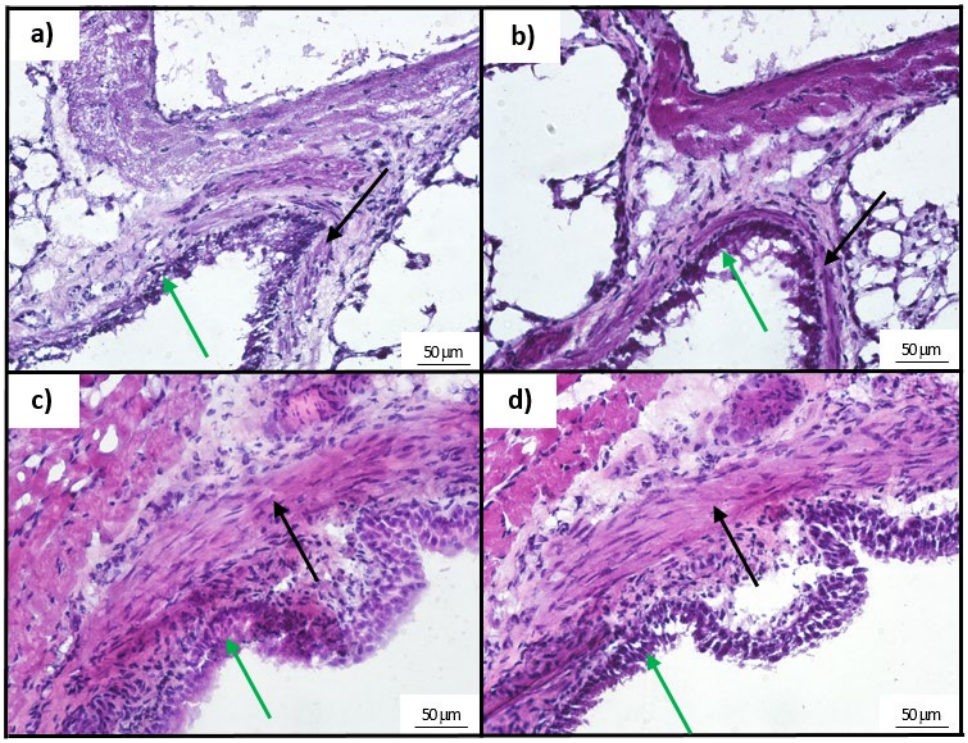

Figure 3.18. Optimisation of fixation method for immunohistochemistry.
Sections of mouse lung (a and b) and trachea (c and b) were fixed in 10% formalin (a and c) or 2% paraformaldehyde (b and d) before counterstaining with haematoxylin and eosin (n=1). Black and green arrows indicate the ASM and the epithelium, respectively.

Therefore, samples for immunohistochemistry techniques were subsequently fixed with 2% paraformaldehyde.

3.2.3.2.1.2 Optimisation of isotype control antibodies

IC antibody controls (*e.g.* general rabbit polyclonal IgG) are used to differentiate non-specific antibody staining from specific antibody staining. ICs are the same antibody class as the primary antibodies and should be prepared in the same way (*e.g.* same concentration and incubation conditions) as the primary antibodies. However, they lack specificity to a target protein. We tested several IC antibodies and different blocking conditions simultaneously.

Firstly, mouse lung sections blocked for 1 hour at RT in BS-D [PBS containing 0.3% Triton X-100 solution and 5% goat serum] were incubated with the rabbit polyclonal IgG IC antibodies from either GeneTex (Bioscience, Nottingham, UK), DAKO (DAKO, Cambridgeshire, UK) or Immunostep (n=1, figure 3.19), all at 6 μg/ml (adjusted to the highest concentration of another primary antibody that was being optimised alongside this experiment). Of these, GeneTex and Immunostep antibodies gave less non-specific immunoreactivity and so these were used for further optimisation However, staining was strong even with these two antibodies, and so sections were blocked for longer in subsequent optimisation.

Next, lung sections were blocked for 2 hours at RT in BS-D 5% goat serum before incubation with the GeneTex and the Immunostep IC antibodies at 4 μg/ml (adjusted to the highest concentration of another primary antibody that was being optimised alongside this experiment). There was less staining with the Immunostep IC antibody than with the GeneTex IC antibody (n=1, figure 3.19). Therefore the Immunostep IC antibody was chosen for use in future experiments.

3.2.3.2.1.3 Optimisation of blocking conditions for DAB staining

Lung sections were blocked in different conditions before being incubated with the Immunostep IC antibody at 3 μg/ml (used at the same concentration as the anti-pancytokeratin primary antibody, which had the highest final concentration of any primary antibody used in this study). 5 different blocking conditions were used: BS-D [PBS containing 0.3% Triton X-100 solution] containing 5% and 10% goat serum (Cell Signalling Technology) at 4°C for 2 hours, BS-D containing 10% goat serum at RT for 2

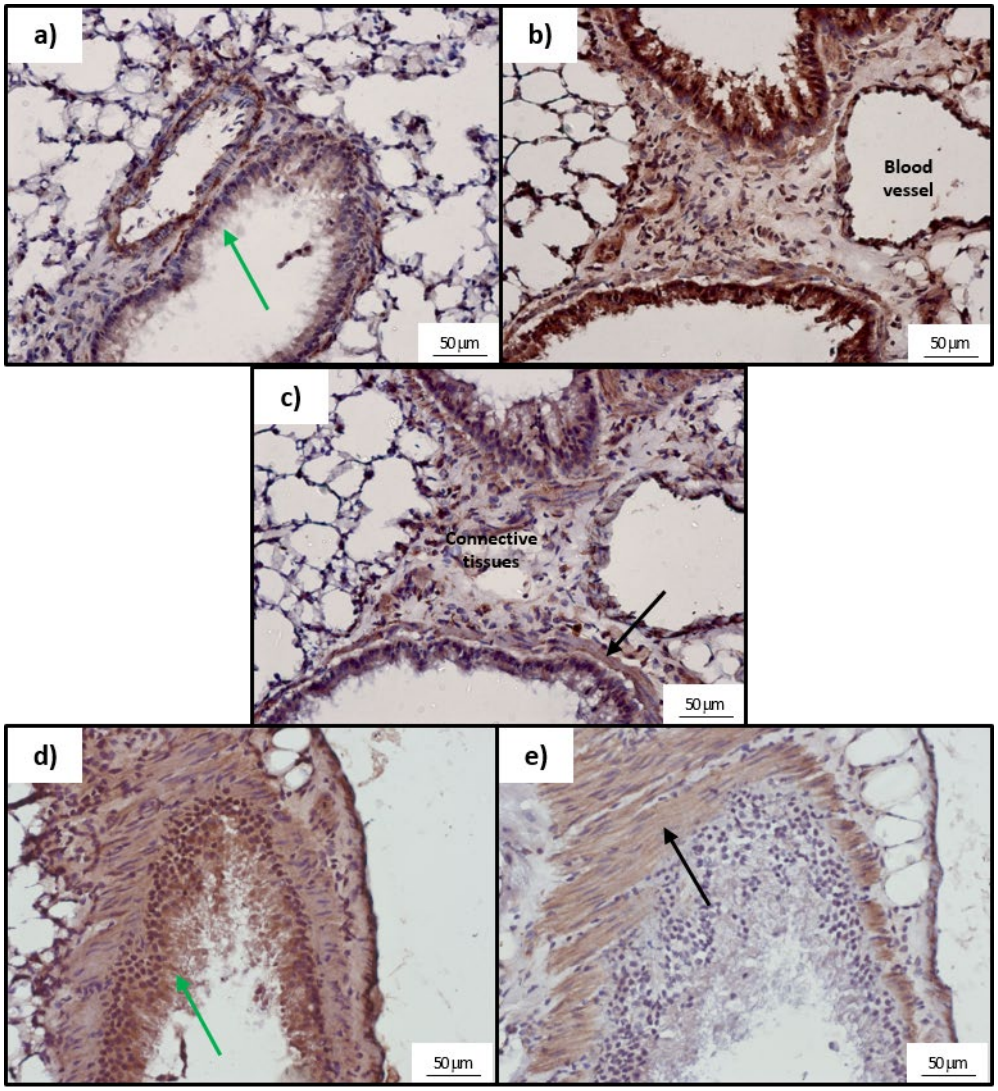

Figure 3.19. Determination of optimal isotype control antibody conditions in DAB staining.

Sections of mouse lung were blocked for 1 hour in BS-D containing 5% goat serum at RT before immunostaining with either the a) GeneTex, b) DAKO or c) Immunostep IC antibodies at 6 µg/ml (n=1). Sections of lung from another mouse were blocked for 2 hours in BS-D containing 5% goat serum at RT before immunostaining with d) GeneTex or e) Immunostep IC antibodies at 4 µg/ml (n=1). Black and green arrows indicate the ASM and the epithelium, respectively.

hours and either BS-D containing 10% goat serum or AFB-BS [AFB 1x containing 0.3% Triton X-100 in dH2O] at 4°C overnight. Blocking at 4°C for 2 hours in BS-D containing 10% goat serum resulted in less background staining than blocking in BS-D containing 5% goat serum for the same time. Blocking in BS-D containing 10% goat serum at RT for 2 hours reduced the staining further, and gave comparable staining when the same solution was used BS-D at 4°C overnight. However, blocking with AFB-BS gave the least amount of non-specific staining (n=1, figure 3.20) out of all blocking conditions tested. Therefore, for all following experiments, sections were blocked in AFB-BS overnight at 4°C.

3.2.3.2.1.4 Optimisation of primary antibody concentrations for DAB staining

For immunohistochemistry techniques, antibody concentrations must be optimised so that good staining can be obtained without losing specificity and increasing background staining. To do this, mouse lung sections were incubated with different primary antibodies at various concentrations.

For P2X1 receptor, immunoreactivity was observed on the sections of mouse lung for all anti-P2X1 antibody concentrations tested (2, 1.3 and 1 μg/ml). However, the respective ICs gave non-specific staining at the highest concentration (2 μg/ml) whilst the lower concentrations didn't result in any visible staining (n=1, figure 3.21). Therefore, anti-P2X1 antibodies were used at 1.3 μg/ml to give specific staining whilst not preventing the observation of potentially weak signals (*i.e.* low P2X1 receptor protein expression).

When mouse lung sections were probed with anti-P2X4 antibodies at 1.42, 1.06 and 0.85 μg/ml, strong immunoreactivity was observed in all tissues whilst no non-specific staining was observed with any of the ICs (n=1, figure 3.22). Therefore, the lowest concentration (0.85 μg/ml) was used to give strong, specific staining.

Sections incubated with anti-P2X7 antibodies at 1.3, 1 and 0.8 μg/ml resulted in comparable immunoreactivity. No immunoreactivity was observed with the IC antibody at any concentration (n=1, figure 3.23). Therefore, anti-P2X7 antibodies were used at 1.3 μg/ml.

Lung sections were also incubated with anti-α-smooth muscle actin antibodies (which stain smooth muscle) at 5, 3.33 and 2.5 μg/ml. Immunoreactivity was observed

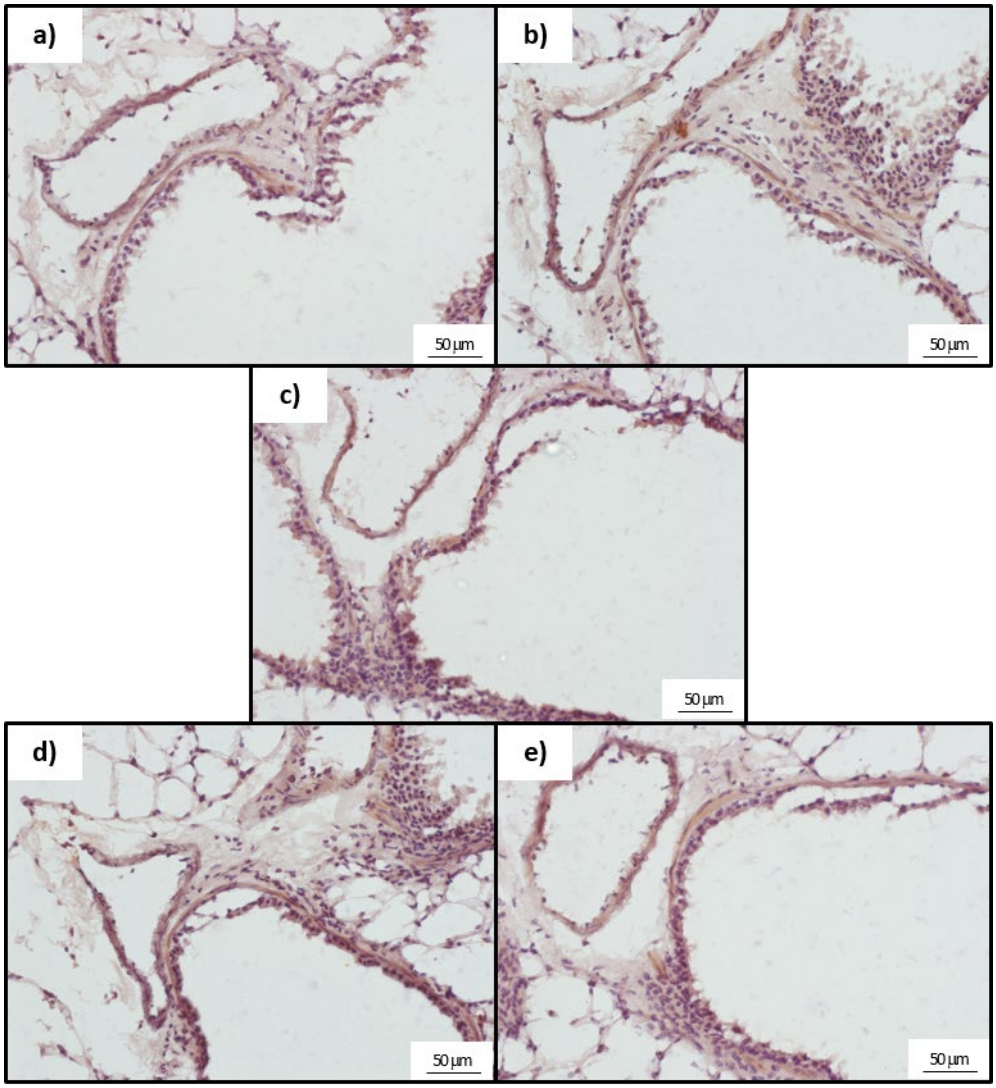

Figure 3.20. Determination of optimal blocking conditions for immunohistochemistry.

Sections of mouse lung were blocked in BS-D containing either a) 5% or b) 10% goat serum at 4°C for 2 hours, c) BS-D containing 10% goat serum at RT for 2 hours, d) BS-D containing 10% goat serum at 4°C overnight or in e) AFB-BS at 4°C overnight. Sections were then probed with the Immunostep IC antibody at 3 µg/ml (n=1).

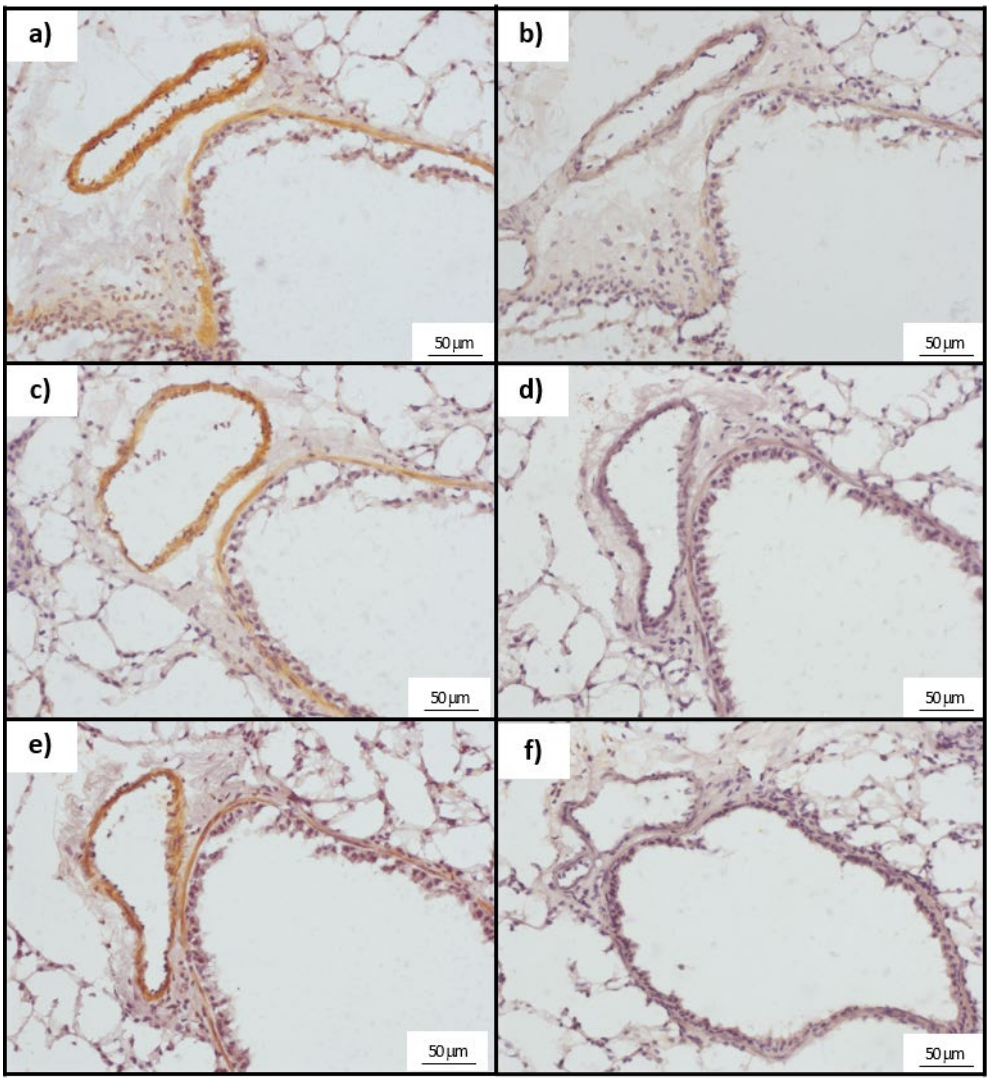

Figure 3.21. Optimisation of anti-P2X1 antibody concentration for immunohistochemistry.

Sections of mouse lung were immunostained with anti-P2X1 antibodies at a) 2 µg/ml, c) 1.3 µg/ml and e) 1 µg/ml, along with their corresponding isotype controls at the same concentrations [b), d) and f), respectively] (n=1).

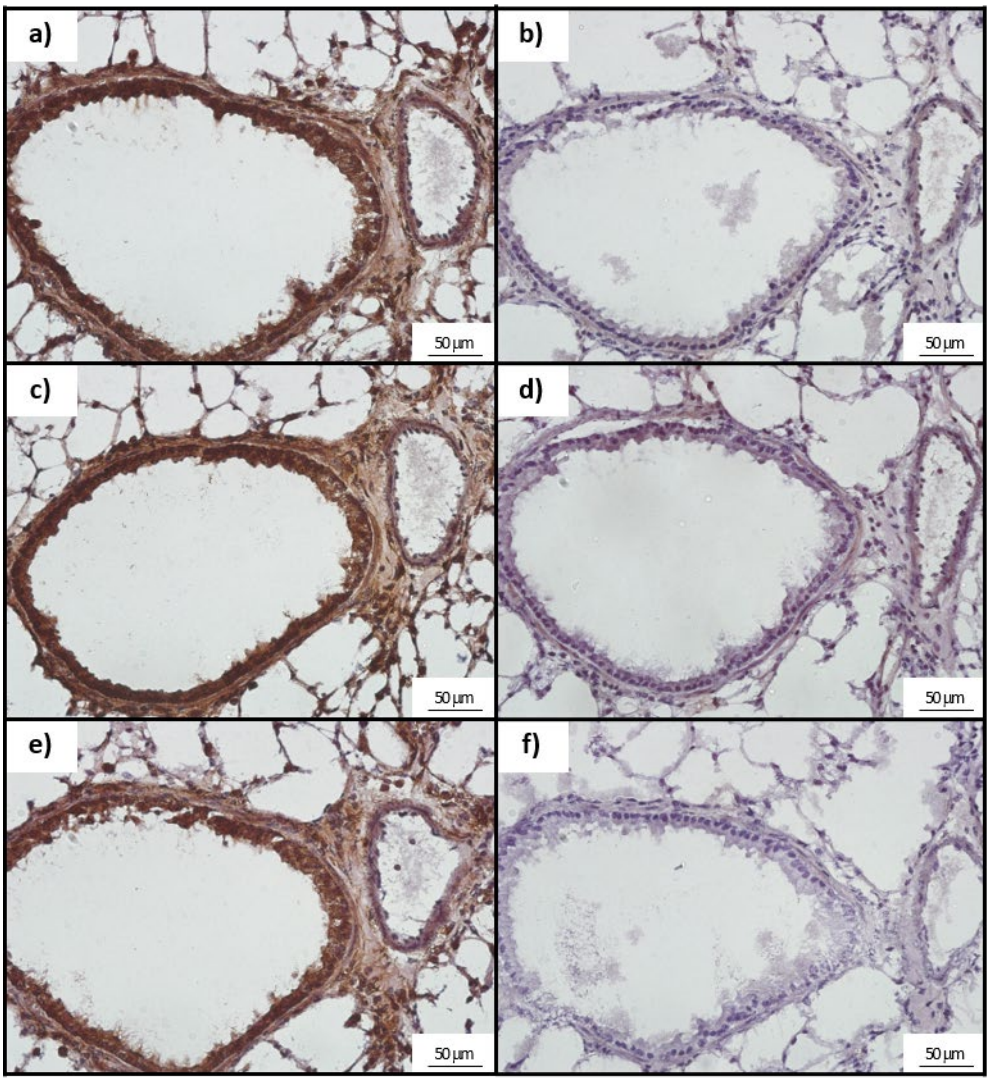

Figure 3.22. Optimisation of anti-P2X4 antibody concentration for immunohistochemistry.

Sections of mouse lung were immunostained with anti-P2X4 antibodies at a) 1.42 µg/ml, c) 1.06 µg/ml and e) 0.85 µg/ml, along with their corresponding isotype controls at the same concentrations [b), d) and f), respectively] (n=1).

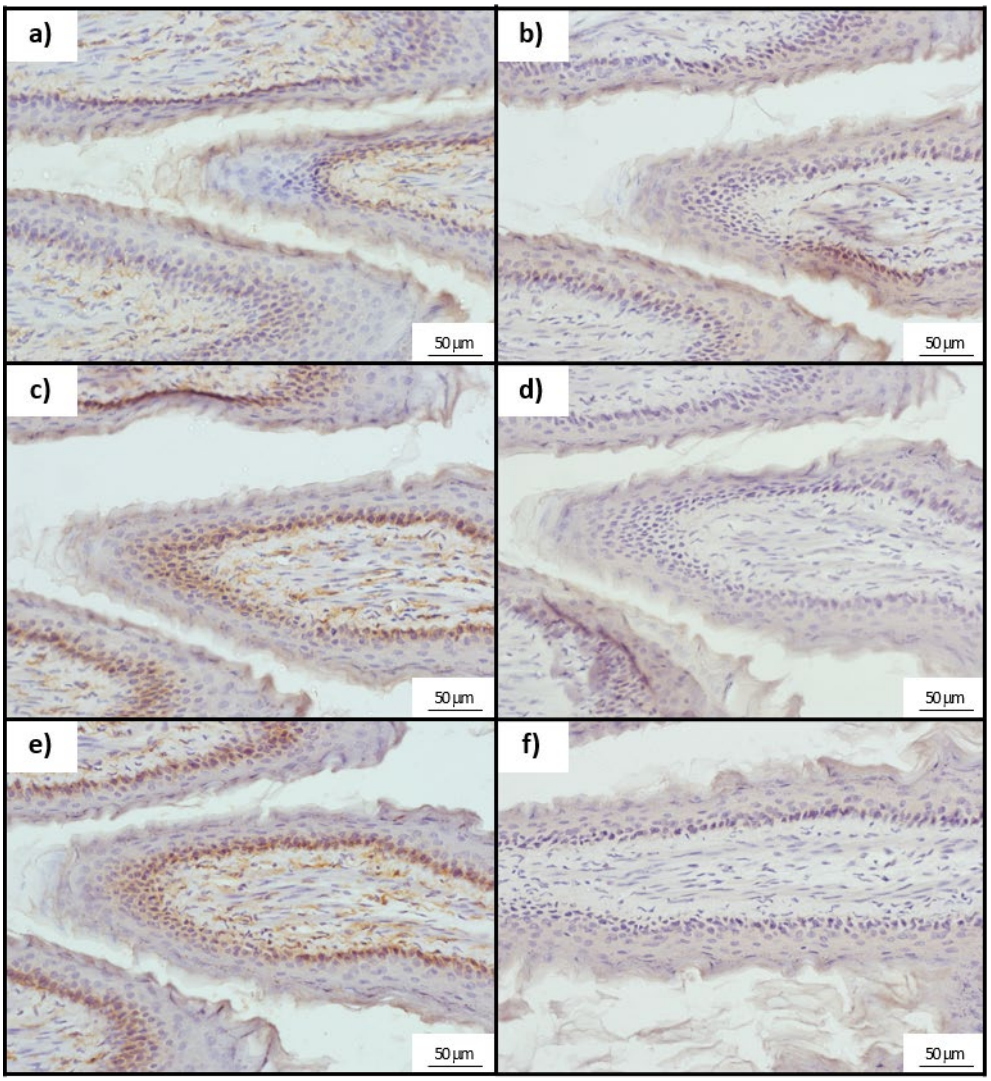

Figure 3.23. Optimisation of anti-P2X7 antibody concentration for immunohistochemistry.

Sections of mouse lung were immunostained with anti-P2X7 antibodies at a) 1.3 µg/ml, c) 1 µg/ml and e) 0.8 µg/ml, along with their corresponding isotype controls at the same concentrations [b), d) and f), respectively] (n=1).

in the epithelium in addition to the ASM at all but the lowest concentration, which gave strong staining only in the ASM (n=2, figure 3.24). Therefore, anti-α-smooth muscle actin antibodies were used at 2.5 μg/ml.

Finally, lung sections were also incubated with anti-pancytokeratin antibodies (which stain the epithelium) at 6, 4 and 3 μg/ml (n=2, figure 3.24). Strong immunoreactivity was observed in the ASM and connective tissues in the 2 highest concentrations whilst only negligible non-specific staining was observed in the lowest concentration. Therefore, anti-pancytokeratin antibodies were used at 3 μg/ml.

3.2.3.2.1.5 Antigen retrieval

The crosslinking of peptides by fixation can mask protein epitopes and therefore prevent antibodies from binding to their specific binding sites. Antigen retrieval can restore masked epitopes by partially breaking some of the methylene crosslinks formed between the peptides whilst maintaining the fixed state of the tissue. Bathing sections in 1% SDS (in PBS) is a common antigen retrieval technique for frozen sections (Brown et al., 1996), and this approach was tested here to see if it affected antibody staining.

Sections of mouse lung were probed with antibodies against P2X1 and P2X4 receptors. Staining was comparable between those samples which had received antigen retrieval and those which had not (n=1, figure 3.25). Therefore, IHC experiments with DAB staining were performed without antigen retrieval.

3.2.3.2.1.6 Summary of the experimental conditions after optimisation

After optimisation, all DAB experiments were performed as follows: tissue sections were fixed in 2% paraformaldehyde for 10 min at RT, no antigen retrieval was performed, sections were blocked overnight at 4°C in AFB, sections were incubated with primary antibodies for 5 hours at RT to ensure maximal antibody-antigen hybridisation, the Immunostep IC antibody was used at 1.3 mg/ml (adjusted to the highest concentration of the P2 receptor primary antibodies used).

3.2.3.2.2 P2X receptor protein expression in mouse trachea

In sections of mouse trachea, immunoreactivity for P2X1 receptor was observed in the ASM but not in the epithelium, whilst immunoreactivity for P2X4 and P2X7 receptors was present in both the ASM and the epithelium. No staining was observed either in the smooth muscle or the epithelium in the presence of the IC antibody, whilst

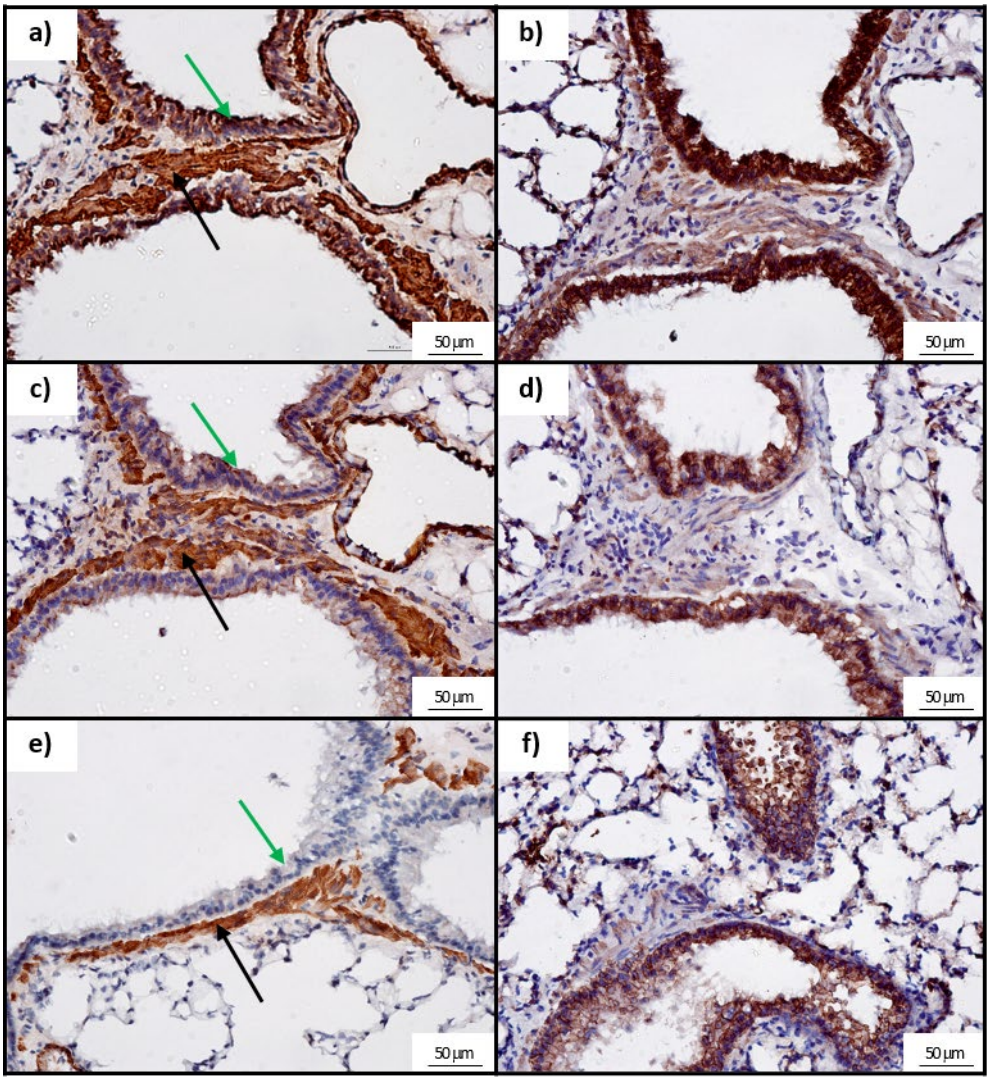

Figure 3.24. Optimisation of anti-α-smooth muscle actin and anti-pancytokeratin antibody concentrations for immunohistochemistry.

Sections of mouse lung were probed with either anti-α-smooth muscle actin antibodies at a) 5 µg/ml, c) 3.33 µg/ml and e) 2.5 µg/ml or anti-pancytokeratin antibodies at b) 6 µg/ml , d) 4 µg/ml and f) 3 µg/ml (n=2 for each). Black and green arrows indicate the ASM and the epithelium, respectively.

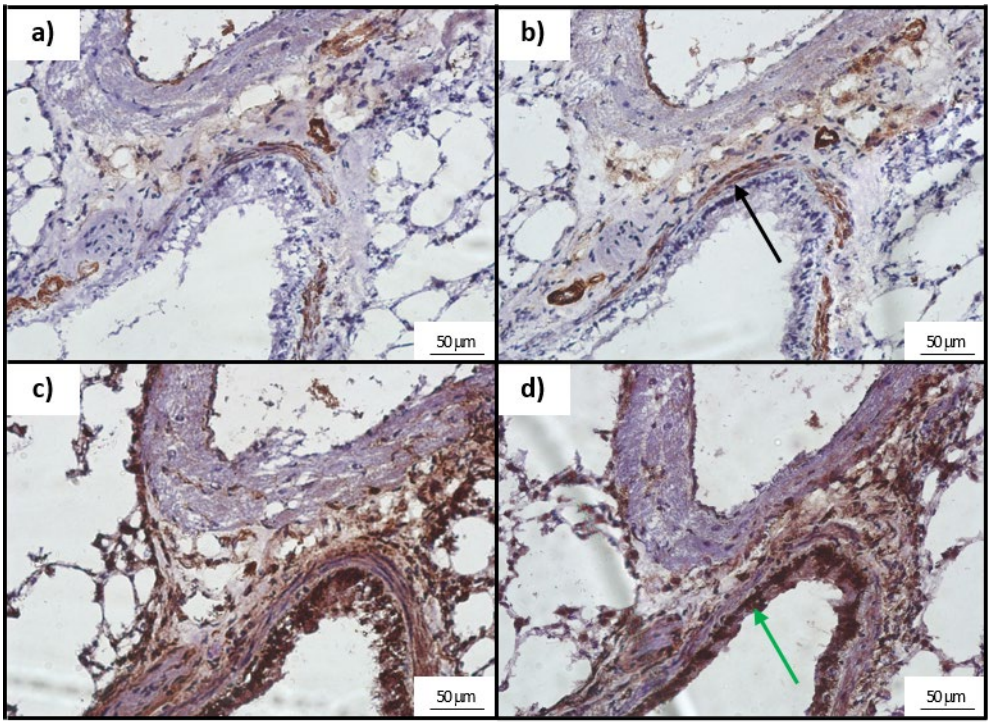

Figure 3.25. Antigen retrieval is not necessary for P2X1 and P2X4 immunostaining.
Sections of mouse lung were probed with anti-P2X1 (a and b) and anti-P2X4 (c and d) antibodies at 1.3 µg/ml and 0.85 µg/ml, respectively, without (a and c) and with (b and d) treatment in 1% SDS in PBS (n=1 for each). Black and green arrows indicate the ASM and the epithelium, respectively.

incubation with anti-α-smooth muscle actin and anti-pancytokeratin antibodies labelled the ASM and the epithelium, respectively (n=3, figure 3.26). These observations support our previous qPCR and western blotting data suggesting that P2X1, P2X4 and P2X7 transcripts and proteins are expressed in the smooth muscle of mouse trachea.

3.2.3.2.3 P2X receptor protein expression in mouse lung

In mouse lung sections, immunoreactivity for P2X1 receptor was present in the ASM but not in the epithelium, whilst immunoreactivity for P2X4 and P2X7 receptors was observed in both the ASM and the epithelium. There was no staining in either the smooth muscle or the epithelium in the presence of the IC antibody, whilst incubation with anti-α-smooth muscle actin and anti-pancytokeratin antibodies labelled the ASM and the epithelium, respectively (n=3, figure 3.27). These observations support our previous qPCR and western blotting data suggesting that P2X1, P2X4 and P2X7 transcripts and proteins are expressed in the smooth muscle of mouse lungs.

3.3 Discussion

This chapter aimed to determine the expression of P2 receptors in ASM. To do this, several experimental approaches were undertaken, including qPCR (to determine the relative transcript expression levels), western blotting (to determine the protein expression of P2X1, P2X4 and P2X7 receptors), and immunohistochemistry (to establish the localisation of these P2 receptors in airway samples). These experiments were performed using samples from mouse and human airways, in addition to cells cultured from these samples, to enable the comparison of data from an animal model with that of humans.

Before performing the qPCR experiments to look at P2 receptor transcript expression in ASM, primers were first designed and validated to ensure that each primer pair amplified only their target genes as single products. Primers were not designed for murine P2Y11 receptor due to a lack of both genetic and functional evidence for a P2Y11 receptor in mice (von Kügelgen and Harden, 2011, Dreisig and Kornum, 2016).

qPCR experiments (and following experiments) made use of cmT-ASM cells. It was necessary to characterise these cells before their use to ensure that they had the correct phenotype corresponding to ASM cells. As discussed in section 1.2.6, a common

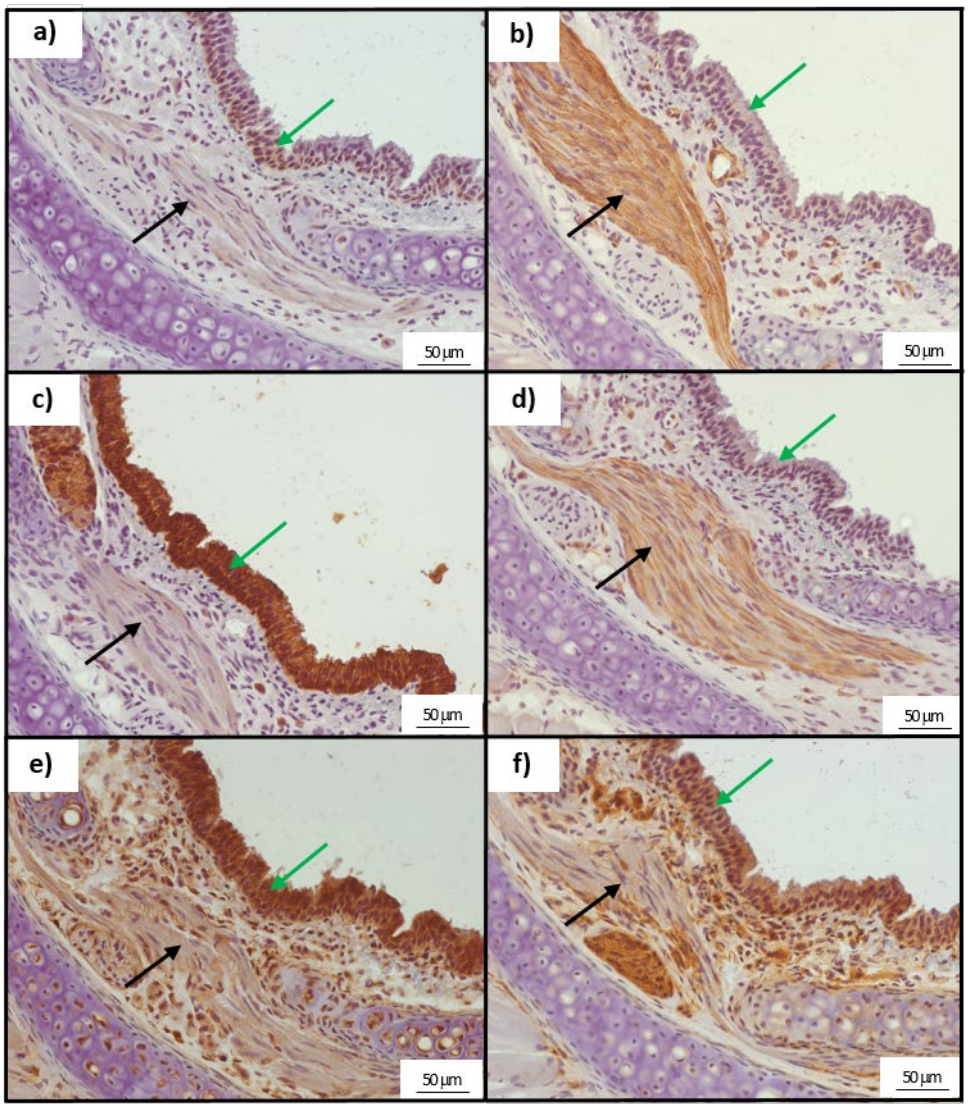

Figure 3.26. P2X1, P2X4 and P2X7 receptor proteins are expressed in mouse tracheal ASM.

Sections of mouse trachea were incubated in the presence of a) the IC antibody (1.3 µg/ml) and antibodies raised against b) α-smooth muscle actin (2.5 µg/ml), c) pancytokeratin (3 µg/ml), d) P2X1 (1.3 µg/ml), e) P2X4 (0.85 µg/ml) and f) P2X7 (1.3 µg/ml) (n=3). Black and green arrows indicate the ASM and the epithelium, respectively.

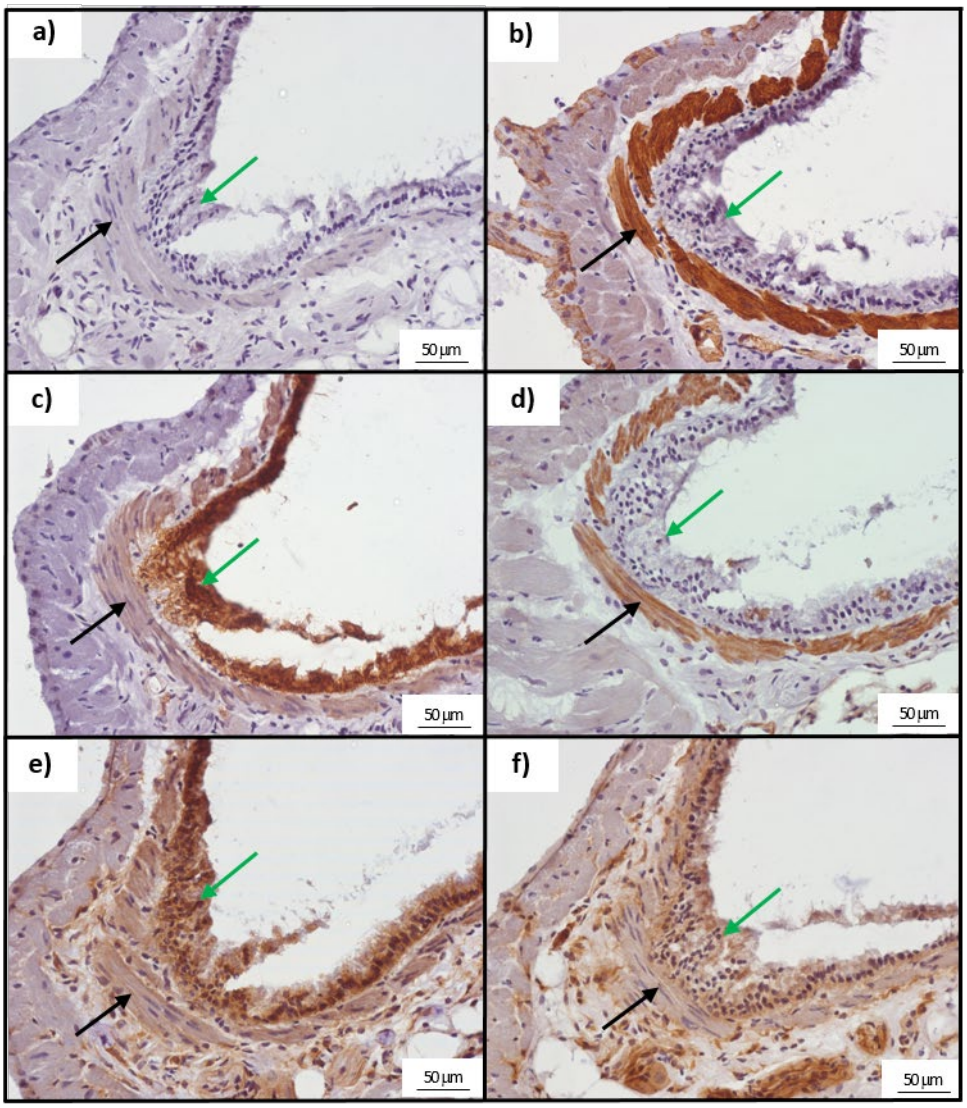

Figure 3.27. P2X1, P2X4 and P2X7 receptor proteins are expressed in mouse lung ASM.

Sections of mouse lung were incubated in the presence of a) the IC antibody (1.3 µg/ml) and antibodies raised against b) α-smooth muscle actin (2.5 µg/ml), c) pancytokeratin (3 µg/ml), d) P2X1 (1.3 µg/ml), e) P2X4 (0.85 µg/ml) and f) P2X7 (1.3 µg/ml) (n=3). Black and green arrows indicate the ASM and the epithelium, respectively.

smooth muscle cell marker is α-smooth muscle actin (Chamley-Campbell et al., 1979, Campbell and Campbell, 1993). Several studies confirm the presence of a pure population of smooth muscle cells by observing positive staining for α-smooth muscle actin and negative staining pan-cytokeratin, a marker of the common contaminating epithelial cell (Michoud et al., 1997, Michoud et al., 1999, Michoud et al., 2002, Liu et al., 2014). Using qPCR and immunohistochemistry, these cells were confirmed to express α-smooth muscle actin and not pan-cytokeratin for the large part. Therefore, these cells were satisfactorily characterised as ASM cells and used for subsequent experiments.

Following the validation of the primer pairs and the characterisation of the cmT-ASM cells, optimisation of qPCR reference genes was performed for each tissue type used in order to ensure the accurate and reliable quantification of relative P2 receptor expression. Often, gene expression in published studies is normalised to single genes that are considered typical 'housekeeping genes', such as GAPDH. However, it is becoming more recognised that this approach is not satisfactory, particularly when dealing with human-derived samples where variation between samples is common (Glare et al., 2002, Tricarico et al., 2002). Therefore, for this study, a panel of potential reference genes was tested to determine their expression stability in each tissue using GeNorm analysis software, which recommends a cut-off of 1.5 M (measure of stability) (Vandesompele et al., 2002). All tested genes were below this cut-off, and the 3 most stably expressed genes in each tissue were used for normalization.

Conditions for western blotting and immunohistochemistry experiments, including blocking conditions and antibody concentrations, were also optimised to enable the accurate and reliable detection of P2 receptor proteins, which could then be compared with P2 receptor transcript expression.

Final qPCR experiments demonstrated that P2 receptor transcripts are expressed in the tested samples, and the expression profiles are summarised in table 3.5. There is limited information in the current literature regarding P2 receptor transcript expression in ASM. For example, P2X4 receptor transcript and protein have been detected in porcine T-ASM cells (Nagaoka et al., 2009), and indeed is the dominant P2X receptor transcript expressed in rat ASM, whilst P2X1 receptor transcript is lowly expressed (Gui et al., 2011). Additional studies have investigated the expression of P2Y

receptors in ASM, identifying that P2Y2, P2Y4 and P2Y6 receptor transcripts are expressed in cultured rat T-ASM cells (Michoud et al., 2002) and cHASM cells (Govindaraju et al., 2005). P2Y1 receptor transcript was also detected in the latter study. Despite the limitations of these studies, such as the use of several different species and the fact that the expression of all P2 receptor has not been investigated in one single model, our findings presented here generally agree with the current literature. One small difference is that P2Y4 receptor transcript could not be confidently detected in mouse lung samples in this study despite its presence in low abundance in strips of mT-ASM, whilst it was expressed in rat T-ASM cells (Michoud et al., 2002). However, despite both species being rodents, this difference could be species-specific.

Arguably the most important observation from the qPCR data, however, is that P2X1 receptor transcript expression is low in all samples. Considering the dominant role that P2X1 receptor plays in the contraction of other types of smooth muscle (Mulryan et al., 2000, Vial and Evans, 2000b, Vial and Evans, 2002), this finding is surprising although it does agree with current literature (Gui et al., 2011). Investigations into the presence of P2X1 receptor protein positively identified its protein in airway samples, yet suggested that its expression was low. Although the specificity of this anti-P2X1 antibody has been previously questioned due to the observation of positive immunoreactivity using this antibody in the central nervous system of P2X1-knockout mice (Ashour et al., 2006), these data correlate well with the qPCR data. Indeed, low expression of P2X1 receptor in mouse lungs could be the reason that immunofluorescence experiments could not detect P2X1 receptor protein there, particularly as ASM layers surrounding airways in the lung tend to be thin compared with those in the trachea, where P2X1 receptor protein expression was more obvious. This issue could have been exacerbated by the high background fluorescence of the lung samples which could have masked the weak P2X1 receptor signal. By using DAB staining as an alternative approach to immunohistochemistry, the potential issue of high autofluorescence was circumvented and allowed the visualisation of P2X1 receptor expression localised to the ASM in both mouse trachea and lung. Western blotting and DAB staining data also confirmed the expression of P2X4 and P2X7 receptor protein in ASM from murine trachea and lungs. To our knowledge, the only report regarding P2X

receptor protein expression in ASM demonstrated that P2X4 receptor protein is present in porcine T-ASM cells (Nagaoka et al., 2009), which corroborates our findings.

Protein expression is known to change along the airway tree (Berg et al., 2014, Singhania et al., 2017), and the study of P2 receptor transcript expression in mouse samples from T-ASM, and upper, middle and peripheral lung allowed the discrimination between the different areas. However, there were no observable changes in P2 receptor transcript expression in samples descending the airway tree. These data correlate well with western blotting data where there is no discernible difference in the expression of P2X1, P2X4 or P2X7 receptor protein between the samples of mouse lung, although data could suggest that there is reduced protein expression in samples of mT-ASM strips compared to mouse lung.

Culturing cells from mT-ASM strips allowed the performance of a wider range of functional assays than using freshly isolated cells alone (see section 4). Cell culture is known to induce changes (e.g. increases and decreases) in transcript and protein expression in many cell types compared to uncultured cells (Neumann et al., 2010, Januszyk et al., 2015), including smooth muscle cells (Chang et al., 2014, Hashemi Gheinani et al., 2015). P2 receptor expression, specifically, is known to change due to cell culture in several cell types including murine microglia (Crain et al., 2009) and rat aortic smooth muscle cells (Pacaud et al., 1995, Govindan et al., 2010). Comparing the P2 receptor transcript expression profiles from strips of mT-ASM and cmT-ASM cells demonstrated that despite small differences in the relative expression of some transcripts, such as a decrease in the expression of P2X1, P2Y1 and P2Y4 receptor transcripts in cmT-ASM cells compared to mT-ASM strips, the P2 receptor transcript expression profiles are extremely similar. This similarity adds confidence to the fact that the cmT-ASM cells are indeed ASM cells and not contaminating cells such as myofibroblasts due to the effect of cell culture. Western blotting data demonstrated that P2X1, P2X4 and P2X7 receptor proteins are still expressed in cmT-ASM cells.

Regarding P2 receptor expression in cHASM cells, the qPCR data suggested that there were no significant differences between the transcript expression levels between healthy and asthmatic individuals at this sample size (n=4 for each). To our knowledge, no study has previously compared P2 receptor expression in ASM from healthy and asthmatic individuals. These data could imply that any contribution of P2 receptors to

asthma pathogenesis is not due to differences in receptor expression, although changes in P2 receptor expression have been previously associated with disease pathogenesis. For example, P2X1, P2X4, P2X5 and P2X7 receptor transcripts are upregulated in paediatric acute myeloid leukaemia patients compared to healthy controls (Chong et al., 2010), and P2Y1, P2Y2 and P2Y4 receptor proteins are upregulated in the brains of patients with intractable epilepsy compared to healthy controls (Sukigara et al., 2014). There were, however, some small differences in transcript expression between the phenotypes. The largest difference suggested a reduced relative expression of P2Y1 receptor transcripts in cHASM cells from asthmatic donors compared to those from healthy individuals when data were analysed using an unpaired *t*-test (P=0.16). There were also similar, yet weaker, trends for P2Y2 and P2Y6 receptor transcript expression. Increasing these sample sizes could clarify the data further, and would potentially decrease inter-sample variation.

Comparisons of the P2 receptor transcript expression profiles in cHASM cells from healthy donors and those in fresh mT-ASM strips and cmT-ASM cells suggest that the profiles are very similar, again supporting the idea that the samples consist of the same cell type. To our knowledge, no single published study has compared the expression of P2 receptors in the same tissue from mouse and human samples. Here, the main difference regarding P2 receptor expression between the two tested species is that P2Y4 receptor transcripts appear well expressed in human samples compared to mouse samples. This difference, and potential differences in the function and physiological roles of P2 receptors between the species, should be considered when comparing data from mouse models for P2 receptor study to human models.

In short, this chapter determined that most P2 receptor transcripts are expressed in the tested samples. To our knowledge, no single published article has investigate the expression of all P2 receptor transcripts in ASM before. Furthermore, no study has compared the expression of P2 receptor transcript in ASM from healthy and asthmatic donors. The P2 receptor transcript expression profiles were very similar between all of the samples we tested, including those from cHASM cells derived from both healthy and asthmatic donors. This suggests that any contribution of P2 receptors to asthma pathogenesis is unlikely to be the result of differential expression between the phenotypes. Protein expression studies here focussed on P2X1, P2X4 and P2X7

receptors. This is primarily due to the restricted expression of P2X2 and P2X3 receptors to sensory neurones (Chen et al., 1995, Lewis et al., 1995, Collo et al., 1996, Ralevic and Burnstock, 1998) and the lack of functional P2X5 and P2X6 receptors in native tissues (Collo et al., 1996, Lê et al., 1997, Bo et al., 2003, Jones et al., 2004, Duckwitz et al., 2006, Ormond et al., 2006). Investigations into the expression of P2Y receptor proteins were not performed in this study. Antibodies directed against GPCRs are notoriously non-selective (Michel et al., 2009) and, despite their continued improvement and use in published studies (Brass et al., 2012), the selectivity of P2Y receptor antibodies, such as those for P2Y6 receptor (Yu et al., 2013), are still in question.

There are arguably huge limitations to the studies which make up the current literature regarding P2 receptor expression in ASM, particularly in that they only investigate the expression of either P2X or P2Y receptors, and often only select receptors. On the other hand, the present study investigated the transcript expression of all P2 receptors and then investigated the protein expression of select receptors. However, there are also limitations to this study. For example, data regarding P2 receptor expression in humans only investigated a small sample size, and are derived solely from a cultured cell system. Although the data correlates well with the data from mice, future work could investigate the expression in fresh tissue, perhaps using material isolated from HASM cells by laser capture microdissection (Kelly et al., 2005, Cook et al., 2017). Other future work could include the clarification of P2Y4 receptor protein expression in mouse lung samples despite the low transcript expression, although this would depend still on antibody selectively. Furthermore, densitometry could be applied to enable the semi-quantification of protein expression across samples, particularly across cHASM cells from healthy and asthmatic donors to confirm that there is no difference in P2X1, P2X4 or P2X7 receptor protein expression between the two phenotypes.

The data presented in this chapter indicate that P2 receptors are expressed in ASM, suggesting that functional P2 receptors could be expressed in ASM. This possibility will be explored in the next chapter.

4 Determination of functional P2 receptor subtypes in cultured ASM cells

4.1 Introduction

The functional expression of P2 receptors in ASM has previously been investigated by a number of research groups. These studies have typically focussed on select P2 receptor subtypes, attempting to study these receptors in isolation. However, it is difficult to interpret these data because one nucleotide (e.g. ATP) can activate multiple P2 receptors (North, 2002, von Kügelgen, 2006). Additionally, the different approaches used between studies make it difficult to build a complete representation of functional P2 receptors in ASM.

In the previous chapter, the expression of P2 receptor subtypes was determined in fresh airway tissues from mice in addition to cmT-ASM cells from WT mice and cHASM cells from both healthy and asthmatic human donors. Due to the pivotal role of Ca^{2+} in classical smooth muscle functions such as cell contraction (see section 1.2.4), this chapter will focus on identifying the functional P2 receptor subtypes in ASM which regulate $[Ca^{2+}]_i$. These subtypes could be either P2X receptors or the Gq-coupled P2Y receptor subtypes.

The use of calcium imaging techniques (Flexstation and widefield epifluorescence microscopy [WEM]) as opposed to other functional approaches such as electrophysiology (patch clamping), allows the study of calcium responses with potential functional contributions by both P2X and P2Y receptor subtypes. By using pharmacological tools (e.g. selective agonists and antagonists) the same approach can be used to elucidate the receptor subtype(s) contributing to nucleotide-induced intracellular Ca^{2+} responses. Calcium imaging by WEM was used for most experiments with cmT-ASM cells whilst Flexstation was used for all calcium imaging experiments with cHASM cells. This is because WEM requires fewer cells than Flexstation experiments, allowing cmT-ASM cells to be maintained at low passage numbers to maintain a near-native phenotype. For reasons inherent to the laboratory, cHASM cells are passaged several times to give a large number of cells and so cell number is rarely limiting, allowing the use of the Flexstation which allows multiple conditions to be run simultaneously. To allow comparison of responses with cHASM cells, a small number of experiments with cmT-ASM cells were performed using Flexstation.

In this way, this chapter will explore the functional expression of P2 receptors in cultured ASM cells from WT mice and from both healthy and asthmatic volunteers. This should indicate which, if any, P2 receptor subtypes function abnormally in the ASM of asthmatic donors and so contribute to the asthmatic phenotype.

4.2 Results

4.2.1 $[Ca^{2+}]_i$ rises in cmT-ASM cells are induced by P2 receptor activation

4.2.1.1 Nucleotides induce $[Ca^{2+}]_i$ rises in cmT-ASM cells

Using the Flexstation™ microplate reader, the peak $[Ca^{2+}]_i$ increases induced by ATP, UTP, ADP and UDP (100 μM each) were measured in cmT-ASM cells (passage 1) [251±38 nM, 238±10 nM, 177±25 nM and 178±7 nM for ATP, UTP, ADP and UDP (100 μM each) respectively] (n=4 for all). The peak $[Ca^{2+}]_i$ rises induced by injections of saline buffer alone or bradykinin (5 μM) were 25±4 nM and 95±14 nM respectively (n=4 for both, figure 4.1).

All following calcium-imaging experiments with cmT-ASM cells used WEM. Using this approach, applications of ATP, UTP, ADP and UDP (100 μM for each) to cmT-ASM cells (passage 1) induced transient rises in $[Ca^{2+}]_i$ with distinct response profiles, whose peak amplitudes in $[Ca^{2+}]_i$ were 304±44% (n=29), 210±30% (n=20), 55±13% (n=13) and 70±13% (n=11) respectively compared to the peak amplitude of bradykinin 5 μM (figure 4.1).

The application of ATP (1 mM) induced a biphasic rise in $[Ca^{2+}]_i$, consisting of a fast increase followed by a sustained response. The peak response was 584±100% of the control response whilst the amplitudes of the responses measured at 5 and 10 min after ATP application were 194±34% and 149±30% of the bradykinin 5 μM control response, respectively (n=10). The $[Ca^{2+}]_i$ returned to baseline upon washout of the ATP. The peak response was significantly different than the 5 minute and 10 minute responses (P=0.042 and P<0.0001, respectively) (figure 4.2).

4.2.1.2 Regulation of $[Ca^{2+}]_i$ by P2X receptors in cmT-ASM cells

P2X2 and P2X3 receptors are typically only expressed in cells of nervous tissues (Chen et al., 1995, Lewis et al., 1995, Collo et al., 1996, Ralevic and Burnstock, 1998) and the qPCR data in section 3.2.1 suggests that the relative expression of these transcripts in cultured ASM cells from both mouse and human donors is low compared to the other

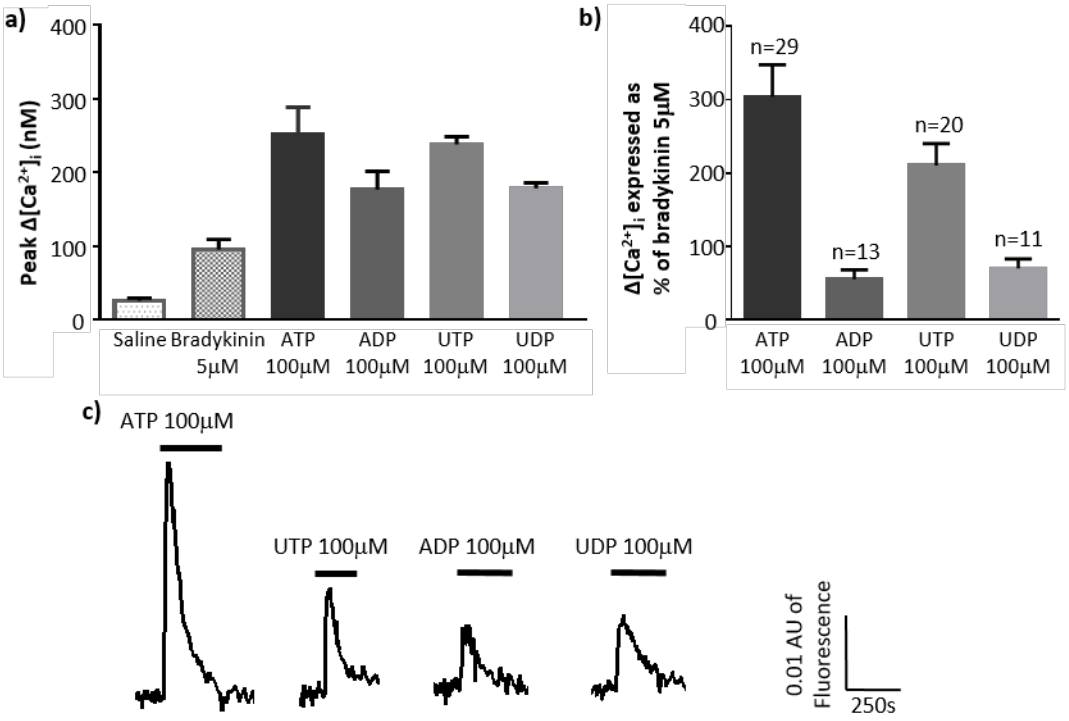

Figure 4.1. cmT-ASM cells express functional P2 receptors.
Nucleotides induced $[Ca^{2+}]_i$ rises in cmT-ASM cells (passage 1). a) A summary of the peak $\Delta[Ca^{2+}]_i$ (nM) induced by different stimuli, measured using the Flexstation™ (n=4). b) A summary of the $\Delta[Ca^{2+}]_i$ induced by different nucleotides, expressed as a % of the control response, bradykinin 5 μM, using WEM. (n) numbers are listed above each column. c) Representative response profiles induced by different nucleotides using WEM. AU of fluorescence indicates arbitrary units of fluorescence. Horizontal lines represent nucleotide application durations. Bars represent mean data ± SEM.

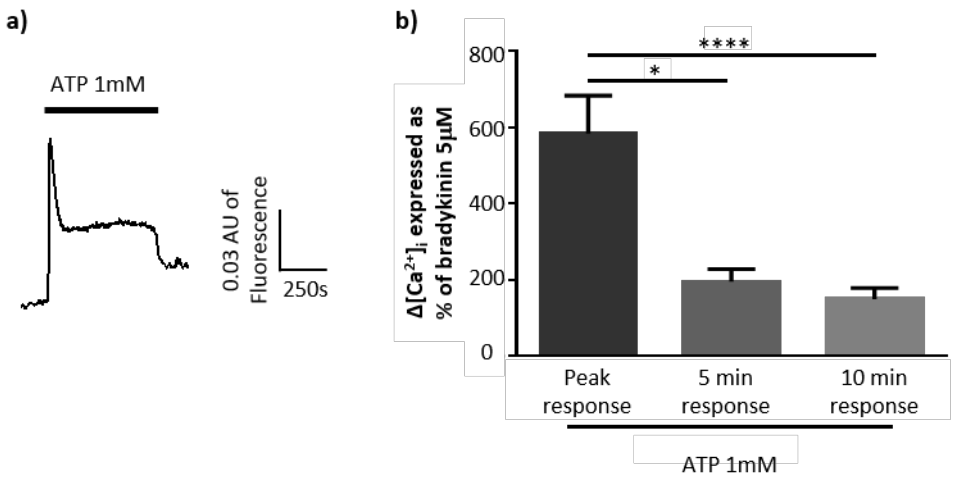

Figure 4.2. ATP (1 mM) induced a biphasic $[Ca^{2+}]_i$ rise in cmT-ASM cells.
*a) A representative response profile induced by ATP 1 mM in cmT-ASM cells (passage 1), performed using WEM. b) Summary of the increase in $[Ca^{2+}]_i$ induced by 1 mM ATP at the peak amplitude and at 5 and 10 min after application, measured using WEM (n=10). Horizontal lines represent nucleotide application. Bars represent mean data ± SEM. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test (*P<0.05, ****P<0.0001).*

P2 receptors. Additionally, there is a lack of both selective drugs and data regarding function in native tissues in current literature for P2X5 and P2X6 receptor subtypes (Collo et al., 1996, Lê et al., 1997, Bo et al., 2003, Jones et al., 2004, Duckwitz et al., 2006, Ormond et al., 2006). Therefore, preliminary experiments did not specifically search for the functional presence of these receptor subtypes.

4.2.1.2.1 ATP-induced $[Ca^{2+}]_i$ responses in cmT-ASM cells are partially dependent on the presence of extracellular Ca^{2+}

cmT-ASM cells were superfused with 0Ca-ES for 2 min before the application of 100 μM ATP in the same buffer. The absence of Ca^{2+}_e significantly decreased the ATP (100 μM) response from 397±63% to 304±36% of the control response (P=0.027, n=9) (figure 4.3). The amplitude of the peak response induced by 1 mM ATP was not significantly affected by the removal of Ca^{2+}_e [671±136% and 482±83% of the control response in the presence and absence of Ca^{2+}_e respectively (P=0.163, n=6)] whilst the sustained response was abolished at 5 min (200±39% and 7±4% of control response in the presence and absence of Ca^{2+}_e respectively, P=0.005, n=6) and 10 min (159±30% and 3±3% of the control response in the presence and absence of Ca^{2+}_e respectively, P=0.004, n=6) (figure 4.3).

4.2.1.2.2 P2X1, P2X3 and P2X4 receptors do not induce a change in $[Ca^{2+}]_i$

The application of the P2X1 and P2X3 receptor agonist α,β–meATP (10 μM) did not increase $[Ca^{2+}]_i$ in cmT-ASM cells (4±2% of the control response, n=5) (figure 4.4).

The use of the P2X4-selective antagonist, 5-BDBD (20 μM), had no significant effect on the ATP (100 μM)-induced response (424±182% and 406±175% of the control response in the presence and absence of 5-BDBD respectively, P=0.748, n=5). Neither did PSB 12054 (10 μM) (Glixx Laboratories, Southborough, MA, USA), another P2X4-selective antagonist (458±102% and 457±127% of the control response without and with PSB 12054 respectively, P=0.983, n=5) (figure 4.4). The application of DMSO as a vehicle control for both 5-BDBD and PSB 12054 (1:1,000) did not induce any apparent changes in baseline fluorescence in any condition (figure 4.5).

These data suggest that P2X1, P2X3 and P2X4 receptors are not functional in cmT-ASM cells.

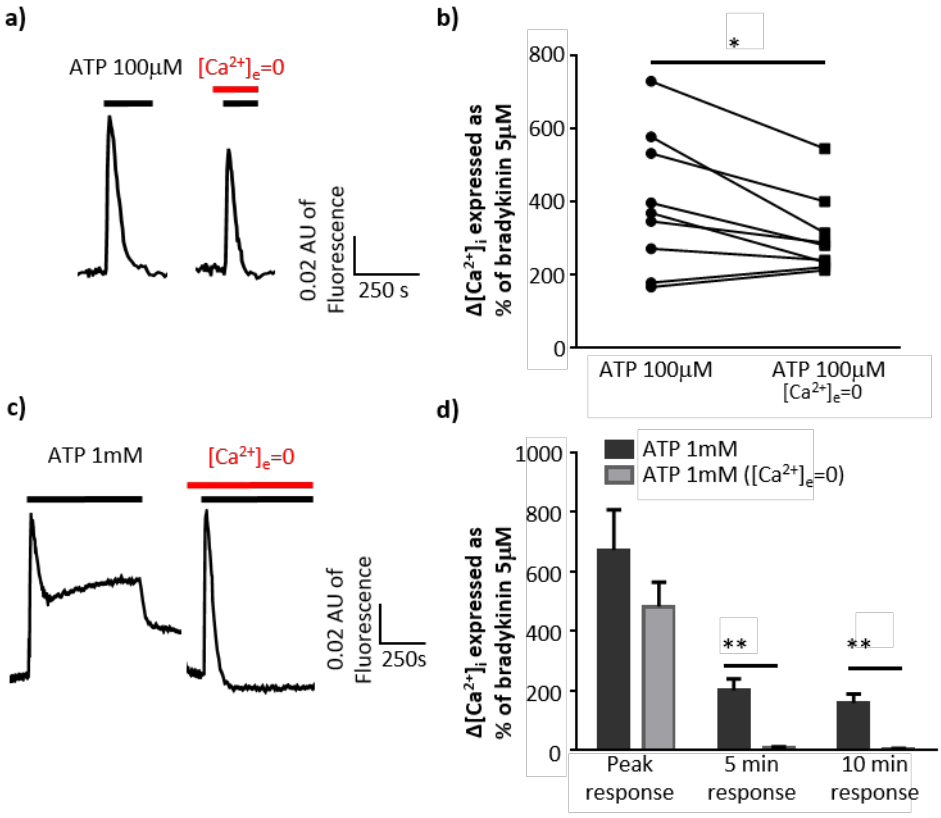

Figure 4.3. $[Ca^{2+}]_i$ rises induced by ATP in cmT-ASM cells are partially dependent on extracellular calcium.

*a) Representative profiles of the $[Ca^{2+}]_i$ responses induced in cmT-ASM cells (passage 1) when 100 µM ATP was applied in the presence and absence of extracellular calcium ($[Ca^{2+}]_e$=0), performed using WEM. These data are summarised in b) (n=9). c) Representative profiles of the $[Ca^{2+}]_i$ responses in cmT-ASM cells (passage 1) when 1 mM ATP was applied in the presence and absence of extracellular calcium ($[Ca^{2+}]_e$=0), performed using WEM. These data are summarised in d) (n=6). Horizontal lines represent the duration of the drug applications. Bars represent mean data ± SEM. Statistical significance was determined by Student's paired t-test (*P<0.05, **P<0.01).*

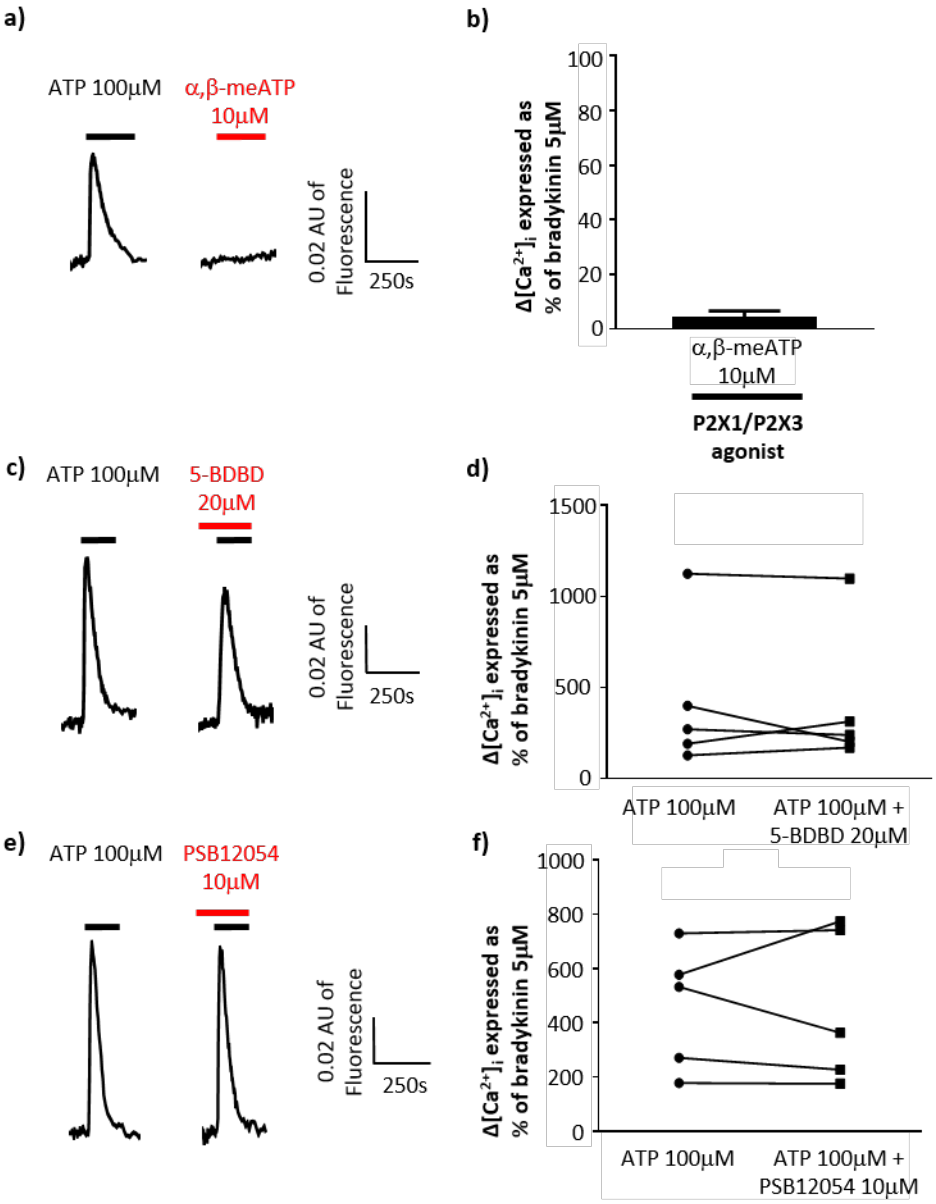

Figure 4.4. P2X1, P2X3 and P2X4 receptors do not directly contribute to $[Ca^{2+}]_i$ rises in cmT-ASM cells.

a) Representative profiles of $[Ca^{2+}]_i$ response induced by 100 µM ATP and the P2X1/P2X3 agonist α,β-meATP (10 µM) in cmT-ASM cells (passage 1), performed using WEM. The response induced by α,β-meATP (10 µM) is summarised in b) (n=5). Representative profiles of $[Ca^{2+}]_i$ responses induced by ATP (100 µM) in cmT-ASM cells (passage 1) in the absence or presence of c) 5-BDBD (20 µM) or e) PSB12054 (10 µM), performed using WEM. These data are summarised in d) (n=5) and f) (n=5), respectively. Horizontal lines represent the duration of the drug applications. Bars represent mean data ± SEM. Statistical significance was determined by Student's paired t-test.

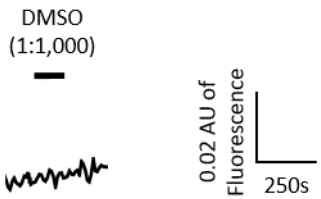

Figure 4.5. DMSO application does not modify $[Ca^{2+}]_i$ in cmT-ASM cells.
Representative profile of $[Ca^{2+}]_i$ response induced by DMSO (1:1,000), used as a vehicle control for 5-BDBD, PSB12054 and AR-C 118925XX. Horizontal lines represent the duration of the drug applications.

4.2.1.2.3 P2X7 receptor contributes to the ATP (1mM) response

Pre-incubation of cmT-ASM cells with the P2X7-selective antagonist AZ10606120 (1 μM) (Bio-Techne Ltd., Abingdon, UK) for 5 min had no significant effect on the amplitude of the ATP (1 mM) peak responses (786±176% and 707±133% of the control response without and with treatment respectively, P=0.478, n=6) (figure 4.6). However, the amplitude of the response at 5 min was significantly decreased in the presence of AZ10606120 (1 μM) treatment (247±35% and 15±10% of the control response without and with treatment respectively, P=0.003, n=6). The same observation was made for the amplitude of the response at 10 min (144±37% to 14±10% of the control response without and with AZ10606120 (1 μM) treatment respectively, P=0.026, n=6). These results, combined with the data obtained in the absence of Ca^{2+}_e (section 4.2.1.2.1), suggest that P2X7 receptor activation contributes to the sustained rise in $[Ca^{2+}]_i$, but not to the fast and transient peak response.

4.2.1.3 Regulation of $[Ca^{2+}]_i$ by P2Y receptors in cmT-ASM cells

4.2.1.3.1 P2Y1 receptor activation increases $[Ca^{2+}]_i$

ADP (100 μM), a known agonist of P2Y1 receptor (Waldo and Harden, 2004), induced an $[Ca^{2+}]_i$ rise in cmT-ASM cells which was significantly reduced by a 5 minute pre-treatment with the P2Y1-selective antagonist MRS2179 (10 μM) (Bio-Techne Ltd.) (47±7% and 30±7% of the control response for untreated and treated cells respectively, P=0.043, n=8) (figure 4.7), suggesting the presence of functional P2Y1 receptors in cmT-ASM cells.

4.2.1.3.2 P2Y2 receptor activation increases $[Ca^{2+}]_i$

Pre-incubation of cmT-ASM cells for 5 min with the P2Y2-selective antagonist AR-C 118925XX (10 μM) (Bio-Techne Ltd.) significantly decreased the amplitude of the $[Ca^{2+}]_i$ rises induced by ATP (100 μM) (141±22% and 36±8% of the control response without and with antagonist treatment respectively, P=0.004, n=6) and UTP (100 μM) (197±42% and 31±10% of the control response without and with treatment respectively, P=0.004, n=6). The same observation was also made for the amplitude of the $[Ca^{2+}]_i$ rises induced by ADP (100 μM) (46±9% and 16±6% of the control response without and with treatment respectively, P=0.003, n=5) and UDP (100 μM) (80±16% and 32±14% of the control response without and with treatment respectively,

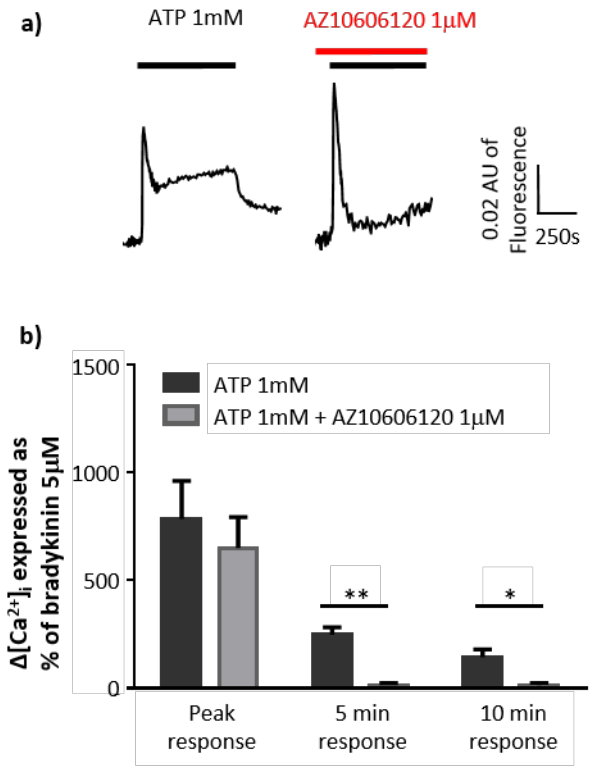

Figure 4.6. P2X7 receptor activation contributes to the ATP (1 mM)-induced rise in $[Ca^{2+}]_i$ in cmT-ASM cells.

*a) Representative response profiles induced by ATP 1 mM in cmT-ASM cells (passage 1) in the absence and presence of the P2X7-selective antagonist AZ10606120 at 1 µM, performed using WEM. b) Summary of the effect of AZ10606120 1 µM on the ATP 1 mM-induced changes in $[Ca^{2+}]_i$ (n=6). Horizontal lines represent the duration of the drug applications. Bars represent mean data ± SEM. Statistical significance was determined by Student's paired t-test (*P<0.05, **P<0.01).*

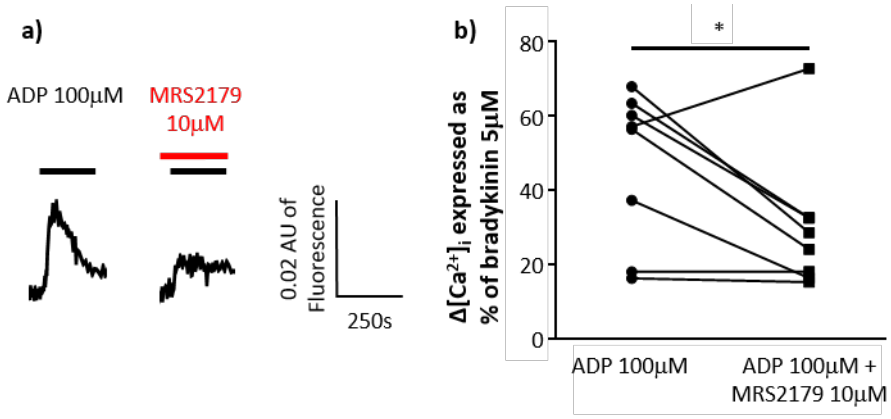

Figure 4.7. P2Y1 receptor activation contributes to the ADP (100 µM)-induced rise in $[Ca^{2+}]_i$ in cmT-ASM cells.

*Representative response profiles induced by ADP (100 µM) in cmT-ASM cells (passage 1) in the absence and presence of the P2Y1-selective antagonist MRS2179 (10 µM), performed using WEM. b) Summary of the effect of MRS2179 (10 µM) on the ADP (100 µM)-induced changes in $[Ca^{2+}]_i$ (n=8). Horizontal lines represent the duration of the drug applications. Bars represent mean data ± SEM. Statistical significance was determined by Student's paired t-test (*P<0.05).*

P=0.009, n=5) (figure 4.8). The application of DMSO as a vehicle control for AR-C 118925XX (1:1,000) did not induce any apparent changes in baseline fluorescence in any condition (figure 4.5). These data suggest that P2Y2 receptor activation induces a rise in $[Ca^{2+}]_i$.

Concentration-response curves for UTP (n=5) and ATP (n=7) were established. Data were analysed using a non-linear single exponential fit with a variable slope. For UTP (figure 4.9, panel a), this gave a value for R-square of 0.81, a Hill slope of 0.92 and EC_{50} of 10.3 μM. For ATP, the single exponential fit gave a value for R-square of 0.57, a Hill slope of 0.18 and EC_{50} of 116 mM (figure 4.9, panel b). Although this could indicate that there are several binding sites, this fit was not suited for ATP concentration-response curve. Therefore, a non-linear regression using 2 sites – fit was used (figure 4.9, panel c). This gave a value for R-square of 0.58 and 2 EC_{50} values of 3 μM and 421 μM, potentially indicating that there is more than one P2 receptor subtype responding to ATP present in cmT-ASM cells. Analysing the ATP concentration-response curve up to only 100 μM with a non-linear single exponential fit with a variable slope (figure 4.9, panel d) gave a value for R-square of 0.57, a Hill slope of 1.1 and an EC_{50} of 4 μM, which is very similar to the EC_{50} of 3 μM of the ATP concentration-response curve up to 1 mM using the non-linear regression fit.

Desensitization experiments were performed by pre-treating the cells with either ATP followed by application of UTP (100 μM), or with UTP followed by application of ATP (100 μM). When pre-treated with ATP 100 μM or 300 μM, the amplitude of the $[Ca^{2+}]_i$ rise induced by UTP (100 μM) was significantly decreased from 336±96% (n=6) to 57±15% (P=0.037, n=6) and 26±6% (P=0.037, n=6) of the control response, respectively. Similarly, when the cells were pre-treated with UTP 100 μM or 300 μM, the amplitude of the $[Ca^{2+}]_i$ rise induced by ATP (100 μM) was significantly decreased from 302±48% (n=4) to 39±8% (P=0.014, n=4) and 16±6% (P=0.019, n=4) of the control response, respectively (figure 4.10). These data could suggest that the same receptor subtype is responding to both ATP and UTP at 100 μM, although there is a possibility that the initial nucleotide application is depleting intracellular Ca^{2+} stores and this could explain the decrease in the subsequent response. Further experimentation is required to clarify this point and is discussed further in section 4.3.

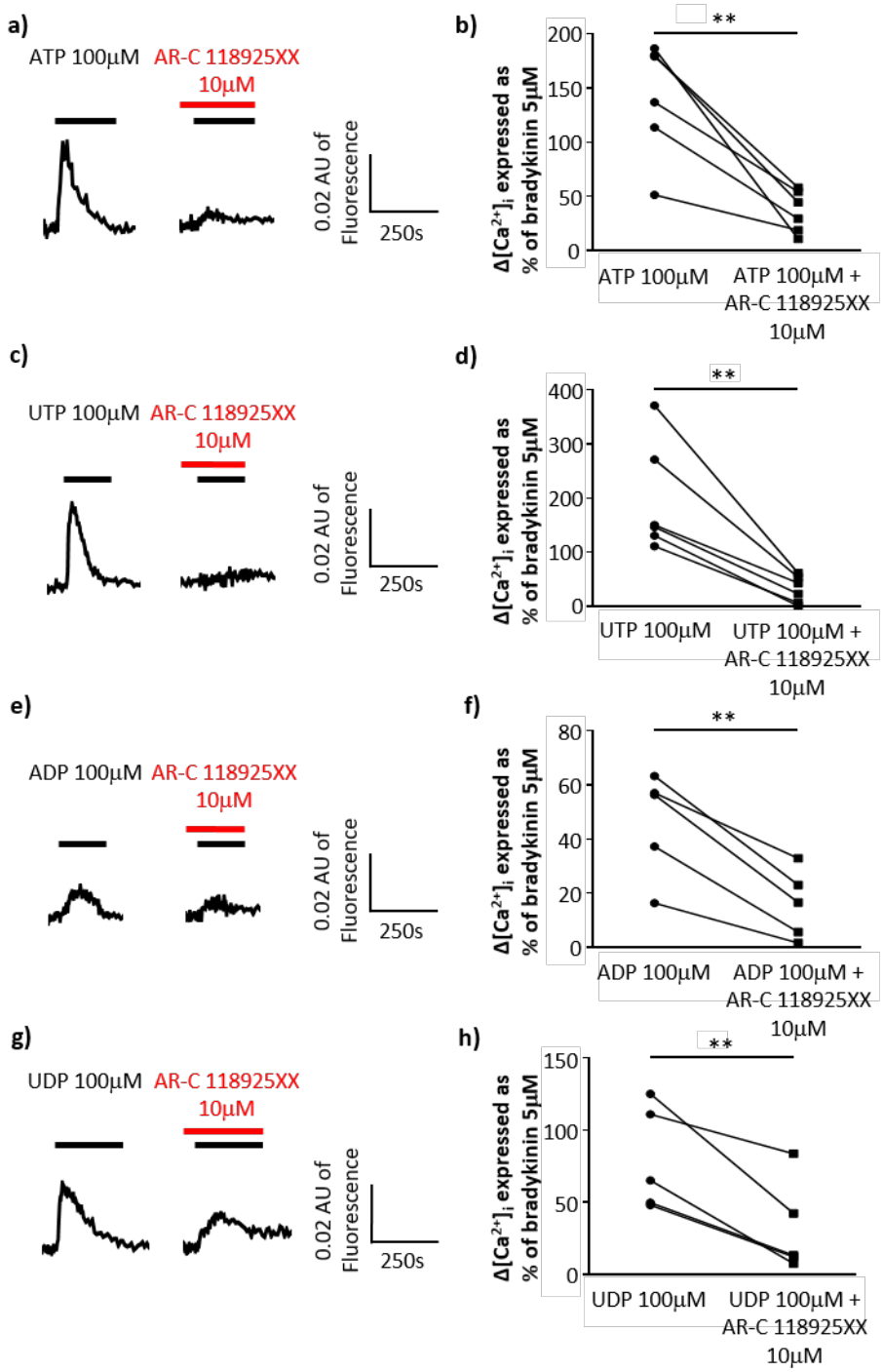

Figure 4.8. P2Y2 receptor activation regulates $[Ca^{2+}]_i$ in cmT-ASM cells.

*Representative response profiles induced by a) ATP, c) UTP, e) ADP and g) UDP (all at 100 µM) in the absence or presence of the P2Y2-selective antagonist AR-C 118925XX (10 µM) in cmT-ASM cells (passage 1), performed using WEM. These data are summarised in b) ATP (n=6), d) UTP (n=6), f) ADP (n=5) and h) UDP (n=5, 100 µM for all). Horizontal lines represent the duration of the drug applications. Statistical significance was determined by Student's paired t-test (**P<0.01).*

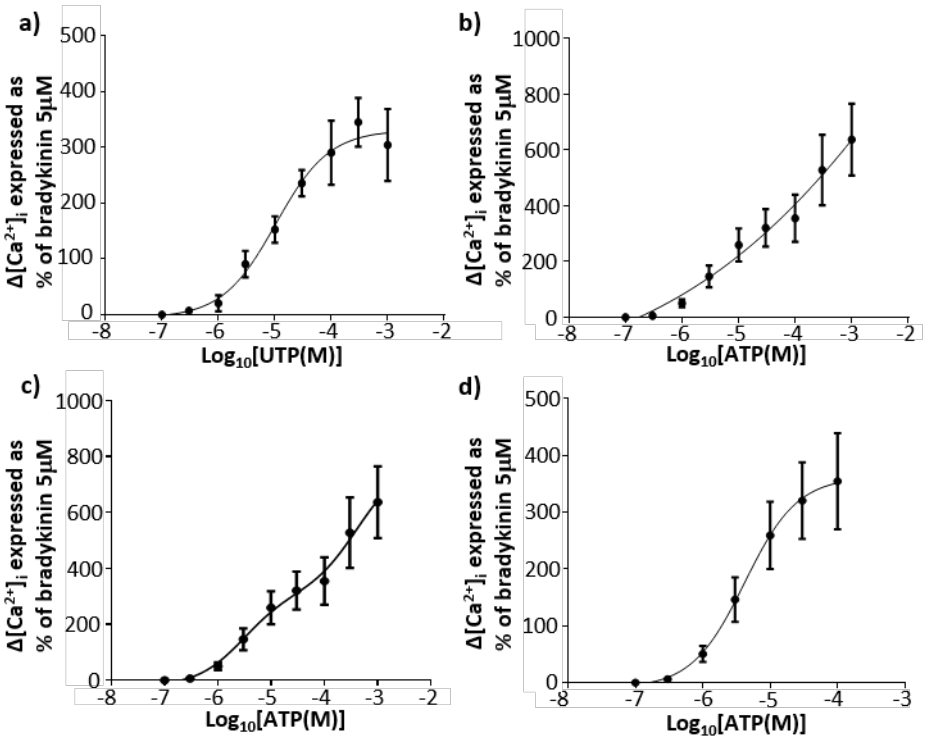

Figure 4.9. UTP and ATP regulate $[Ca^{2+}]_i$ in cmT-ASM cells in a concentration-dependent manner.

Concentration response curves were performed for a) UTP (n=5) and b-d) ATP (n=7) in cmT-ASM cells (passage 1) using WEM. The data were analysed using a non-linear single exponential fit with a variable slope (panels a, b and d) and with a non-linear regression using 2 sites – fit (panel c). *Bars represent mean data ± SEM.*

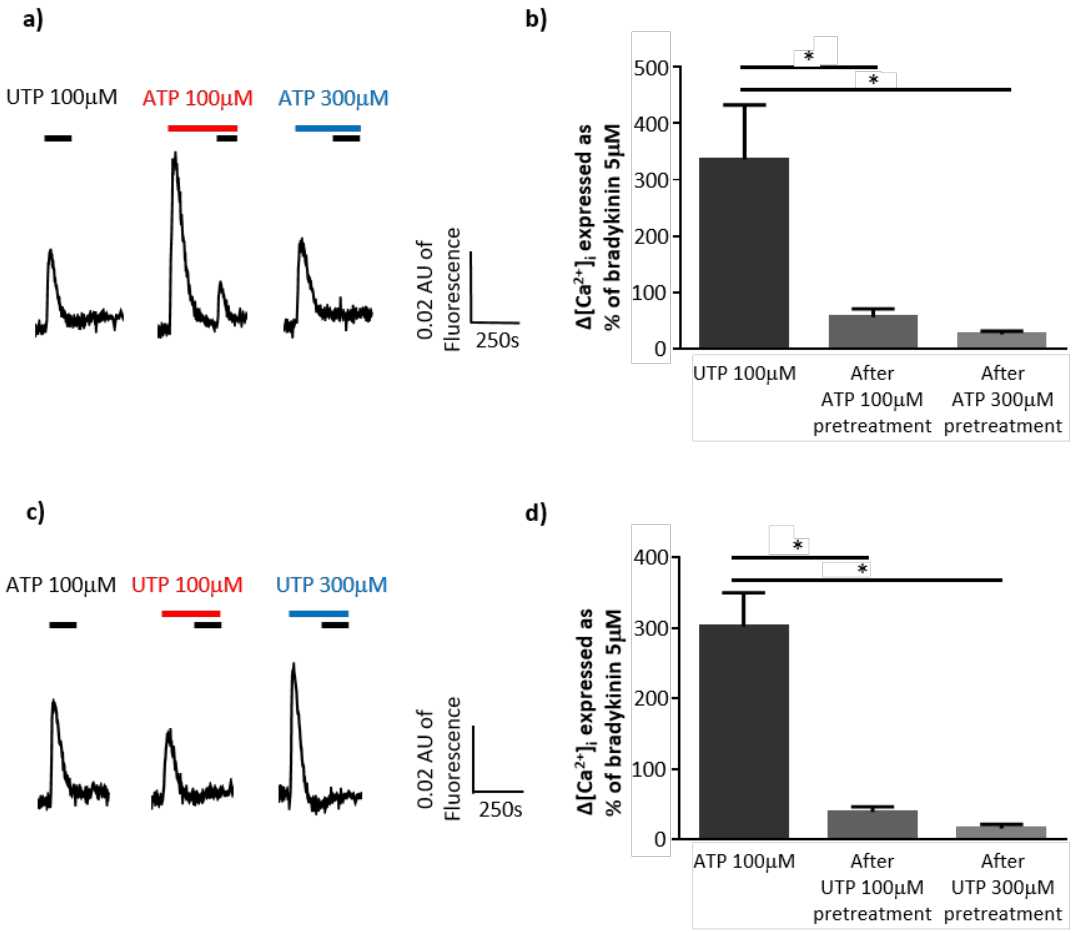

Figure 4.10. Pre-treatment with UTP or ATP significantly reduces subsequent ATP- or UTP-induced rises in $[Ca^{2+}]_i$ in cmT-ASM cells respectively.

*Representative response profiles in cmT-ASM cells (passage 1) of a) UTP (100 µM) application without and with pre-treatment with ATP (100 µM or 300 µM) and c) ATP (100 µM) application without and with pre-treatment with UTP (100 µM or 300 µM), performed using WEM. These data are summarised in panels b) (n=6 for all) and d) (n=4 for all), respectively. Horizontal lines represent the duration of the drug applications. Bars represent mean data ± SEM. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test (*P<0.05).*

4.2.1.3.3 P2Y6 and P2Y14 receptors do not directly influence $[Ca^{2+}]_i$ changes

In cmT-ASM cells, the application of the P2Y6-selective agonist MRS 2693 (10 μM) (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) and the P2Y14-selective agonist UDP-glucose (100 μM) failed to induce a rise in $[Ca^{2+}]_i$ (1±1% of the control response, n=5 and 9±7% of the control response, n=3 respectively) (figure 4.11), indicating that both receptors are unlikely to be functional in cmT-ASM cells.

4.2.2 P2 receptor activation increases $[Ca^{2+}]_i$ in cHASM cells from healthy and asthmatic donors

Using cmT-ASM cells as a model to investigate P2 receptor functions allows the passage number to be kept low, maintaining near-native cells. Although these data are informative regarding basal P2 receptor function in WT mice, and could contribute to murine models of P2 receptors, the data is not directly relatable to humans. Therefore, cHASM cells from healthy and asthmatic donors were also used to allow the identification of functional differences between P2 receptors in ASM from healthy and asthmatic individuals, potentially suggesting dysfunctional P2 receptor subtypes which could contribute to, or be a result of, asthma pathogenesis. However these cells, as they are obtained via a shared facility, were used at higher passage numbers compared to cmT-ASM cells.

4.2.2.1 Nucleotides and nucleosides induce $[Ca^{2+}]_i$ rises in cHASM cells

Using the Flexstation™ microplate reader, the peak $[Ca^{2+}]_i$ increases induced in cHASM cells from healthy individuals (passages 2-5) by 1 mM ATP (146±36 nM, n=5) and ATP (126±26 nM, n=9), UTP (56±9 nM, n=9), ADP (71±15 nM, n=9), UDP (78±18 nM, n=9) and adenosine (37±12 nM, n=5) (100 μM for each) were measured. Using cHASM cells from asthmatic individuals, the peak $[Ca^{2+}]_i$ increases induced by the same compounds were also measured: ATP (1 mM) (180±49 nM, n=5), ATP (105±31 nM, n=10), UTP (42±9 nM, n=10), ADP (77±24 nM, n=10), UDP (32±6 nM, n=10) and adenosine (24±4 nM, n=5) (100 μM for each). The peak $[Ca^{2+}]_i$ rises induced by injections of saline buffer alone were 18±3 nM (n=9) and 16±2 nM (n=10) in cHASM cells from healthy and asthmatic individuals respectively. Representative profiles of these $[Ca^{2+}]_i$ rises are given in figure 4.12 and these data are summarised in figure 4.13.

A significant difference was observed between the peak $[Ca^{2+}]_i$ rises induced by

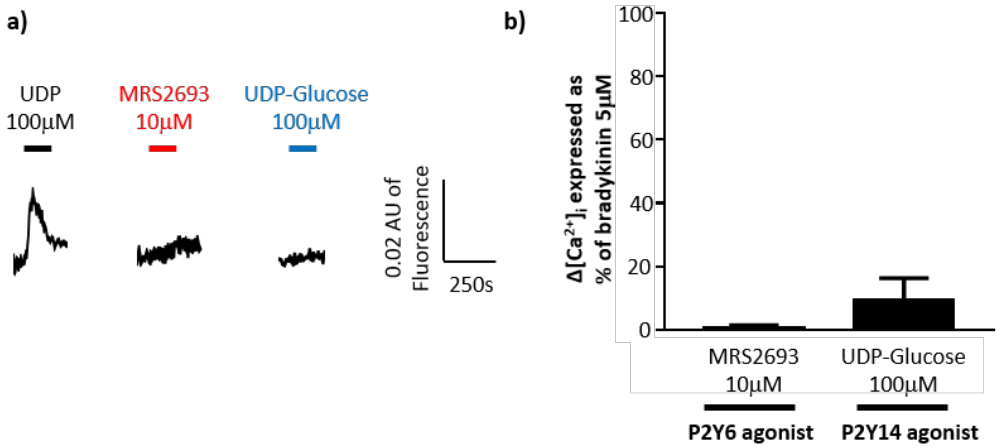

Figure 4.11. P2Y6 and P2Y14 receptor activation does not directly increase $[Ca^{2+}]_i$ in cmT-ASM cells.

a) Representative profiles of $[Ca^{2+}]_i$ responses induced by UDP (100 µM), the P2Y6 receptor-selective agonist MRS2693 (10 µM) and the P2Y14 receptor-selective agonist UDP-Glucose (100 µM) in cmT-ASM cells (passage 1), performed using WEM. These data are summarised in b) for both MRS2693 (n=5) and UDP-glucose (n=3). Horizontal lines represent the duration of the drug applications. Bars represent mean data ± SEM.

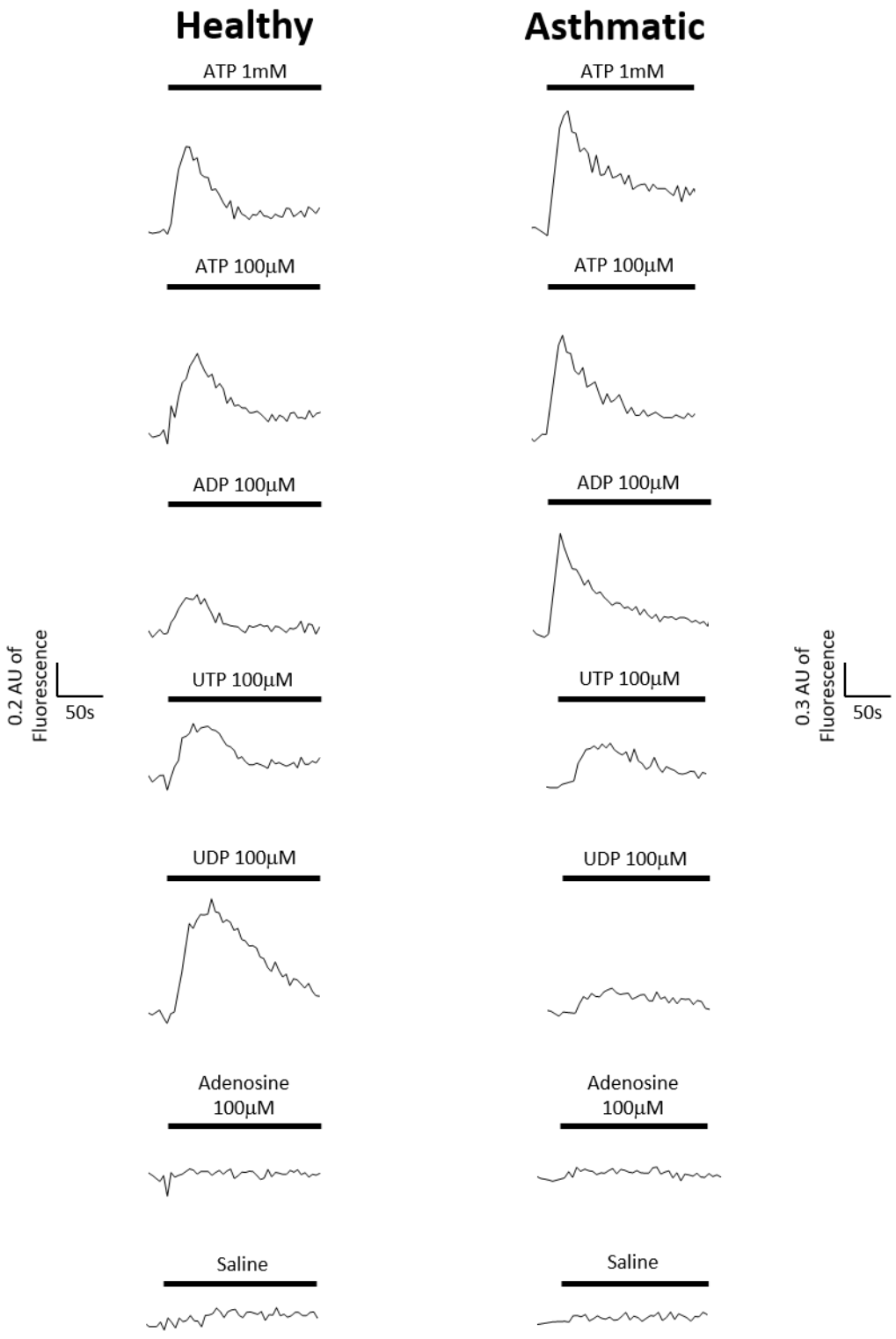

Figure 4.12. $[Ca^{2+}]_i$ response profiles induced by nucleotides/nucleosides in cHASM cells from healthy and asthmatic individuals.

Representative $[Ca^{2+}]_i$ response profiles in cHASM cells (up to passage 5) from healthy and asthmatic individuals following the application of compounds and saline, using the Flexstation™. Horizontal lines represent the duration of compound/saline application.

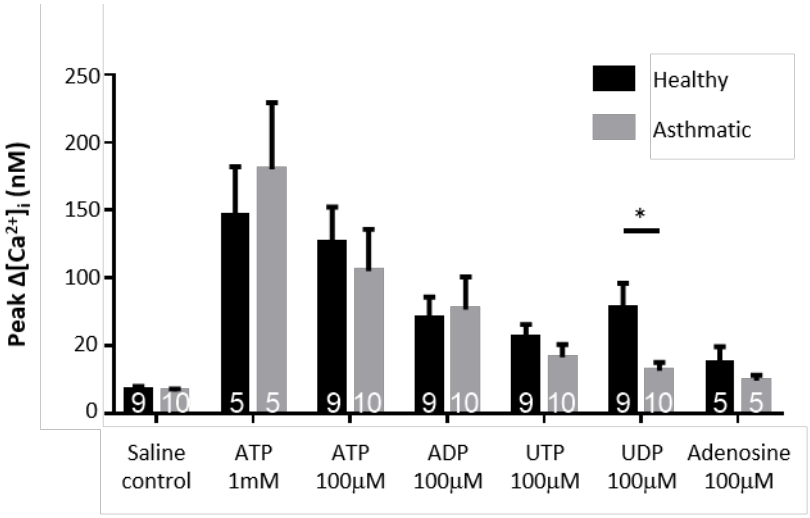

Figure 4.13. cHASM cells from healthy and asthmatic individuals express functional P2 receptors.

A summary of the peak $\Delta[Ca^{2+}]_i$ (nM) induced by different nucleotides, and the nucleoside adenosine, in cultured HASM cells from healthy and asthmatic individuals (up to passage 5). Measurements were performed using the Flexstation™. Bars represent mean data ± SEM and *(n) numbers for each condition are given on each column. Statistical significance was determined using* Student's unpaired *t*-tests *(*P<0.05)*.

UDP (100 μM) in cHASM cells from healthy donors compared to asthmatic individuals (P=0.023 using unpaired *t*-test) (figure 4.13).

4.2.2.2 Regulation of $[Ca^{2+}]_i$ by P2X receptors in cHASM cells

4.2.2.2.1 ATP (100 μM)-induced $[Ca^{2+}]_i$ responses in cHASM cells are partially dependent on the presence of extracellular Ca^{2+}

ATP (100 μM) was applied in FS or 0Ca-FS and induced a peak $[Ca^{2+}]_i$ rise of 220±14 nM and 120±17 nM in cHASM cells from healthy volunteers (P=0.005, n=5) and 130±46 nM and 71±29 nM in cHASM cells from asthmatic donors (P=0.028, n=7) (figure 4.14). The significant decreases observed in these responses in the absence of Ca^{2+}_e suggest that a portion of the ATP (100 μM)-induced $[Ca^{2+}]_i$ rises in these cells is dependent on Ca^{2+}_e.

4.2.2.2.2 P2X1 and/or P2X3 receptors activation does not induce significant $[Ca^{2+}]_i$ rises in cHASM cells from healthy or asthmatic donors

The application of α,β-meATP (10 μM), a selective agonist for P2X1 and P2X3 receptors, to cHASM cells from healthy and asthmatic individuals induced $[Ca^{2+}]_i$ rises of 68±38 nM (n=5) and 18±3 nM (n=5), respectively. The respective saline buffer controls induced $[Ca^{2+}]_i$ rises of 22±3 nM (n=5) and 21±2 nM (n=5). Compared to the controls, α,β-meATP (10 μM) did not induce significant $[Ca^{2+}]_i$ rises in cHASM cells from either healthy (P=0.261) or asthmatic (P=0.383) donors (figure 4.15). These data suggest that P2X1 and P2X3 receptors are not functional in cHASM cells from either healthy or asthmatic individuals.

4.2.2.2.3 P2X4 receptor plays a significant role in $[Ca^{2+}]_i$ regulation in cHASM cells from asthmatic donors but not healthy individuals

The peak $[Ca^{2+}]_i$ rise induced in cHASM cells from healthy volunteers by ATP (100 μM) was not significantly reduced when applied in the presence of the P2X4-selective antagonist PSB 12054 (10 μM) (156±32 nM and 116±28 nM without and with treatment, respectively) (P=0.105, n=8). However, PSB 12054 (10 μM) significantly reduced the ATP (100 μM)-induced $[Ca^{2+}]_i$ response in cHASM cells from asthmatic donors (130±46 nM and 114±49 nM without and with treatment, respectively) (P=0.030, n=7) (figure 4.16). The application of DMSO (1:1,000) as a vehicle control did not appear to affect $[Ca^{2+}]_i$ in cHASM cells from either healthy or asthmatic donors (figure 4.17). These data suggest

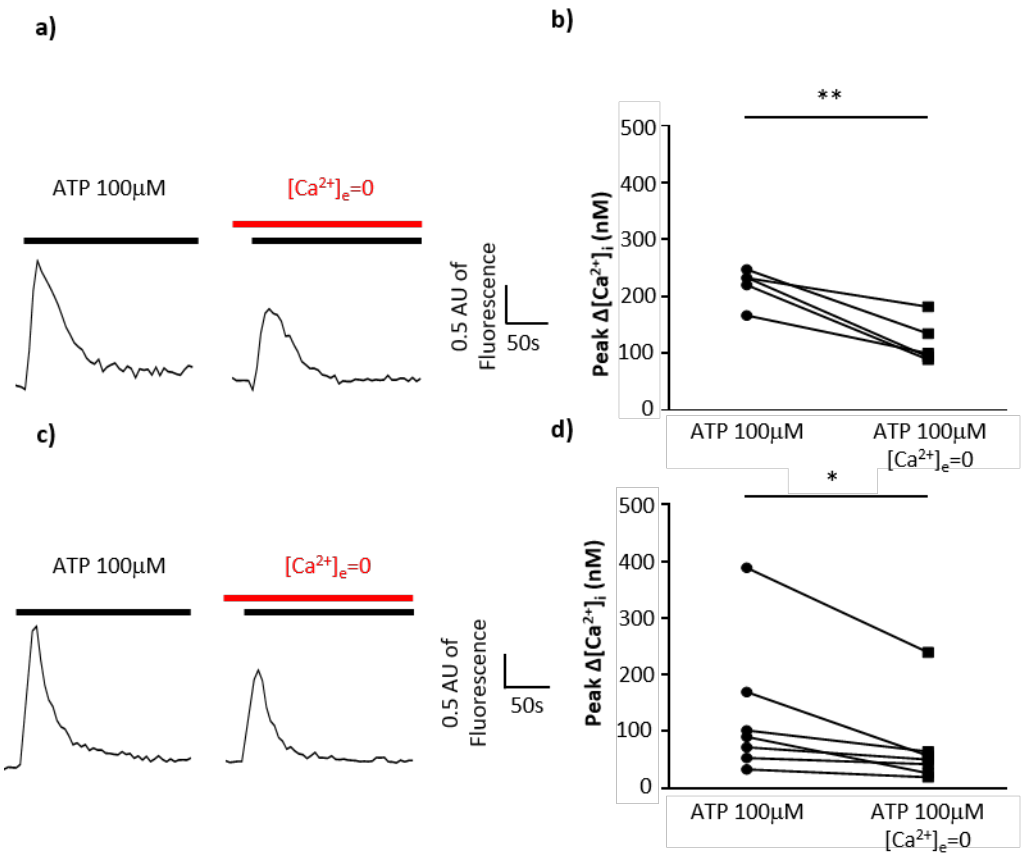

Figure 4.14. The absence of extracellular Ca^{2+} reduces the $[Ca^{2+}]_i$ rise induced by ATP (100 µM)

Representative $[Ca^{2+}]_i$ response profiles in cHASM cells (up to passage 5) from a) healthy and c) asthmatic individuals following the application of ATP (100 µM) in the presence or absence of extracellular Ca^{2+}. Horizontal lines represent the duration of the drug applications. Summaries of the peak $[Ca^{2+}]_i$ rises in b) healthy and d) asthmatic groups, measured using the Flexstation™ [n=5 for (b) and n=7 for (d)]. Statistical significance was determined using Student's paired *t*-tests *(*P<0.05, **P<0.01).*

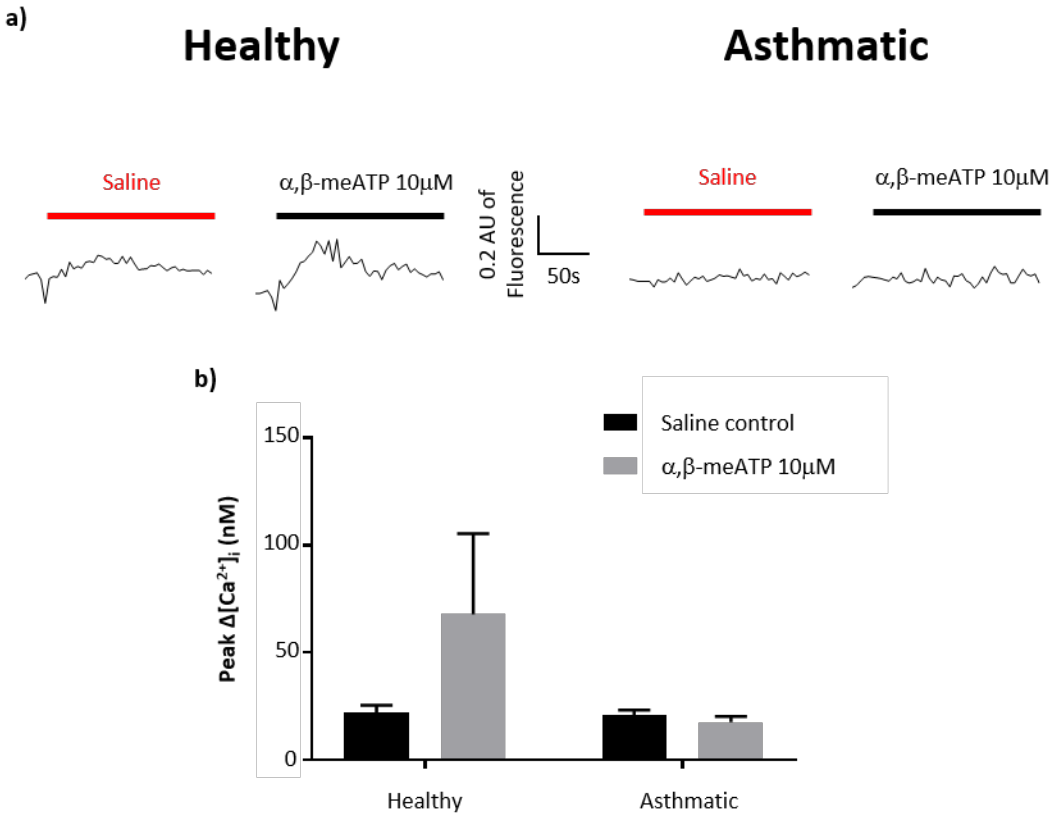

Figure 4.15. The activation of P2X1 or P2X3 receptors does not induce significant $[Ca^{2+}]_i$ rises in cHASM cells from healthy or asthmatic individuals.

a) Representative $[Ca^{2+}]_i$ response profiles in cHASM cells (up to passage 5) from healthy and asthmatic individuals as indicated of the applications of α,β-meATP (10 μM), a selective agonist for P2X1 and P2X3 receptors, with their respective saline controls. Horizontal lines represent the duration of the drug applications. b) *Summary of peak $[Ca^{2+}]_i$ rises in healthy and asthmatic groups, measured using the Flexstation™ (n=5 for all). Bars represent mean data* ± SEM. *Statistical significance was determined using* Student's paired *t*-tests.

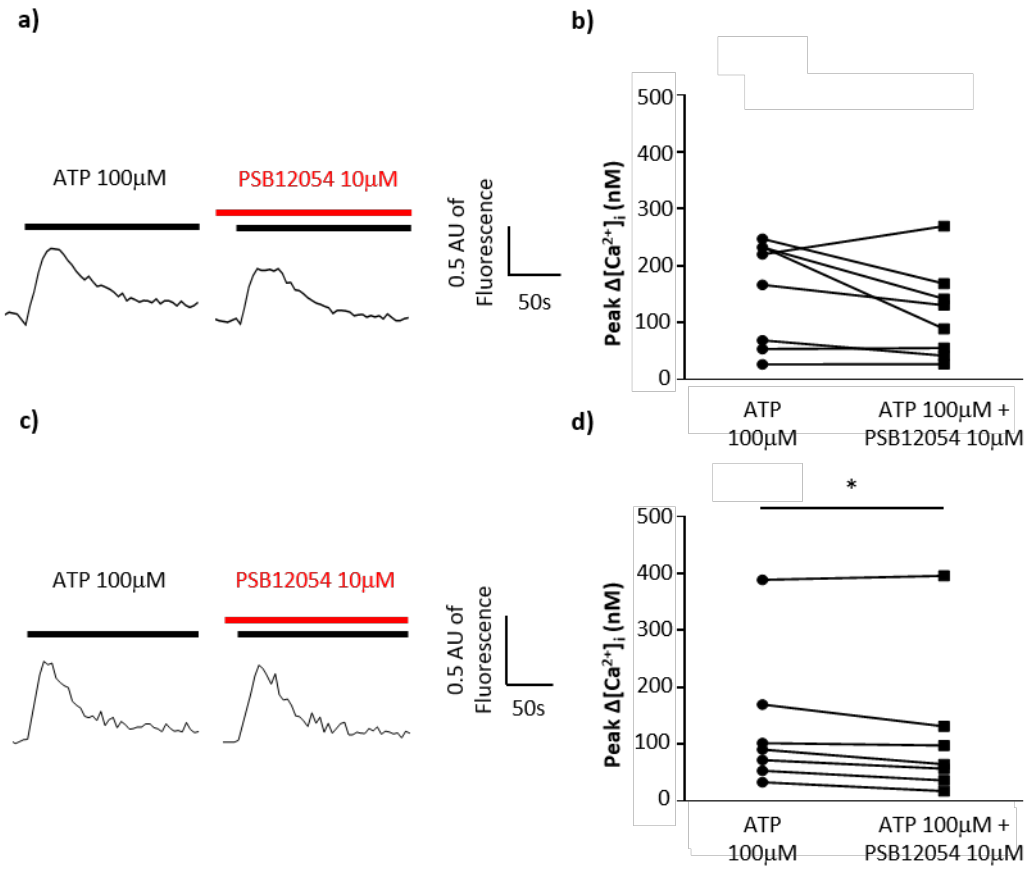

Figure 4.16. P2X4 receptor significantly regulates $[Ca^{2+}]_i$ in cHASM cells from asthmatic, but not healthy, donors.

Representative $[Ca^{2+}]_i$ response profiles in cHASM cells cultured (up to passage 5) from a) healthy and c) asthmatic individuals following the application of ATP (100 µM) in the absence and presence of the P2X4-selective antagonist PSB12054 (10 µM). Horizontal lines represent the duration of the drug applications. Summaries of the peak $[Ca^{2+}]_i$ rises b) in healthy and d) asthmatic groups, measured using the Flexstation™ [n=8 for (b) and n=7 for (d)]. Statistical significance was determined using Student's paired *t*-tests *(*P<0.05)*.

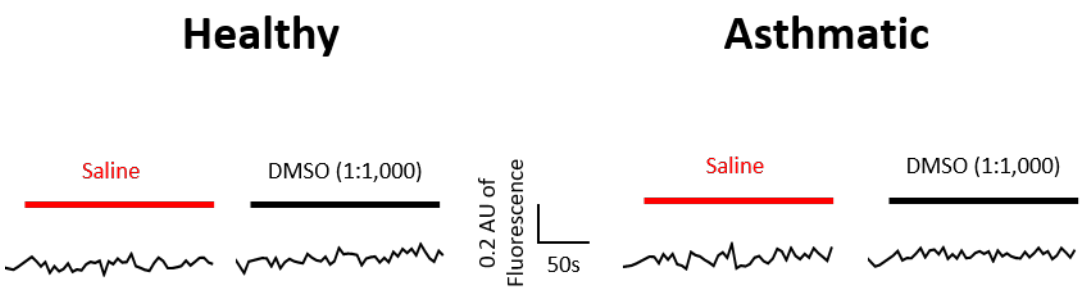

Figure 4.17. DMSO application does not modify $[Ca^{2+}]_i$ in cHASM cells.
Representative profile of $[Ca^{2+}]_i$ response induced by DMSO (1:1,000), used as a vehicle control for PSB12054 and AR-C 118925XX. Horizontal lines represent the duration of the drug applications.

that P2X4 receptor is functional in cHASM cells from asthmatic donors, but not those from healthy volunteers.

4.2.2.2.4 P2X7 receptor does not influence $[Ca^{2+}]_i$ in cHASM cells

No difference in the peak $[Ca^{2+}]_i$ rises induced by ATP (1mM) was observed in cHASM cells from healthy and asthmatic individuals following pre-incubation with the P2X7-selective antagonist AZ10606120 (1 μM) (146±36 nM and 143±44 nM for healthy donors without and with treatment, respectively, P=0.816, n=5) (180±49 nM and 197±64 nM for asthmatic donors without and with treatment, respectively, P=0.426, n=5) (figure 4.18), suggesting that P2X7 receptor is not involved in the regulation of $[Ca^{2+}]_i$ in cHASM cells.

4.2.2.3 Regulation of $[Ca^{2+}]_i$ by P2Y receptors in cHASM cells

4.2.2.3.1 P2Y1 receptor does not influence $[Ca^{2+}]_i$ in cHASM cells

The P2Y1-selective antagonist MRS2179 (10 μM) did not significantly reduce the amplitudes of the ADP (100 μM)-induced $[Ca^{2+}]_i$ rises in cHASM cells from healthy or asthmatic individuals (101±17 nM and 103±23 nM for healthy volunteers without and with treatment, respectively, P=0.842, n=5) (93±28 nM and 76±14 nM for asthmatic donors without and with treatment, respectively, P=0.319, n=8) (figure 4.19), implying that P2Y1 receptor is not functional in these cells.

4.2.2.3.2 P2Y2 receptor activation increases $[Ca^{2+}]_i$ in cHASM cells

The use of the P2Y2-selective antagonist AR-C 118925XX (10 μM) significantly reduced the amplitude of the $[Ca^{2+}]_i$ rises induced by the following nucleotides (all at 100 μM) in cHASM cells from healthy donors: ATP (109±23 nM and 64±20 nM, P=0.006, n=9), UTP (62±10 nM and 24±7 nM, P=0.001, n=7), ADP (59±14 nM and 43±12 nM, P=0.025, n=5) and UDP (55±11 nM and 16±4 nM, P=0.009, n=5) (all shown without and with treatment, respectively) (figure 4.20). When cHASM cells from asthmatic individuals were pre-treated with the same antagonist, the peak amplitudes of the $[Ca^{2+}]_i$ rises induced by the following nucleotides (all at 100 μM) were all significantly decreased: ATP (96±31 nM and 65±28 nM, P<0.001, n=9), UTP (45±11 nM and 14±1 nM, P=0.028, n=8) and ADP (57±13 nM and 29±9 nM, P=0.005, n=5) (all shown without and with treatment, respectively). However, pre-treatment of cHASM cells from asthmatic donors with AR-C 118925XX did not significantly reduce the $[Ca^{2+}]_i$ rise induced by UDP

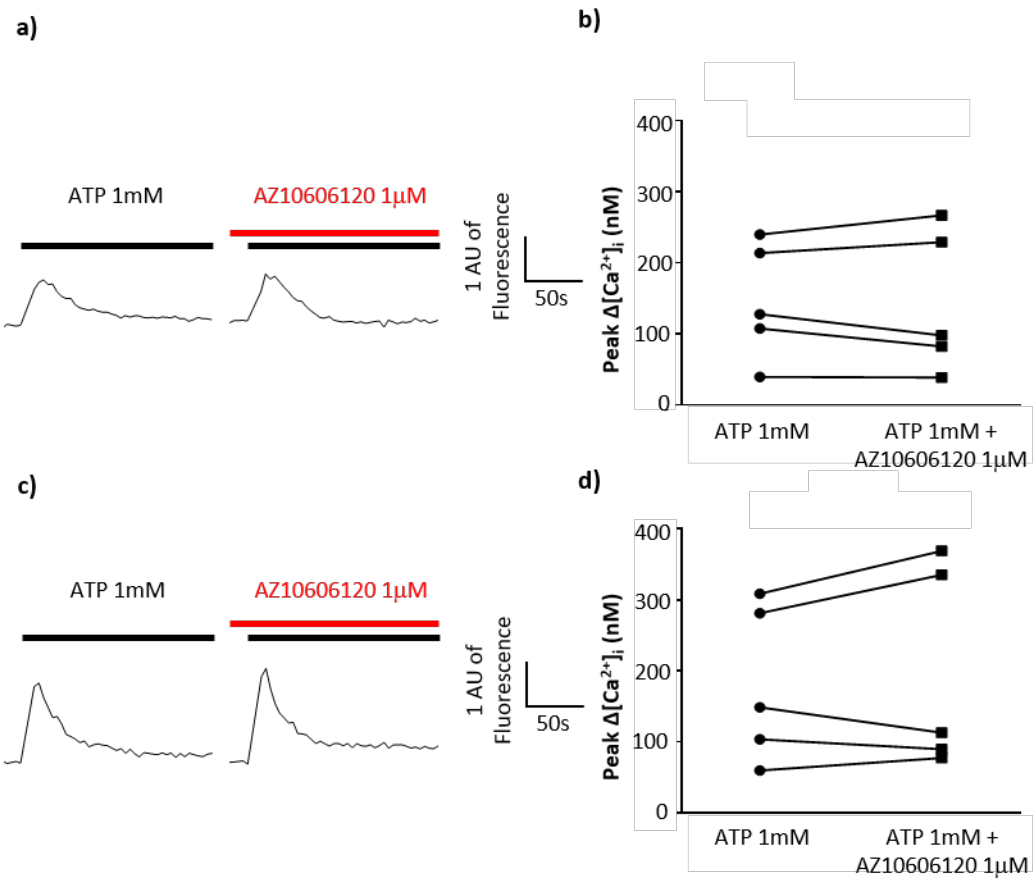

Figure 4.18. P2X7 receptor does not regulate $[Ca^{2+}]_i$ in cHASM cells from healthy or asthmatic individuals.

Representative $[Ca^{2+}]_i$ response profiles in cHASM cells (up to passage 5) from a) healthy and c) asthmatic individuals of the application of ATP (1 mM) in the absence and presence of the P2X7-selective antagonist AZ10606120 (1 µM). Horizontal lines represent the duration of the drug applications. Summaries of the peak $[Ca^{2+}]_i$ rises in b) healthy and d) asthmatic groups, measured using the Flexstation™ (n=5 for all). Statistical significance was determined using Student's paired *t*-tests.

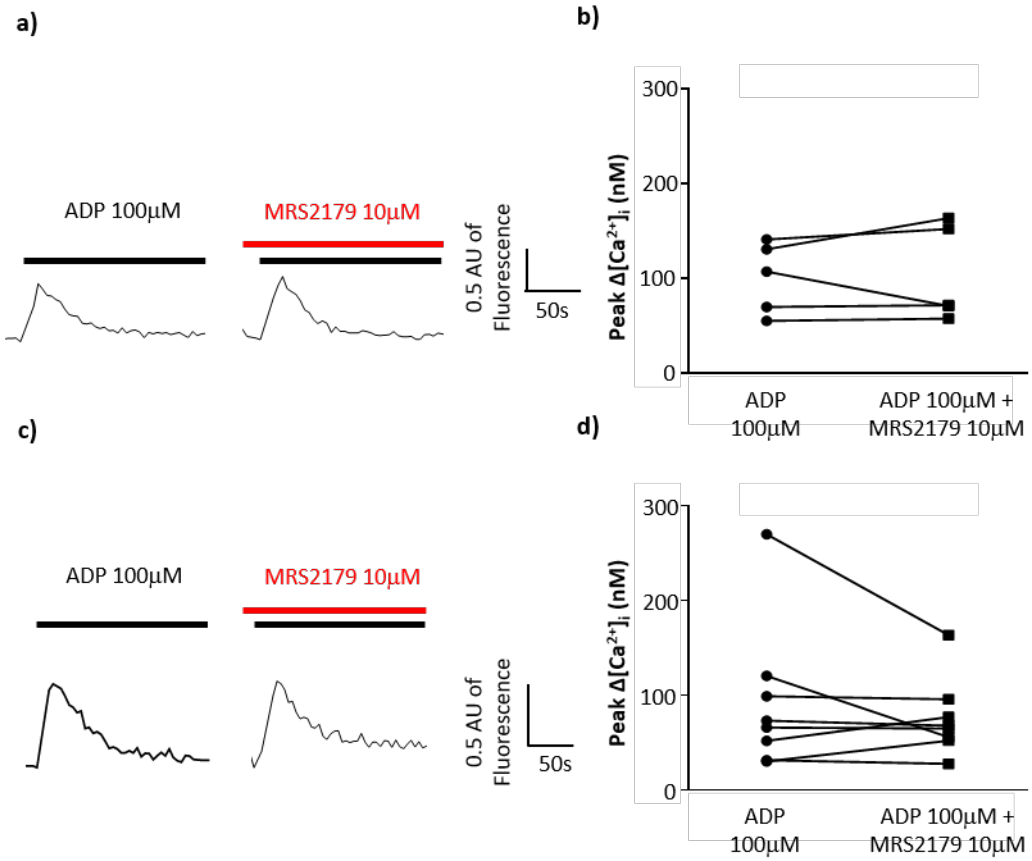

Figure 4.19. P2Y1 receptor does not regulate $[Ca^{2+}]_i$ in cHASM cells from healthy or asthmatic individuals.

Representative $[Ca^{2+}]_i$ response profiles in cHASM cells (up to passage 5) from a) healthy and c) asthmatic individuals of the application of ADP (100 µM) in the absence and presence of the P2Y1-selective antagonist MRS2179 (10 µM). Horizontal lines represent the duration of the drug applications. Summaries of the peak $[Ca^{2+}]_i$ rises in b) healthy (n=5) and d) asthmatic (n=8) groups, measured using the Flexstation™ (n=5 for all). Statistical significance was determined using Student's paired *t*-tests.

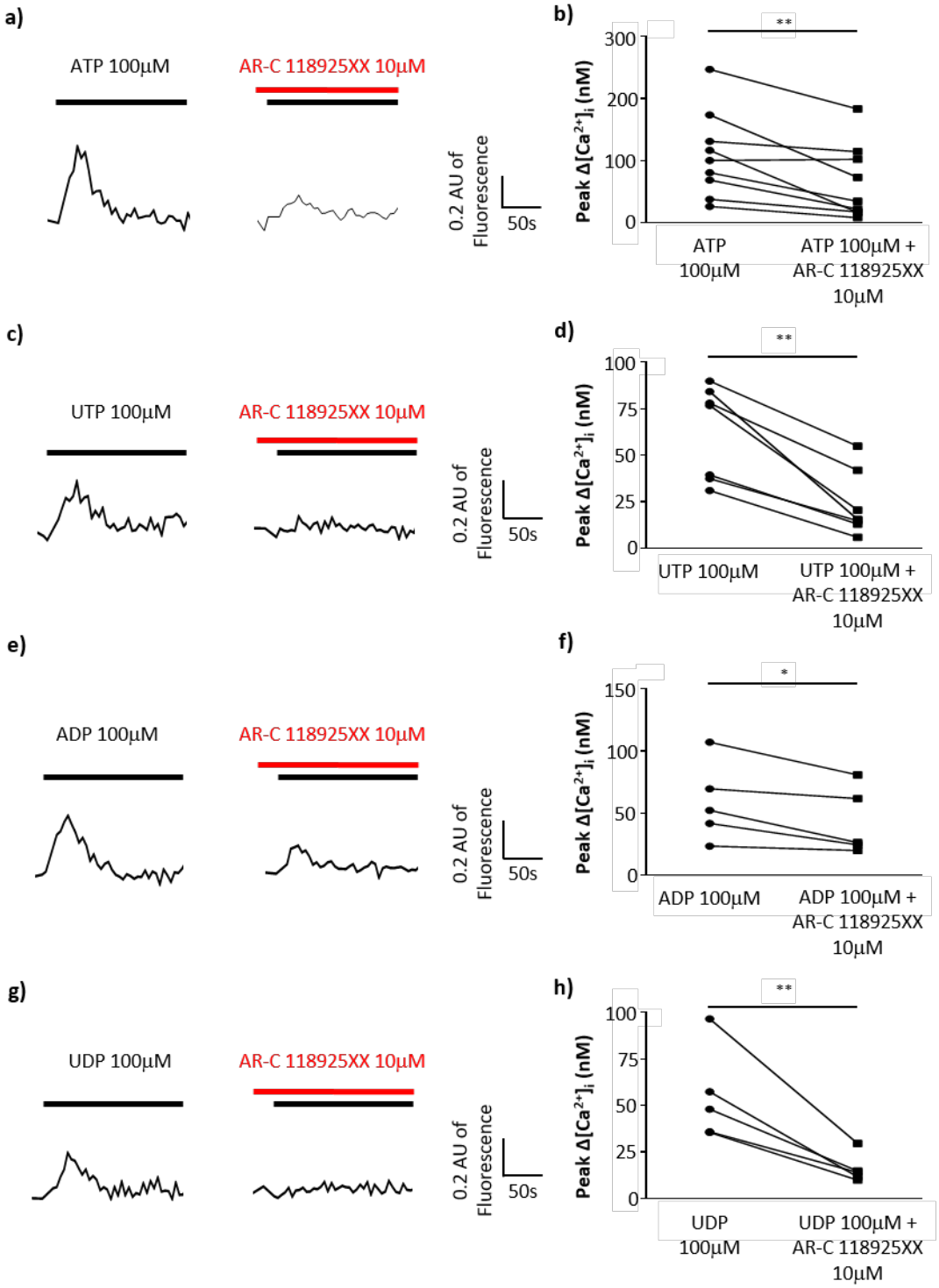

Figure 4.20. P2Y2 receptor regulates $[Ca^{2+}]_i$ in cHASM cells from healthy individuals.
Representative $[Ca^{2+}]_i$ response profiles in cHASM cells (up to passage 5) from healthy individuals of ATP (a), UTP (c), ADP (e) and UDP (g) applications with and without pre-treatment with the P2Y2-selective antagonist AR-C 118925XX (10 µM). Horizontal lines represent the duration of the drug applications. Summaries of the peak ATP- (b, n=9), UTP- (d, n=7), ADP- (f, n=5) and UDP- (h, n=5) (100 µM for all) induced $[Ca^{2+}]_i$ rises, measured using the Flexstation™. Statistical significance was determined using Student's paired *t*-tests (* P<0.05, ** P<0.01).

(100 μM), although a trend suggesting a reduction with antagonist treatment was observed (51±12 nM and 14±2 nM without and with treatment, respectively, P=0.056, n=4) (figure 4.21). Furthermore, the application of DMSO (1:1,000) as a vehicle control did not appear to affect $[Ca^{2+}]_i$ in cHASM cells from either healthy or asthmatic donors (figure 4.17). These data suggest that P2Y2 receptor is functional in HASM cells from both healthy and asthmatic donors and is activated by ATP, UTP, ADP and UDP (all at 100 μM).

4.2.2.3.3 The activation of P2Y4 and P2Y6 receptors does not induce significant $[Ca^{2+}]_i$ rises in cHASM cells from either healthy or asthmatic individuals

The P2Y4 receptor-selective agonist MRS 4062 (3 μM) (Bio-Techne Ltd.) induced peak $[Ca^{2+}]_i$ rises of 40±12 nM and 19±3 nM in cHASM cells from healthy and asthmatic donors, respectively (n=5 for both). The respective saline buffer controls induced $[Ca^{2+}]_i$ rises of 22±3 nM and 21±2 nM (n=5 for both). Compared to the controls, MRS 4062 (3 μM) did not induce significant $[Ca^{2+}]_i$ rises in cHASM cells from either healthy (P=0.136) or asthmatic (P=0.609) donors (figure 4.22).

The P2Y6 receptor-selective agonist MRS 2693 (10 μM) induced peak $[Ca^{2+}]_i$ rises of 24±7 nM and 20±2 nM in cHASM cells from healthy (n=6) and asthmatic (n=7) donors, respectively. The respective saline buffer controls induced $[Ca^{2+}]_i$ rises of 17±3 nM (n=6) and 17±2 nM (n=7). Compared to the controls, MRS 2693 (10 μM) did not induce significant $[Ca^{2+}]_i$ rises in cHASM cells from either healthy (P=0.306) or asthmatic (P=0.473) donors (figure 4.22).

These data suggest that neither P2Y4 nor P2Y6 receptor activation induces $[Ca^{2+}]_i$ rises in cHASM cells from either healthy or asthmatic donors.

4.2.2.3.4 P2Y11 receptor does not influence $[Ca^{2+}]_i$ in cHASM cells

The P2Y11-selective antagonist NF340 (10 μM) (Bio-Techne Ltd.) had no significant effect on the peak $[Ca^{2+}]_i$ amplitudes induced by ATP (100 μM) in cHASM cells from healthy (158±39 nM and 164±44 nM without and with treatment, respectively, P=0.788, n=6) or asthmatic (165±58 nM and 156±69 nM without and with treatment, respectively, P=0.625, n=5) donors, implying that P2Y11 receptor does not regulate $[Ca^{2+}]_i$ in these cells (figure 4.23).

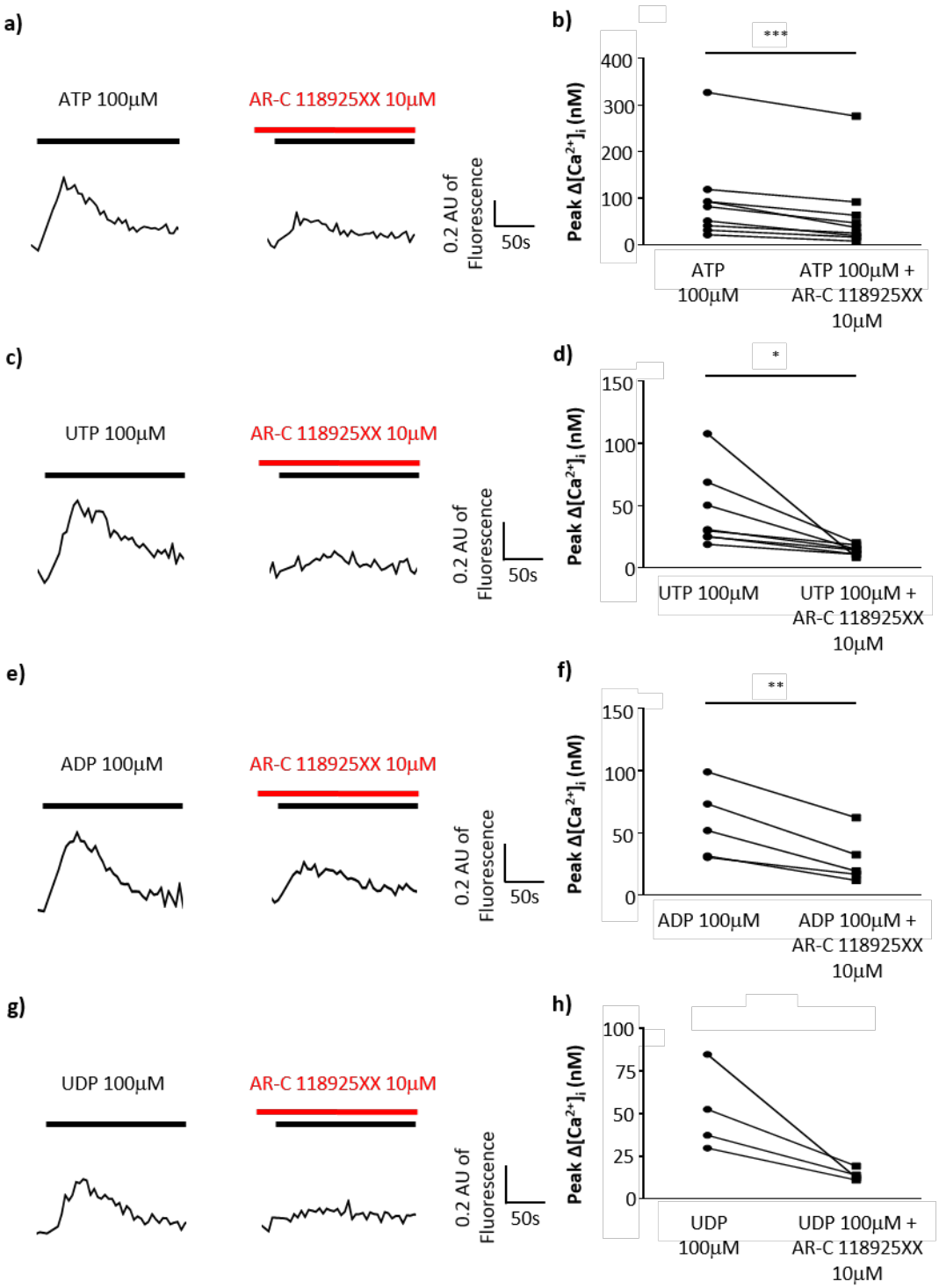

Figure 4.21. P2Y2 receptor regulates $[Ca^{2+}]_i$ in cHASM cells from asthmatic individuals.
Representative $[Ca^{2+}]_i$ response profiles in cHASM cells (up to passage 5) from asthmatic individuals of ATP (a), UTP (c), ADP (e) and UDP (g) applications with and without pre-treatment with the P2Y2-selective antagonist AR-C 118925XX (10 µM). Horizontal lines represent the duration of the drug applications. Summaries of the peak ATP- (b, n=9), UTP- (d, n=8), ADP- (f, n=5) and UDP- (h, n=4) (100 µM for all) induced $[Ca^{2+}]_i$ rises, measured using the Flexstation™. Statistical significance was determined using Student's paired *t*-tests (* P<0.05, ** P<0.01, ***P<0.001).

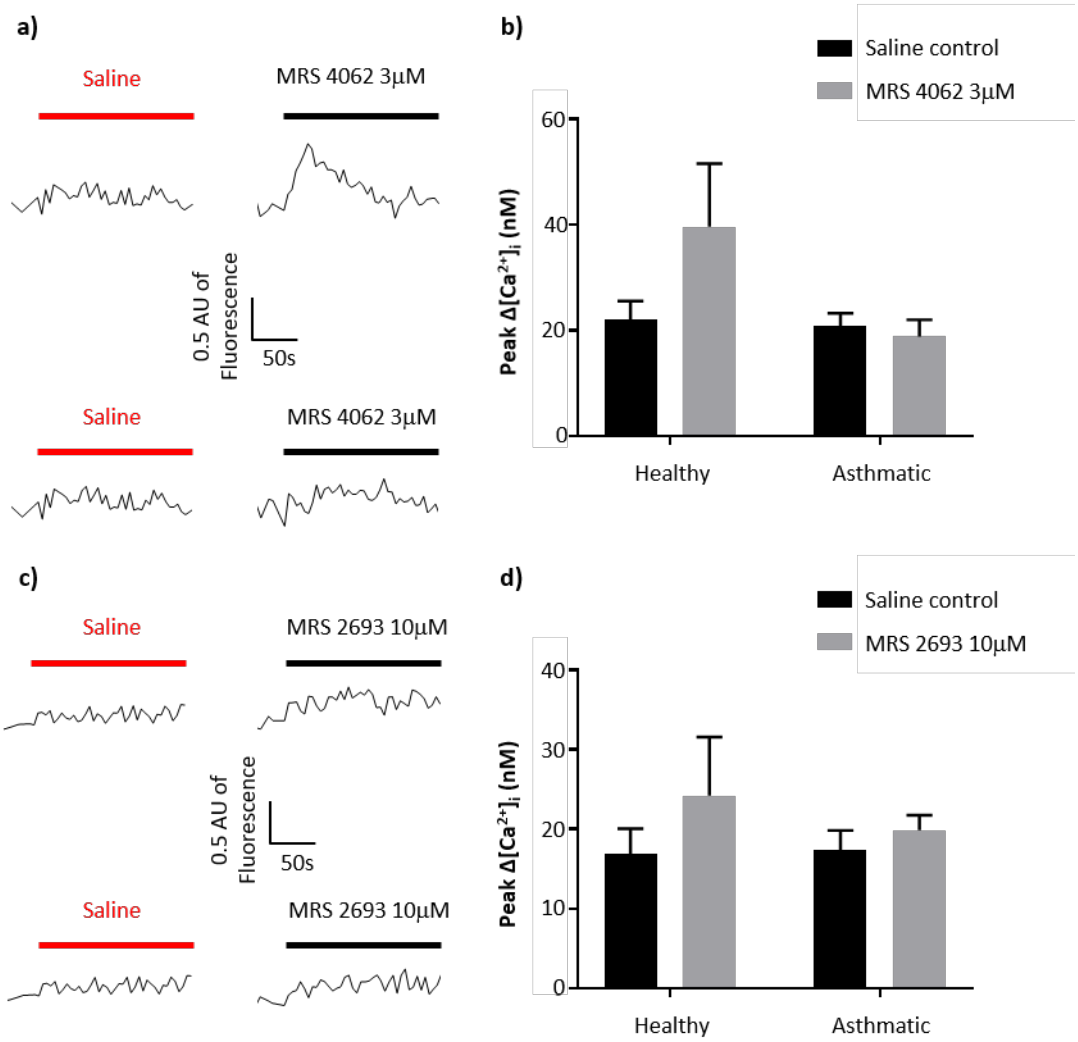

Figure 4.22. Neither P2Y4 nor P2Y6 receptor activation increases $[Ca^{2+}]_i$ in cHASM cells from healthy or asthmatic donors.

Representative $[Ca^{2+}]_i$ response profiles in cHASM cells (up to passage 5) from healthy [top panels of a) and c)] and asthmatic [bottom panels of a) and c)] donors induced by the application of either a) the P2Y4 receptor-selective agonist MRS 4062 (3 µM) or c) the P2Y6 receptor-selective agonist MRS 2693 (10 µM) with their respective saline controls. Horizontal lines represent the duration of the drug applications. These data, summarised in panels b) and d) for MRS 4062 and MRS 2693, respectively, were measured using the Flexstation™ and are expressed as peak $\Delta[Ca^{2+}]_i$ (nM) (n=5 for MRS 4062, n=6 and n=7 for MRS 2693 in healthy and asthmatic groups, respectively). Bars represent mean data ± SEM. *Statistical significance was determined using* Student's paired *t*-tests.

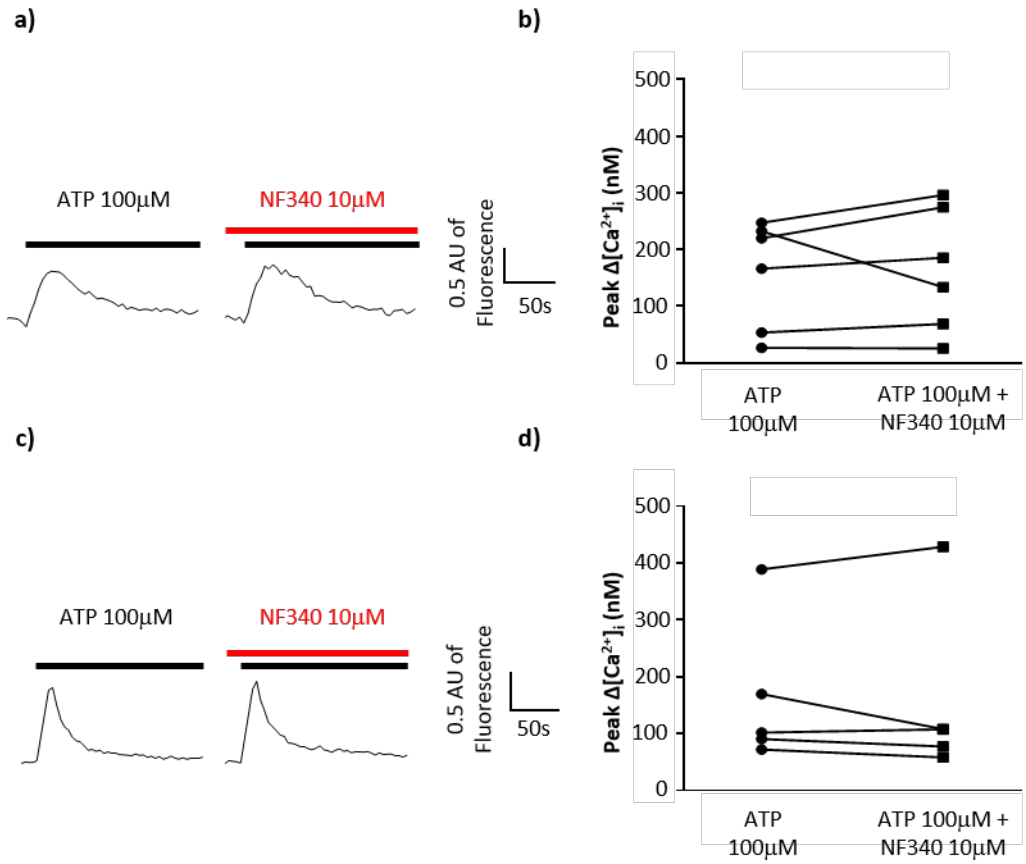

Figure 4.23. P2Y11 receptor does not regulate $[Ca^{2+}]_i$ in cHASM cells from healthy and asthmatic individuals.

Representative $[Ca^{2+}]_i$ response profiles in HASM cells cultured (up to passage 5) from a) healthy and c) asthmatic individuals following the application of ATP (100 µM) in the absence and presence of the P2Y11-selective antagonist NF340 (10 µM). Horizontal lines represent the duration of the drug applications. Summaries of the peak $[Ca^{2+}]_i$ rises in b) healthy and d) asthmatic groups, measured using the Flexstation™ [n=6 for (b) and n=5 for (d)]. Statistical significance was determined using Student's paired *t*-tests.

4.3 Discussion

Typical ASM functions, including contraction, rely heavily on Ca^{2+} (see section 1.2.4) and so calcium imaging assays were performed using the Ca^{2+}-sensitive fluorophore fura 2-AM to study those P2 receptors which regulate $[Ca^{2+}]_i$ in ASM. WEM was used with cmT-ASM cells whilst Flexstation was used with both cmT-ASM cells and cHASM cells. This allowed the investigation of both P2X and P2Y receptors.

Using WEM, the application of the nucleotides ATP, UTP, ADP and UDP (100 μM) induced fast, transient $[Ca^{2+}]_i$ rises in cmT-ASM cells (passage 1). Similar findings have been found in a previously published study where ATP, UTP and ADP application increased $[Ca^{2+}]_i$ in cultured rat ASM cells over a range of concentrations (Michoud et al., 2002). Furthermore, ATP (100 μM) induced Ca^{2+} oscillations in mouse lung slices (Bergner and Sanderson, 2002). When Flexstation was used to quantify the same responses in these cells, the response profile was very similar (ATP>UTP>ADP≈UDP) although the magnitude of the ADP- and UDP-induced $[Ca^{2+}]_i$ rises were larger when measured using Flexstation. This could be explained by the presence of cells more responsive to ADP and UDP in the smaller sample size used for Flexstation compared to WEM experiments. These data suggest that functional P2Y, and potentially functional P2X, receptors are expressed in cmT-ASM cells.

Further experimentation demonstrated that ATP and UTP application to cmT-ASM cells increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Whilst measurements of $[Ca^{2+}]_i$ induced by ATP, UTP and ADP application in cultured rat ASM cells (Michoud et al., 2002), and for ATP, UTP, ADP and UDP in cHASM cells (Govindaraju et al., 2005), were made over a range of concentrations, the EC_{50} values for these responses were not indicated. When analysed using a non-linear single exponential fit with a variable slope, the concentration-dose response curve for UTP had a value for R-square of 0.81, a Hill slope of 0.92 and EC_{50} of 10.3 μM. P2Y2 and P2Y4 receptors are those typically activated by UTP up to 10 μM (Jacobson and Muller, 2016). Due to the low expression of P2Y4 receptor transcripts in these cells (figure 3.8), it could be concluded that P2Y2 receptor is the UTP-responding receptor in these cells. However, further experimentation was required at this time to clarify this.

Analysing the concentration-response curve for ATP with a non-linear regression using 2 sites – fit gave a value for R-square of 0.58 and 2 EC_{50} values of 3 μM and 421 μM. These data could suggest that there are two functional P2 receptors responding to ATP in these cells that are contributing to the measured response, although further experimentation was required to identify thee functional receptor subtypes. The application of ATP (1 mM) induced a biphasic $[Ca^{2+}]_i$ rise with a different response profile compared to that of ATP (100 μM) consisting of a fast, transient peak response and a sustained $[Ca^{2+}]_i$ rise. This could indicate the contribution of several P2 receptors to the ATP (1 mM)-induced $[Ca^{2+}]_i$ rise. Analysing the ATP concentration-dose response curve up to only 100 μM with a non-linear single exponential fit with a variable slope gave a value for R-square of 0.57, a Hill slope of 1.1 and an EC_{50} of 4 μM, which is very similar to the EC_{50} value of 'higher affinity' of the ATP concentration-response curve up to 1 mM using the non-linear regression fit, providing support to the idea that there is a unique binding site for ATP up to 100 μM. All P2X receptors have EC_{50} values between 1-10 μM when expressed in recombinant systems except for P2X7 receptor, which requires much higher concentrations of ATP in order to be activated (Surprenant et al., 1996, Boeynaems et al., 2005, Coddou et al., 2011, Jacobson and Muller, 2016). Of the P2Y receptors, only P2Y2 and P2Y11 receptors are typically activated by ATP at concentrations between 1-10 μM (Jacobson and Muller, 2016). Due to the lack of a functional P2Y11 receptor orthologue in mice (von Kügelgen and Harden, 2011, Dreisig and Kornum, 2016), this receptor was excluded from consideration. Therefore, these data suggest that P2X7 receptor is functional in cmT-ASM cells (passage 1) and that its activation corresponds to the low affinity binding site, particularly as repeated or continued P2X7 receptor activation is known to induce a sustained $[Ca^{2+}]_i$ response (Surprenant et al., 1996, Coddou et al., 2011). However, further experimentation was required at this time in order to fully discern the functional P2 receptor subtypes in these cells.

The observation that UTP, UDP and ADP induced $[Ca^{2+}]_i$ rises in cmT-ASM cells suggests the presence of functional P2Y receptors in these cells. However, although data suggests the presence of P2X7 receptor, the functional expression of P2X receptors in these cells remained to be clarified. The ligand-gated P2X receptor ion channels are

activated by ATP and allow the entry of extracellular cations, including Ca^{2+}, into the cell (North, 2002). To investigate functional P2X receptor expression, ATP was applied ± Ca^{2+}_e. The absence of Ca^{2+}_e significantly reduced the amplitude of the ATP (100 μM)-induced $[Ca^{2+}]_i$ rise, and abolished the sustained portion of the ATP (1 mM)-induced $[Ca^{2+}]_i$ rise. These data suggest that these responses, but not that of the peak ATP (1 mM)-induced $[Ca^{2+}]_i$ rise, are dependent on extracellular Ca^{2+} which could be explained by the contribution of P2X receptors to the ATP (100 μM) response, and to the sustained portion of the ATP (1 mM) response. However, it is also possible that the sustained portion of the ATP (1 mM) response could be attributed to the opening of store-operated Ca^{2+} channels, which are activated upon depletion of intracellular Ca^{2+} stores in order to aid store refilling (Prakriya and Lewis, 2015), following the activation of Gq-coupled P2Y receptors. Indeed, store-operated Ca^{2+} entry is known to occur in ASM cells (Peel et al., 2006, Chen and Sanderson, 2017). Performing experiments whereby the intracellular Ca^{2+} stores are depleted, perhaps by the use of thapsigargin, could clarify this issue. If this approach is taken and the same effect, a sustained Ca^{2+} influx, is observed following the depletion of Ca^{2+} stores, then this reduces the likelihood of P2X receptors contributing to the ATP (1 mM) response. Furthermore, general P2X receptor antagonists, such as pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt, could be used to ensure that influx via P2X receptors would not complicate data interpretation. However, their potency at, and selectivity for, P2X receptor subtypes has been questioned and there have been some published reports that these can act on P2Y receptor subtypes and even inhibit the reverse mode of the NCX in guinea-pig ASM (Kim et al., 2001c, Flores-Soto et al., 2012). However, these data could also suggest the presence of functional P2X7 receptor as its repeated/continued activation is known to induce biphasic, sustained $[Ca^{2+}]_i$ rises (Surprenant et al., 1996, Coddou et al., 2011). It is also plausible that ATP (100 μM) application could activate a small population of P2X7 receptors, which could account for the observed reduction in the amplitude of the ATP (100 μM)-induced $[Ca^{2+}]_i$ rise. Subsequent experiments involved the use of selective pharmacological tools for P2 receptors, as summarised in tables 1.3 and 1.4, to identify the functional P2 receptor subtypes expressed in cmT-ASM cells. These tables also show examples of studies where these compounds were used and for which selective effects

were observed. It can also be confirmed here that none of the selective antagonists used in this study directly affected $[Ca^{2+}]_i$ when applied alone to either cmT-ASM or cHASM cells.

As described, P2X2 and P2X3 receptors are typically expressed only in sensory neurones (Chen et al., 1995, Lewis et al., 1995, Collo et al., 1996, Ralevic and Burnstock, 1998) and data regarding functional P2X5 and P2X6 homomers in native tissues is limited (Collo et al., 1996, Lê et al., 1997, Bo et al., 2003, Jones et al., 2004, Duckwitz et al., 2006, Ormond et al., 2006). Therefore, the investigation into functional P2X receptor expression in cmT-ASM cells focussed on P2X1, P2X4 and P2X7 receptors in the first instance. The application of α,β-meATP (10 μM), a selective agonist for P2X1 and P2X3 receptors, did not induce $[Ca^{2+}]_i$ rises in these cells, suggesting the absence of functional P2X1 and P2X3 receptors despite the fact that P2X1 protein was positively identified in cmT-ASM cells. This could be due to a lack of expression of the protein at the cell membrane, or could in fact be due to a lack of P2X1 receptor protein expression in these cells if the observed staining was non-specific (Ashour et al., 2006). However, it is a possibility that the receptors, if expressed, were desensitized as both P2X1 and P2X3 receptors are known to rapidly desensitize (Ralevic and Burnstock, 1998, North, 2002). Performing experiments without the perfusion system to prevent ATP release, and pre-treating the cells with the commercial ectonucleotidase apyrase, could eliminate the possibility of receptor desensitization in future experiments. The use of the selective P2X4 receptor antagonists 5-BDBD (20 μM) and PSB 12054 (10 μM) did not significantly affect the ATP (100 μM)-induced $[Ca^{2+}]_i$ rise, suggesting the absence of functional P2X4 receptors in cmT-ASM cells, despite its functional presence being indicated in porcine ASM (Nagaoka et al., 2009). Species differences could explain the dissimilarity in observations here. The presence of the P2X7 receptor-selective antagonist, AZ10606120 (1 μM), abolished the sustained portion of the ATP (1 mM)-induced $[Ca^{2+}]_i$ rise without significantly affecting the peak response. These data are extremely similar to those observed in the absence of Ca^{2+}_e which, when considered together, could suggest the presence of functional P2X7 receptor in these cells which contributes to the sustained portion of the ATP (1 mM)-induced $[Ca^{2+}]_i$ rise. However, the similarity between the effects of removing Ca^{2+}_e and pre-incubation with AZ10606120 could

indicate that AZ10606120 blocks Ca^{2+} influx. A control experiment should be performed to clarify this issue. For example, if AZ10606120 pre-treatment inhibited the response of an agonist whose $[Ca^{2+}]_i$ rise was known to be dependent on Ca^{2+} influx then this could suggest non-specific effects of AZ10606120. Future experiments could also apply ATP (100 μM) ± P2X7 receptor-selective antagonist in order to determine if P2X7 receptor contributes to the ATP (100 μM)-induced $[Ca^{2+}]_i$ rise. However, our data could suggest that the sole functional P2X receptor in cmT-ASM cells (passage 1) is P2X7 receptor.

As discussed, our data suggests the presence of functional P2Y receptors which respond to UDP, ADP and UTP in cmT-ASM cells. UDP can typically activate either P2Y6 or P2Y14 receptors (Boeynaems et al., 2005, Jacobson and Muller, 2016). Despite the fact that UDP (100 μM) induced $[Ca^{2+}]_i$ rises in these cells, the selective agonists for P2Y6 and P2Y14 receptors, MRS 2693 (10 μM) and UDP-glucose (100 μM) respectively, did not affect $[Ca^{2+}]_i$, suggesting that these receptors do not regulate $[Ca^{2+}]_i$ in these cells. Therefore, a different P2Y receptor subtype must be responsible for the UDP-induced $[Ca^{2+}]_i$ rise. Further experiments could also be performed to confirm that Gi-coupled P2Y receptors do not regulate $[Ca^{2+}]_i$. For example, pertussis toxin could be applied to the cells to act as an inhibitor of Gi-coupled GPCRs (Mangmool and Kurose, 2011).

ADP classically activates P2Y1, P2Y12 and P2Y13 receptors (Jacobson and Muller, 2016). Due to their coupling to Gi/o proteins (Boeynaems et al., 2005), P2Y12 and P2Y13 are not typically considered to be involved in $[Ca^{2+}]_i$ regulation. Therefore, these receptors were excluded from consideration for the time being. The use of the P2Y1 receptor-selective antagonist MRS2179 significantly reduced the amplitude of the ADP-induced $[Ca^{2+}]_i$ rise by ~40%, suggesting the presence of functional P2Y1 receptors in these cells whilst potentially indicating the presence of another ADP-sensitive receptor.

As discussed, our data suggests that the UTP-responding receptor in cmT-ASM cells must be P2Y2 receptor. This receptor is typically activated by ATP and UTP, but reports have also suggested that it can be activated by ADP and UDP (Zambon et al., 2000, Shen et al., 2004, von Kügelgen, 2006). The selective P2Y2 receptor antagonist, AR-C 118925XX, significantly reduced the amplitudes of the $[Ca^{2+}]_i$ rises induced by ATP, UTP, ADP and UDP (all at 100 μM), suggesting that P2Y2 receptor is functional in these

cells and is activated by each of the four nucleotides tested. Although it has been suggested that reports of P2Y2 receptor activation by ADP and UDP are likely due primarily to contamination of samples by nucleotide triphosphates (Nicholas et al., 1996), our data suggests that P2Y2 receptor is activated by ADP and UDP. Furthermore, P2Y2 receptor activation could account for the high-affinity binding site for ATP and so P2Y2 could be the receptor activated by both ATP and UTP at concentrations up to 100 μM. These data are supported by desensitization experiments whereby $[Ca^{2+}]_i$ responses to either UTP/ATP application were measured after pre-treatment/application of cmT-ASM cells with the other nucleotide. In theory, if these two nucleotides activated different receptors, no desensitization would have been observed and so no reduction in the response amplitude compared to control responses would have been detected. This approach has previously been applied in published studies to investigate the same concept (Cressman et al., 1999). Our data demonstrated a significant reduction in the observed UTP-induced $[Ca^{2+}]_i$ rises after ATP pre-treatment, and vice versa, suggesting that receptor desensitization had occurred and that, at 100 μM, both nucleotides activate the same P2 receptor subtype, P2Y2 receptor. However, there is a possibility that the initial application of the 'desensitization nucleotide' could be depleting Ca^{2+} stores and that this could account for the reduction in the subsequent $[Ca^{2+}]_i$ rise. To test this, control experiments should be performed in future such as the application of ATP (100 μM) followed by that of bradykinin (whose $[Ca^{2+}]_i$ rise is dependent on intracellular Ca^{2+} stores). If ATP depletes intracellular Ca^{2+} stores, then this would reduce the bradykinin-induced $[Ca^{2+}]_i$ rise. Further experiments could also be performed to assess how replete the Ca^{2+} store was, for example by using another Gq-coupled agonist such as carbachol, using a receptor-independent method of depleting Ca^{2+} stores, such as thapsigargin, or even by measuring the $[Ca^{2+}]$ directly in the Ca^{2+} stores by using an ER/SR-targeted Ca^{2+} sensor such as CatchER (Reddish et al., 2017).

In summary, our data suggests that cmT-ASM cells (passage 1) express functional P2X7, P2Y1 and P2Y2 receptors. P2X7 receptor contributes to the sustained portion of the ATP (1 mM)-induced $[Ca^{2+}]_i$ rise, whilst P2Y2 receptor is activated by both ATP and UTP, in addition to UDP and ADP (100 μM). P2Y1 receptor is also activated by ADP (100

μM). To our knowledge, these receptors have not previously been identified as functional in ASM by use of selective tools.

These data were then compared with $[Ca^{2+}]_i$ responses induced by nucleotides in cHASM cells from healthy and asthmatic donors measured using the Flexstation. This approach both allowed the comparison of basal P2 receptor functional in ASM by using ASM cells cultured from both WT murine ASM and healthy donors, and permitted the comparison of P2 receptor function between cHASM cells from healthy and asthmatic donors. This could potentially highlight P2 receptor dysfunction which could contribute to asthma pathogenesis.

The application of ATP (1 mM) and ATP, UTP, ADP and UDP nucleotides (all at 100 μM) to cHASM cells from healthy and asthmatic donors induced $[Ca^{2+}]_i$ rises above saline controls with distinct response profiles, suggesting the presence of functional P2Y, and potentially P2X, receptors. To our knowledge, only one published study has investigated P2 receptor function in HASM. This study observed that ATP, UTP, ADP and UDP application over a range of concentrations induced $[Ca^{2+}]_i$ rises in cHASM cells derived from surgical and lung transplant specimens (Govindaraju et al., 2005). Comparing the amplitudes of these responses with those from cmT-ASM cells, also measured with Flexstation, demonstrated that these nucleotides elicit more Ca^{2+} flux in cmT-ASM cells. This could either be due to species differences or to the lower passage numbers used in cmT-ASM cells. No study has directly compared functional responses of P2 receptor between human and murine samples to our knowledge. In cHASM cells, significant differences were only observed between the amplitudes of the UDP (100 μM)-induced responses from healthy and asthmatic donors, suggesting a functional difference in a UDP-sensitive receptor. ATP (100 μM)-induced $[Ca^{2+}]_i$ rises were partially dependent on Ca^{2+}_e in cHASM cells, as in cmT-ASM cells, supporting the idea of functional P2X receptor expression in cHASM cells from both healthy and asthmatic individuals. In a previously published study, the $[Ca^{2+}]_i$ rises induced by ATP (10 μM) were not dependent on Ca^{2+}_e, although higher concentrations were not tested (Govindaraju et al., 2005). The application of adenosine (100 μM) to cHASM cells from healthy and asthmatic donors induced small $[Ca^{2+}]_i$ rises, suggesting the presence of functional adenosine receptors in these cells, although no further work was carried out

to determine which adenosine receptor subtypes were responsible for these rises. This finding agrees with current literature suggesting that adenosine induces small, yet significant, $[Ca^{2+}]_i$ rises in cHASM cells at concentrations up to 100 μM (Govindaraju et al., 2005).

Pharmacological tools were also used to elucidate the functional P2 receptor subtypes in cHASM cells, as they were in cmT-ASM cells. Regarding the investigation of functional P2X receptor expression in cHASM cells, the selective agonist for both P2X1 and P2X3 receptors, α,β-meATP (10 μM), did not induce significant $[Ca^{2+}]_i$ rises in cHASM cells from either healthy or asthmatic donors, despite the positive identification of P2X1 receptor protein in these cells, although responses measured in cHASM cells from healthy individuals were very variable. These data suggest that neither P2X1, nor P2X3, receptors are functional in these cells and so do not contribute to the ATP-induced $[Ca^{2+}]_i$ rises in these cells. This could reflect a change in the functional expression of either P2X1 or P2X3 receptors due to culture (Pacaud et al., 1995), in addition to typical variation observed in small populations. However, for cHASM cells as in cmT-ASM cells, this could be due to a lack of expression of the protein at the cell membrane or even a lack of P2X1 receptor protein expression in these cells if the observed staining was non-specific (Ashour et al., 2006). Unlike in the WEM experiments performed with cmT-ASM cells, however, cHASM cells were pre-treated with the commercial ectonucleotidase apyrase in Flexstation experiments, reducing the likelihood of P2X1 or P2X3 receptor protein desensitization. Increasing sample sizes could clarify this point further in the future. The presence of the P2X4 receptor-selective antagonist PSB 12054 (10 μM) significantly decreased the amplitude of the ATP (100 μM)-induced $[Ca^{2+}]_i$ rises in cHASM from asthmatic donors by a small amount, and data suggested a similar trend in cHASM from healthy individuals although these differences were not significant at this sample size. These data suggest that P2X4 is functional in these cells, although further donors could be added in future to clarify this. Furthermore, the presence of the P2X7 receptor-selective antagonist AZ10606120 (1 μM) did not significantly affect the peak amplitude of the ATP (1 mM)-induced $[Ca^{2+}]_i$ rise in cHASM cells from either healthy or asthmatic donors. However, the application of ATP (1 mM) to cmT-ASM cells induced a biphasic $[Ca^{2+}]_i$ rise, and data suggested that P2X7 receptor contributed to the

sustained, but not the peak, response. Application of ATP (1 mM) to cHASM cells did not result in a biphasic response when measured with Flexstation, adding further evidence that P2X7 receptor is not functional in cHASM cells as it is in cmT-ASM cells.

The above data suggests that functional P2X receptors are not expressed in cHASM cells from either healthy or asthmatic donors, and functional P2X receptors specifically have not been previously identified in cHASM cells to our knowledge. Therefore, the ATP-induced $[Ca^{2+}]_i$ rises, in addition to those of UTP, ADP and UDP, must be attributed to P2Y receptors. The use of the selective agonists for P2Y4 and P2Y6 receptors, MRS 4062 (3 μM) and MRS 2693 (10 μM) respectively, did not induce significant $[Ca^{2+}]_i$ rises in cHASM cells from either healthy or asthmatic donors compared to saline controls, suggesting that these receptors are not functional in these cells. However, although the data were not significant, there was a trend suggesting that P2Y4 receptor activation increases $[Ca^{2+}]_i$ in cHASM cells from healthy individuals despite the variable responses measured. The addition of more donors could clarify this point in future.

Pre-treatment of cHASM cells with the P2Y1-selective antagonist MRS2179 (10 μM) did not significantly inhibit ADP-induced $[Ca^{2+}]_i$ rises, suggesting that P2Y1 receptor is not functional in cHASM cells as it is in cmT-ASM cells. Furthermore, pre-treatment of cHASM cells with the P2Y11-selective antagonist NF340 failed to inhibit the ATP (100 μM)-induced $[Ca^{2+}]_i$ rise in these cells, suggesting that P2Y11 receptor is not functional in these cells. Our data also suggests that cHASM cells from both healthy and asthmatic individuals express functional P2Y2 receptors which are activated by ATP, UTP, ADP and UDP, as they are in cmT-ASM cells, and therefore that this receptor subtype is responsible for all of the observed nucleotide-induced $[Ca^{2+}]_i$ rises in these cells. As far as we know, the identification of the functional P2Y receptors present in HASM has not been previously determined using selective approaches.

In summary, our data suggests that cHASM cells from both healthy and asthmatic individuals express functional P2Y2 receptors, whilst only those from asthmatic donors express functional P2X4 receptors. Despite the observed significant reduction in UDP (100 μM)-induced $[Ca^{2+}]_i$ rises in cHASM cells from asthmatics compared to healthy donors, these data suggest that differences in P2 receptor

functional expression do not contribute to asthma pathogenesis. Therefore, cHASM cells (passages 2-5) appear to express fewer functional P2 receptors compared to cmT-ASM cells (passage 1), which are most likely due to species differences or the fact that the cmT-ASM cells are passaged fewer times than the cHASM cells.

The data presented in this chapter has several potential limitations. For example, despite the fact that the compounds were used at concentrations carefully chosen to give selective activation/antagonism of receptors as appropriate, experiments relied heavily on the use of pharmacological tools. The use of approaches such as RNA knockdowns, or even P2 receptor knockout animal models, in conjunction with pharmacological tools could be used in future experiments to give more confidence to the data generated. Furthermore, experiments using human samples have been limited by small sample sizes and relatively high passage numbers. Increasing the availability of samples in future studies could combat this issue. Furthermore, as outlined in this discussion, further control experiments are required and should be performed to allow more concrete conclusions to be drawn from the data presented. Without these controls, conclusions are more limited. Yet, despite these limitations, it is evident from the literature that a comprehensive study of P2 receptor function in ASM is greatly needed. This study has tackled this issue by using a variety of selective pharmacological tools to investigate the function of a wider range of P2 receptor subtypes which can directly regulate $[Ca^{2+}]_i$ in ASM from several models. The data presented here also highlights differences in P2 receptor function in ASM from different species, demonstrating that care must be taken when applying data generated from animal models to humans. Now that the expression of functional P2 receptors which regulate $[Ca^{2+}]_i$ in ASM from murine and humans has been determined, the next chapter will explore their physiological roles.

5 The physiological roles of P2 receptors in ASM

5.1 Introduction

Data from the previous results chapter suggested that P2X7, P2Y1 and P2Y2 receptors are functionally expressed in cmT-ASM cells. The data also suggested that P2Y2 receptors are functional in cHASM cells from both healthy and asthmatic donors. In addition, P2X4 receptor was identified as functional in cHASM cells from asthmatic donors, whilst a trend suggested functional P2X4 receptor expression in those cells from healthy individuals.

This chapter aimed to determine the functional contribution of these receptors to key physiological roles of ASM, including contraction and proliferation. Wire myography and collagen gel-contraction assays were used to determine the functional contribution of P2 receptors to the contraction of mouse trachea segments and cHASM cells from both healthy and asthmatic donors, respectively. Using wire myography with excised mouse trachea segments allows the measurement of physiologically relevant data regarding the contraction of mouse trachea, although the presence of contaminating tissues, such as the epithelium, can interfere with the study of P2 receptor function in ASM specifically. On the other hand, using collagen-gel contraction assays with cHASM cells allows the indirect study of the contraction of a pure population of ASM cells via the measurement of the gel area. However, these data rely on cultured cells and so are potentially less physiologically relevant due to phenotypic changes that can occur during culture (Halayko et al., 1996).

Preliminary experiments regarding the effect of extracellular nucleotides on the proliferation of cmT-ASM cells (passage 2) and cHASM cells from both healthy and asthmatic individuals (passages 2-5) were also performed using manual counting assays.

The data in this chapter are presented as preliminary data and, as such, are not ready for publication and cannot be used to draw definitive conclusions. However, they can be used to identify research directions to pursue in future studies regarding the physiological roles of P2 receptors in ASM.

5.2 Results

5.2.1 Preliminary study on the regulation of airway contraction by P2 receptors

5.2.1.1 Regulation of mouse trachea contraction by P2 receptors

5.2.1.1.1 Optimisation of resting tension applied to mouse trachea

Tension was manually applied to excised mouse trachea segments in 0.2 mN increments to determine the tension required to give optimal contraction. A 5-minute application of 60K-PSS was performed at each increment to contract the trachea. The contraction with the largest peak amplitude was observed when the trachea was under 0.8 mN tension (n=3) (figure 5.1).

5.2.1.1.2 Carbachol contracts mouse trachea in a concentration-dependent manner

The application of carbachol [an M3 muscarinic receptor-mediated bronchoconstrictor of ASM (Fryer and Jacoby, 1998)] induced the contraction of mouse trachea segments in a concentration-dependent manner with a Hill slope of 1.247 and an EC_{50} of 0.27 μM when analysed with a non-linear single exponential fit with a variable slope (n=5) (figure 5.2).

5.2.1.1.3 Extracellular nucleotides do not induce large contractions of mouse trachea

The application of ATP, ADP, UTP and UDP (all at 100 μM) to mouse trachea segments induced contractions measured at 8.1±1.6% (n=15), 1.8±0.7% (n=8), 1.1±0.6% (n=3) and 5.6±4.8% (n=2) of 60KPSS-induced contraction, respectively. The corresponding carbachol (0.3 μM)-induced contractions were 105.1±6.0% (n=15), 92.0±6.1% (n=8), 114.0±2.3% (n=3) and 120.3±39.8% (n=2) of 60KPSS-induced contraction, respectively (figure 5.3). These data suggest that P2 receptor activation is not a key component of mouse trachea contraction.

5.2.1.1.4 Pre-treatment of mouse trachea with ADP (100 μM) significantly potentiates carbachol-induced contraction

Extracellular nucleotides (Kitazawa et al., 1991a) and P2 receptors (Yu et al., 2013, Oguma et al., 2007) have been implicated in the sensitization of ASM contraction. Furthermore, P2 receptor activation in the airways can induce ASM relaxation in some instances (Marcet et al., 2007). Therefore, to investigate if extracellular nucleotides regulate the sensitization of murine ASM contraction, mouse trachea segments were

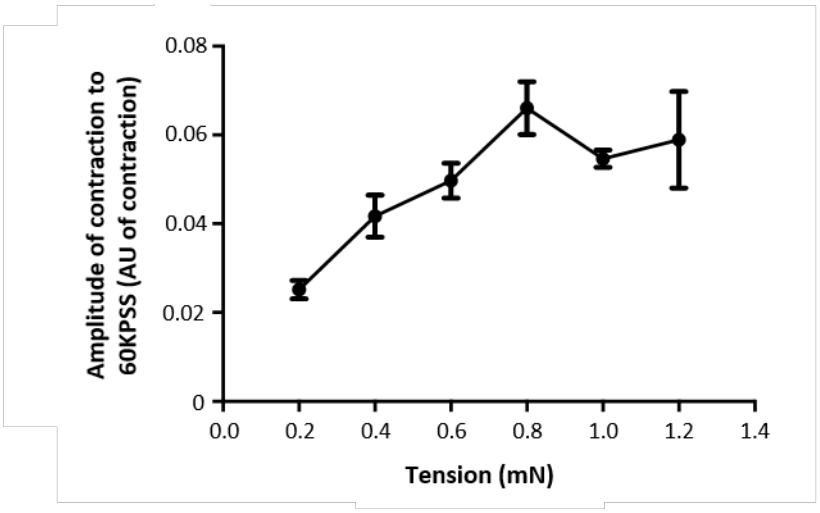

Figure 5.1. The amplitude of 60K-PSS-induced contraction in mouse trachea is dependent on its initial resting tension.

The peak amplitude of contractions (AU of contraction) induced by 60KPSS in mouse trachea segments (measured by wire myography) is dependent on the initial resting tension of the trachea (n=3 different mice). Bars represent mean data ± SEM.

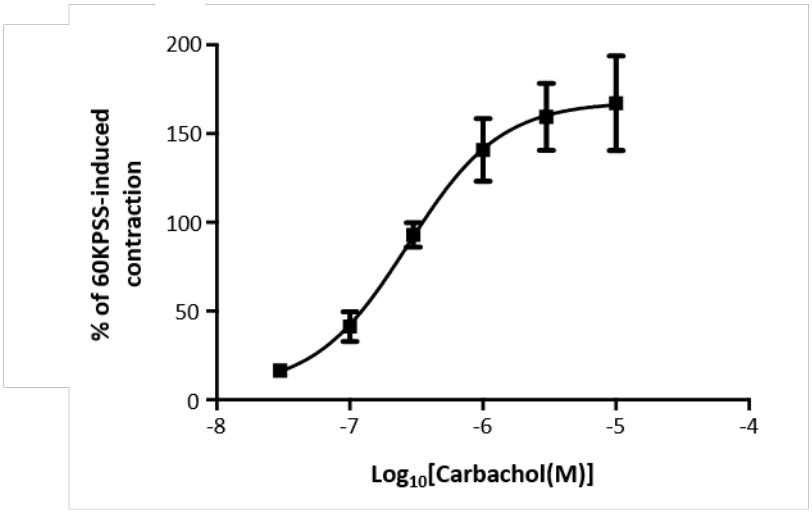

Figure 5.2. Carbachol induces contraction of mouse trachea in a concentration-dependent manner.

The peak amplitude of contractions induced by the muscarinic receptor agonist carbachol in mouse trachea segments (measured by wire myography) is concentration-dependent. When analysed with a non-linear single exponential fit with a variable slope, a Hill slope of 1.247 and an EC_{50} of 0.27μM were observed (n=5 different mice). Data are expressed as a % of the 60KPSS-induced contraction. Bars represent mean data ± SEM.

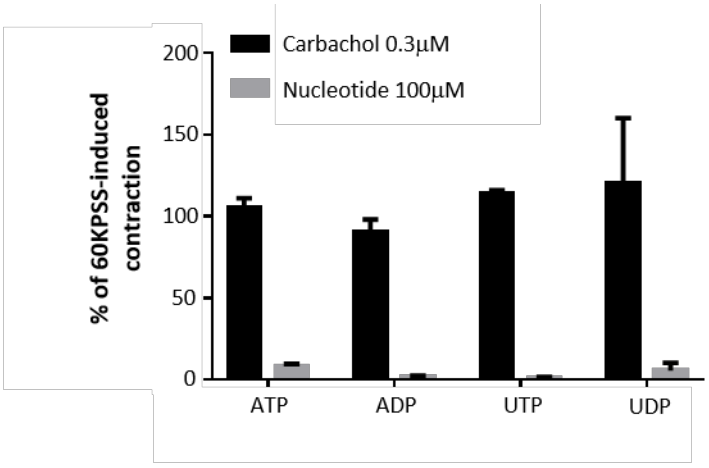

Figure 5.3. Extracellular nucleotides do not induce contraction of mouse trachea.

The peak amplitude of contractions induced the application of the nucleotides ATP (n=15), ADP (n=5), UTP (n=3) and UDP (n=2) (all at 100µM) to mouse trachea segments were measured by wire myography. The respective positive control contractions induced by carbachol (0.3mM) are also shown. Data are expressed as a % of the 60KPSS-induced contraction. Bars represent mean data ± SEM

pre-treated with extracellular nucleotides for 5 min before the application of carbachol at its EC_{50} (0.3 μM). The contraction amplitudes were then compared with the first carbachol-induced contractions (control) so that any potentiation or reduction of the contraction amplitude due to extracellular nucleotide pre-treatment could be observed. A final carbachol application was used to determine if carbachol-induced contractions returned to the same peak amplitude as the control carbachol-induced contractions. Here, 45 min were left between each application. A schematic for this protocol is given in figure 5.4a.

ATP (100 μM) pre-treatment of mouse trachea segments increased the peak amplitude of carbachol-induced contractions from 105.2±14.1% to 159.1±6.5% of the 60KPSS-induced contraction, which then decreased to 135.3±24.8% upon reapplication of carbachol (0.3 μM) (n=2) (figure 5.5, panels a and b). With UTP (100 μM) pre-treatment, the peak amplitude of carbachol-induced contractions increased from 114.4±3.8% to 144.4±0.1% of the 60KPSS-induced contraction, which then decreased to 115.4±0.1% (n=2) (figure 5.5, panels c and d). After UDP (100 μM) pre-treatment, the peak amplitude of the carbachol-induced contraction increased from 80.5% to 122.5% of the 60KPSS-induced contraction, which then decreased to 118.3% (n=1) (figure 5.5, panels e and f). When mouse trachea segments were pre-treated with ADP (100 μM), carbachol-induced contractions significantly increased from 92.0±6.1% to 132.2±8.2% of the 60KPSS-induced contraction (P=0.004), which then decreased significantly to 105.7±7.9% (n=8, P=0.013) (figure 5.5, panels g and h). There was also a trend suggesting that the second carbachol (0.3 μM)-induced contraction was increased compared to the first (P=0.065).

Although conclusions cannot be drawn regarding the effects of ATP, UTP and UDP pre-treatment on carbachol-induced contraction at this time, and that further control experiments should be performed, these data could suggest that the pre-treatment of mouse trachea segments with ADP (100 μM) potentiates carbachol (0.3 μM)-induced contractions.

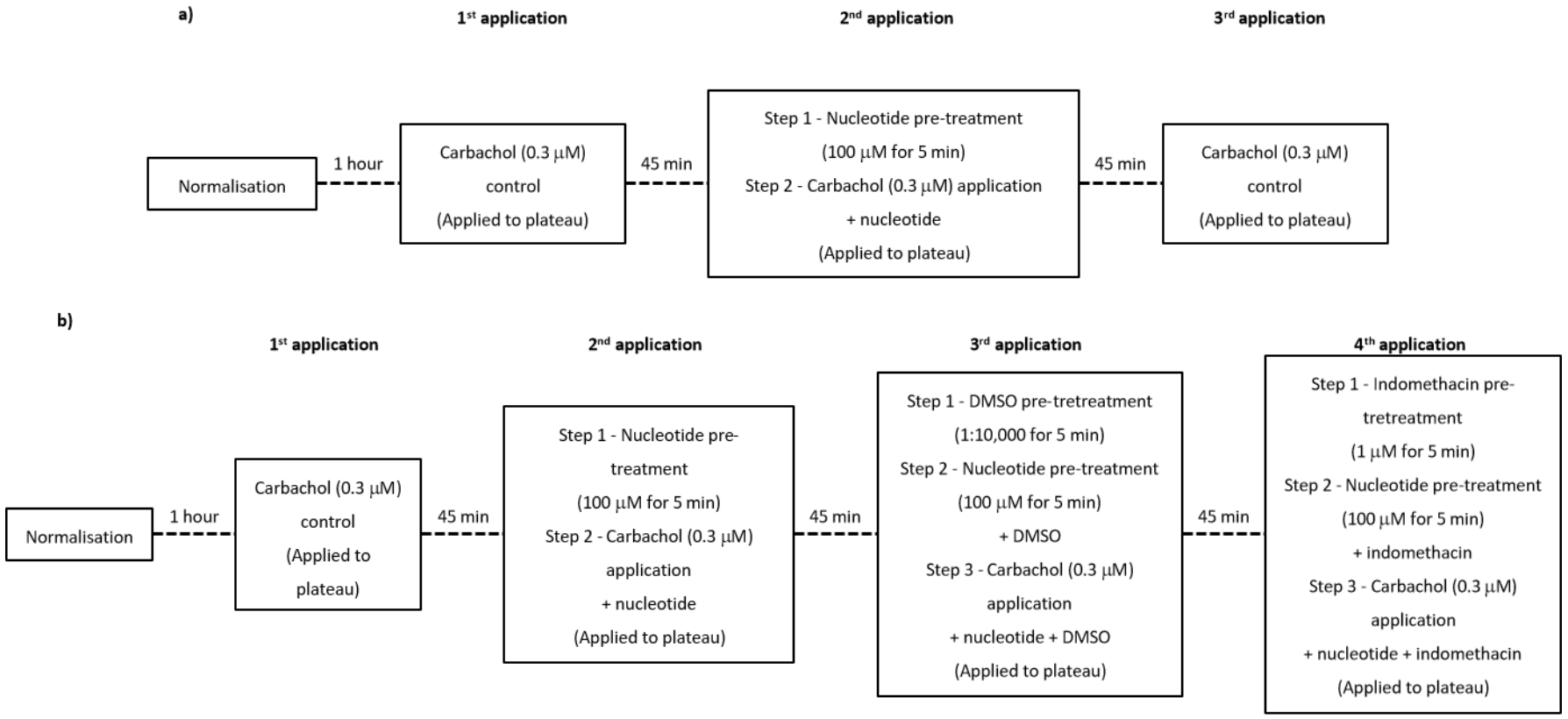

Figure 5.4. Schematic of initial myography protocols.

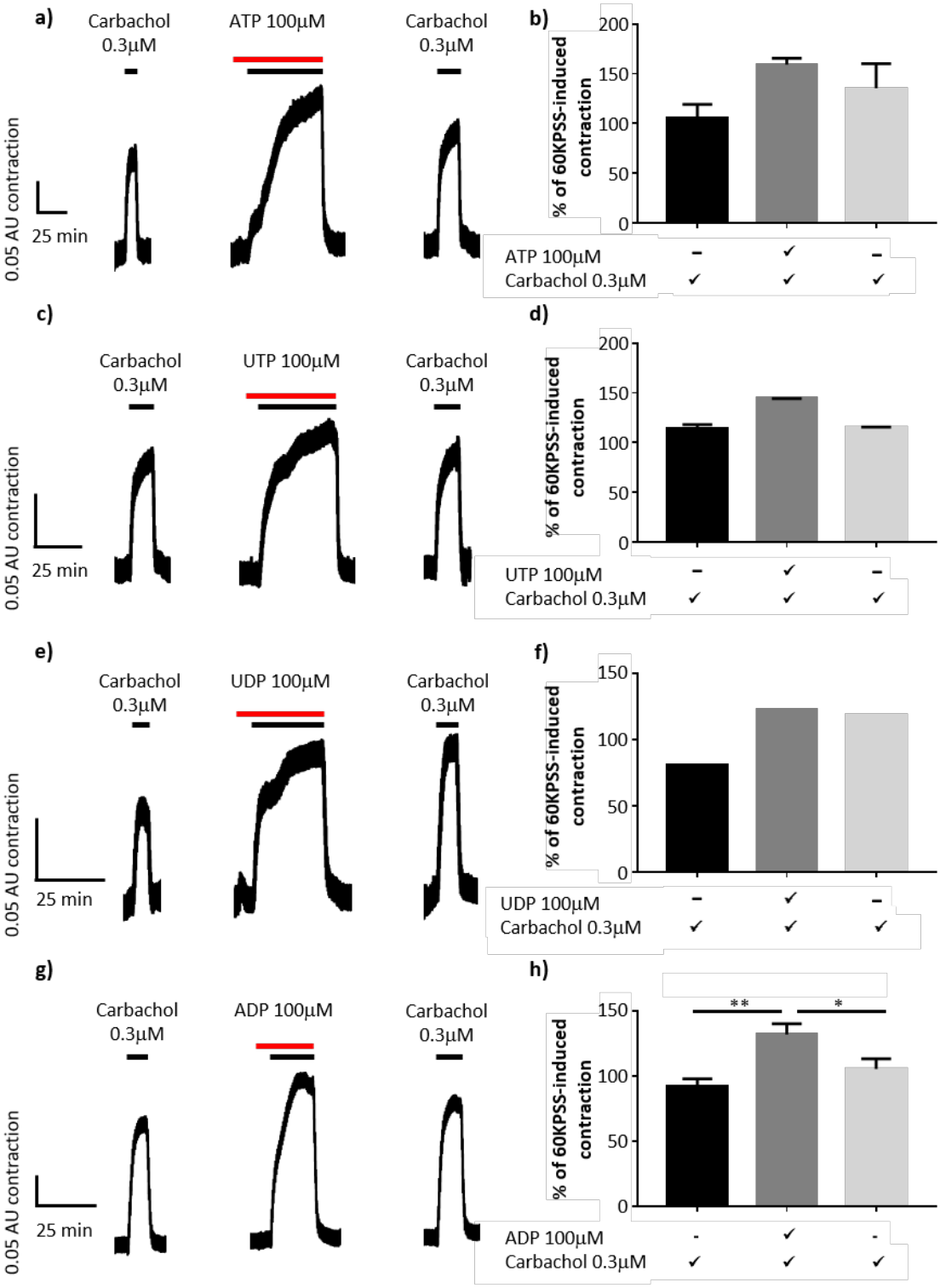

Figure 5.5. Pre-treatment of mouse trachea segments with ADP (100 µM) significantly potentiates carbachol-induced contractions.

*Representative contraction profiles induced in mouse trachea segments by carbachol (0.3µM) ± pre-treatment with a) ATP, c) UTP, e) UDP or g) ADP (all at 100µM), measured by wire myography. Summary of the peak amplitude of contractions of mouse trachea segments induced by carbachol 0.3µM ± pre-treatment with b) ATP (n=2), d) UTP (n=2), f) UDP (n=1) and h) ADP (n=8) (all at 100µM). Applications were made 45 min apart as outlined in figure 5.4. Horizontal lines represent drug application durations as indicated. Data are expressed as a % of the 60KPSS-induced contraction. Bars represent mean data ± SEM. Statistical significance was determined by one-way ANOVA followed by Sidak's multiple comparison test (*P<0.05, **P<0.01).*

5.2.1.1.5 Pre-treatment of mouse trachea with indomethacin (1 μM) does not affect ADP (100 μM)-potentiated carbachol-induced contraction

In section 5.2.1.1.4, ADP (100 μM) was shown to significantly potentiate carbachol (0.3 μM)-induced contractions of mouse trachea segments. However, the contraction profile changed and the time for the contraction to reach its peak amplitude appeared to increase. As extracellular nucleotides have previously been linked to ASM relaxation through P2Y2 receptor activation and the subsequent COX-mediated prostaglandin release (Marcet et al., 2007), further investigations were performed to determine if ADP modifies the carbachol-induced contraction of mouse trachea segments in a COX-mediated manner. The protocol in this section was performed according to figure 5.4b.

The pre-treatment of mouse trachea segments with ADP (100 μM) significantly increased the peak amplitudes of carbachol (0.3 μM)-induced contractions from 92.0±6.1% to 132.2±8.2% of the 60KPSS-induced contraction (n=8, P=0.006). ADP pre-treated contractions were not significantly affected by further pre-treatment with DMSO (1:10,000) as a vehicle control (138.0±12.7%) (n=8, P=0.973). The pre-treatment of mouse trachea segments with indomethacin (1 μM), a COX pathway inhibitor (Blanco et al., 1999), did not significantly affect the peak amplitudes of the ADP pre-treated carbachol (0.3 μM)-induced contractions, increasing them to 154.8±11.2% of the 60KPSS-induced contraction when compared to vehicle control responses (n=8, P=0.094). Compared to the carbachol control, the pre-treatment of mouse trachea segments with indomethacin (1 μM) and then ADP (100 μM) significantly increased carbachol-induced contractions (n=8, P=0.005). However, pre-treatment of mouse trachea segments with indomethacin (1 μM) did not further increase ADP potentiated carbachol-induced contractions compared to control responses without indomethacin (n=8, P=0.054). These data are summarised in figure 5.6, panels a and b. These data suggest that neither DMSO nor indomethacin have a significant effect on ADP-potentiated carbachol-induced contraction of mouse trachea, and therefore that the effect of ADP upon carbachol-induced contraction is not mediated by the COX pathway.

Interestingly, the time to peak contraction induced by carbachol (0.3 μM) differed with each condition tested. Control carbachol-induced contractions reached

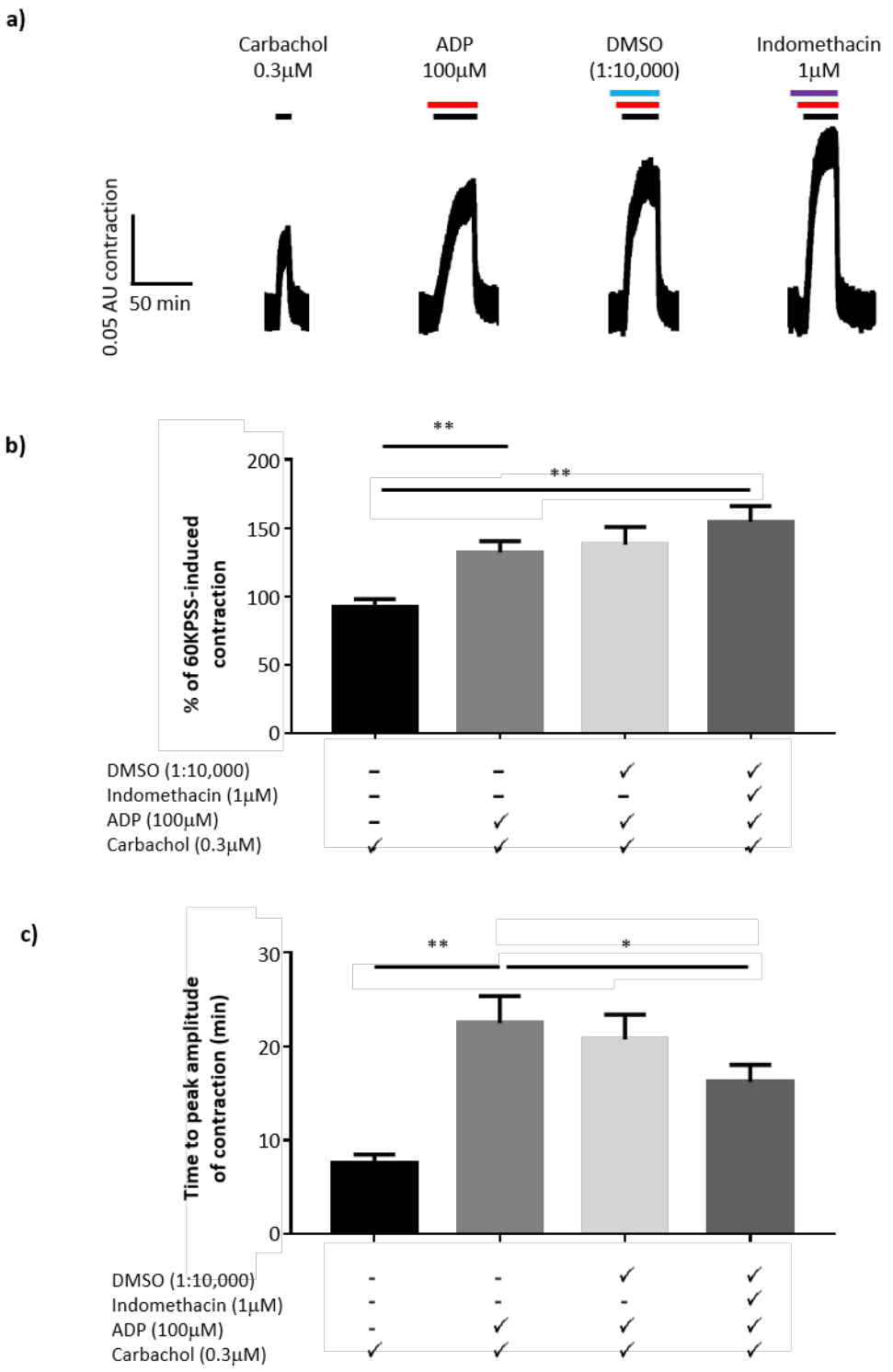

Figure 5.6. The effect of ADP (100µM) pre-treatment of mouse trachea segments on carbachol-induced contractions.

a) Representative contraction profiles induced in mouse trachea segments by carbachol (0.3µM) ± pre-treatment with ADP (100µM) in addition to indomethacin (1µM) or its vehicle control, DMSO (1:10,000). b) Summary of the peak amplitudes of the contractions induced in mouse trachea segments by these applications, measured by wire myography (n=8). c) Summary of the time taken for the contractions induced by these applications to reach their peak amplitudes (n=8). Applications were made 45 min apart as outlined in figure 5.4. Horizontal lines represent drug application durations as indicated. Data are expressed as a % of the 60KPSS-induced contraction. Bars represent mean data ± SEM. Statistical significance was determined by one-way ANOVA followed by Sidak's multiple comparison test (P<0.05, **P<0.01).*

their peak amplitudes in 7.6±0.9 min and, when pre-treated with ADP (100 μM), this time increased significantly to 22.5±2.9 min (n=8, P=0.004). Further pre-treatment of mouse trachea segments with DMSO (1:10,000) as a vehicle control did not significantly modify ADP pre-treated carbachol-induced contractions, which reached their peak amplitudes in 20.8±2.6 min (n=8, P=0.950). When mouse trachea segments were pre-treated with indomethacin (1 μM) followed by ADP (100 μM), carbachol-induced contractions reached their peak amplitudes in 16.2±1.8 min (n=8). Although there was a significant difference when this response was compared to ADP-potentiated carbachol-induced contractions (P=0.029), there was no significant difference when compared to vehicle control contractions (n=8, P=0.123). These data are summarised in figure 5.6, panels a and c. These data suggest that ADP significantly increases the time it takes for carbachol-induced contractions to reach their peak amplitudes. However, neither DMSO nor indomethacin significantly modify this effect any further.

5.2.1.1.6 Effect of ATP (100 μM) pre-treatment of mouse trachea segments on carbachol-induced contraction

The data presented in chapter 4 suggested that the functional P2 receptor subtypes expressed in cmT-ASM cells from mice were P2X7, P2Y1 and P2Y2 receptors, and that P2Y2 receptor was activated by ATP, UTP, ADP and UDP. Although only pre-treatment with ADP, and not ATP, UTP or UDP, appeared to significantly potentiate carbachol-induced contractions of mouse trachea in section 5.2.1.1.4, the preliminary data suggested that with ATP, UTP and UDP pre-treatment might follow the same trend bearing in mind the small sample sizes. As each nucleotide activates the same receptor, P2Y2 receptor, this could add support to this argument. Therefore, experiments were performed to clarify if ATP affects carbachol-induced contractions of mouse trachea and, if an effect was observed, to determine if it was a result of P2Y2 receptor activation. ATP was chosen as it is a widely recognised agonist of P2Y2 receptor (von Kügelgen, 2006), unlike ADP.

In section 5.2.1.1.4, drug applications were made 45 min apart. However, the peak amplitude of contractions of mouse trachea segments induced by carbachol (0.3 μM) did not return to their original magnitude following nucleotide pre-treatment. This suggests that contractions could still be affected by previous applications. Furthermore,

nucleotide pre-treatment increased the time taken for contractions to reach their peak amplitudes, potentially due to prostaglandin release (see section 5.2.1.1.5). To circumvent these issues, 2 hours were left between each application. Also, carbachol controls were applied for approximately the same time that it took for nucleotide pre-treated carbachol-induced contractions to reach their peak amplitudes (~30 min). The schematic of this protocol is summarised in figure 5.7a.

When all applications were made for 30 min, pre-treatment of mouse trachea segments with ATP (100 μM) for 5 min significantly potentiated the peak amplitudes of carbachol-induced contractions from 112.8±9.2% to 163.1±11.1% of the 60KPSS-induced contraction (n=8, P=0.021). When carbachol (0.3 μM) was reapplied following this, the peak contraction amplitude decreased to 146.5±9.1% of the 60KPSS-induced contraction (n=8, P=0.068). The data suggested that the second carbachol-induced contraction was increased compared to the first (P=0.069), although this increase was not significant at this sample size (figure 5.8).

When mouse trachea segments were pre-treated with the P2Y2 receptor-selective antagonist AR-C 118925XX (10 μM) for 5 min before ATP (100 μM) pre-treatment for the same duration, the peak amplitudes of carbachol (0.3 μM)-induced contraction decreased to 119.4±17.46% of the 60KPSS-induced contraction compared to when trachea segments were pre-treated with ATP (100 μM) alone (n=8, P=0.060), although this reduction was not significant at this sample size (figure 5.8). These data suggest that the pre-treatment of mouse trachea segments with ATP (100 μM) significantly potentiates carbachol (0.3 μM)-induced contractions, but it cannot be confirmed at this time whether this effect is due to P2Y2 receptor activation or not.

When two 30 minute applications of carbachol (0.3 μM) were performed with 2 hours rest between the applications, the peak amplitudes of mouse trachea segment contractions measured 135.9±7.2% initially and then 135.4±3.6% of the 60KPSS-induced contractions (n=3, P=0.913) (figure 5.9). The schematic of this protocol is summarised in figure 5.7b. These data demonstrate that reapplication of carbachol (0.3 μM) to mouse trachea segments does not affect the amplitudes of the contraction. Although additional carbachol applications are required to provide control data for the above experiments in their whole parts, these data suggest that the apparent potentiation of

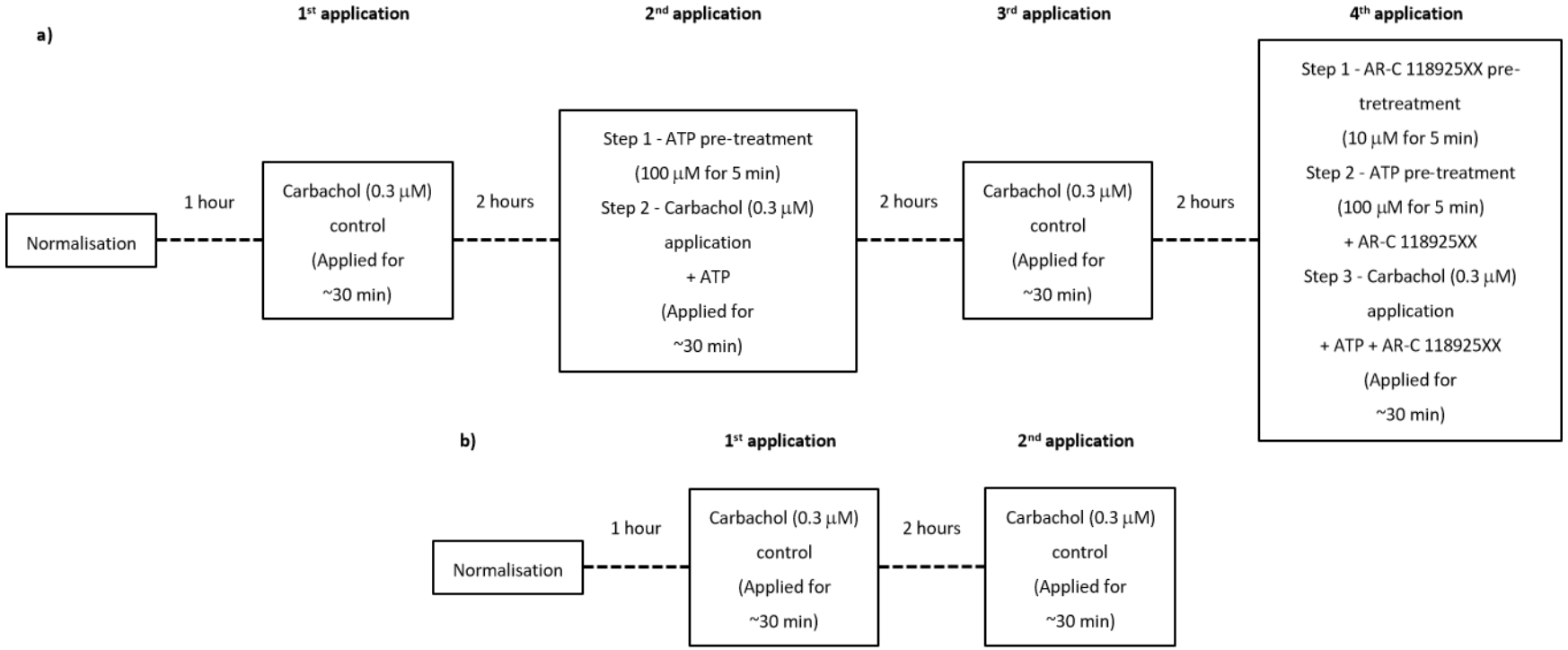

Figure 5.7. Schematic of final myography protocols.

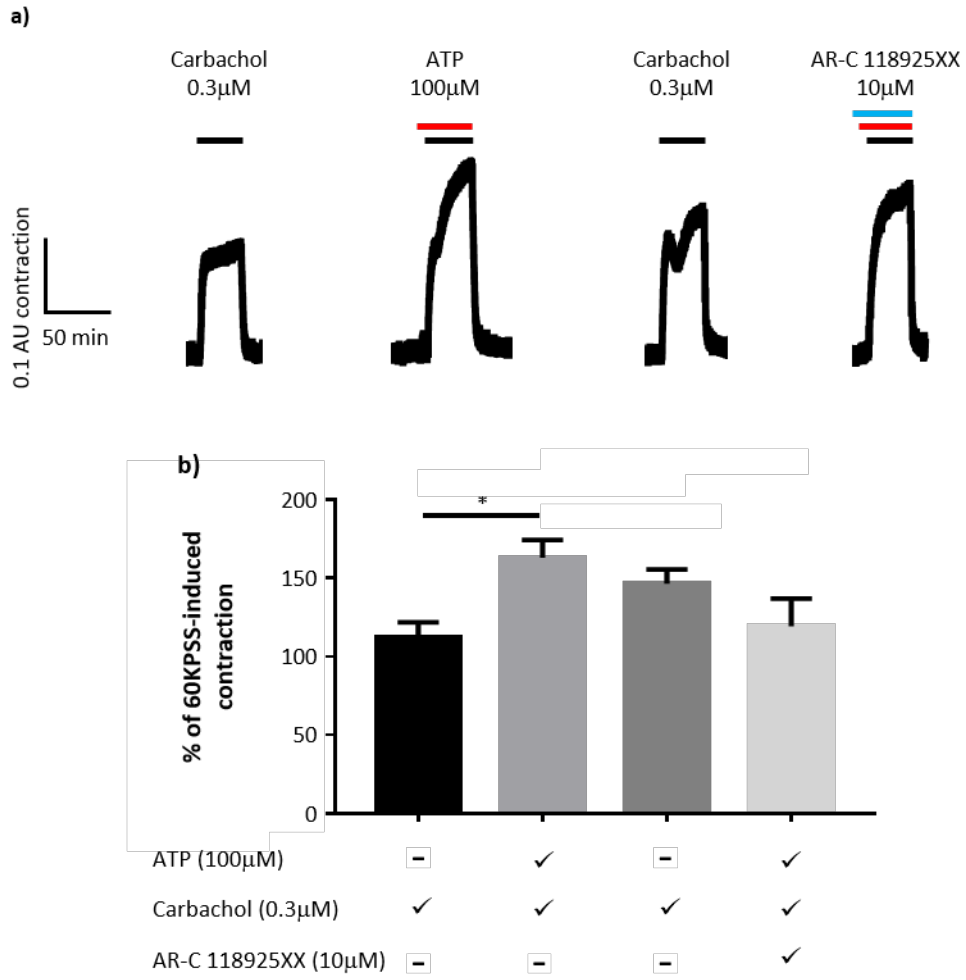

Figure 5.8. Pre-treatment of mouse trachea segments with ATP (100 µM) significantly potentiates carbachol-induced contractions.

*a) Representative contraction profiles induced in mouse trachea segments by carbachol (0.3µM) ± pre-treatment with ATP (100µM) in addition to the P2Y2-selective antagonist AR-C 118925XX (10µM). b) Summary of the peak amplitudes of the contractions induced in mouse trachea segments by carbachol (0.3µM) ± pre-treatment with ATP (100µM) in addition to AR-C 118925XX (10 µM) (n=8). Applications were made 2 hours apart for approximately 30 min as outlined in figure 5.7. Horizontal lines represent drug application durations as indicated. Data are expressed as a % of the 60KPSS-induced contraction. Bars represent mean data ± SEM. Statistical significance was determined by one-way ANOVA followed by Sidak's multiple comparison test (*P<0.05).*

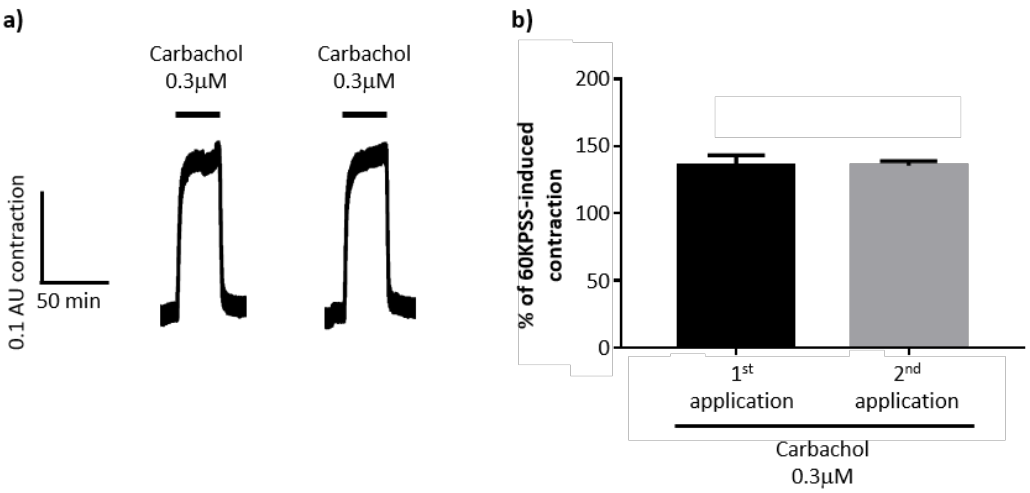

Figure 5.9. Repeated application of carbachol to mouse trachea segments for 30 minutes does not affect the peak amplitudes of contraction.

a) Representative contraction profiles induced in mouse trachea segments by the repeated application of carbachol (0.3µM) for 30 min. b) Summary of the peak amplitudes of the contractions induced in mouse trachea segments by repeated 30 min applications of carbachol (0.3µM), measured by wire myography (n=3). Applications were made 2 hours apart for approximately 30 min as outlined in figure 5.7. Horizontal lines represent drug application durations as indicated. Data are expressed as a % of the 60KPSS-induced contraction. Bars represent mean data ± SEM. Statistical significance was determined by student's paired t-test.

carbachol-induced contractions by ATP (comparing the second response to the first) is due to the effects of ATP itself.

5.2.1.2 The effect of extracellular nucleotides on cHASM cell contraction

As discussed in the introduction to this chapter, the contraction of cHASM cells was studied indirectly using a collagen-gel contraction assay. cHASM cells were seeded into collagen gels and the areas of the gels were measured at different time-points to investigate whether the added compounds had an effect upon cHASM cell contraction. To do this, the area of each gel (all conditions tested at all time-points) was normalised to the area of the H_2O control gels, averaged from the measurements at -10 and -5 min. This gave a single baseline of the gel areas from which all gel areas could be compared, which was important considering that some gels were pre-treated with nucleotides at -10 min. It also allowed the observation of the effect of H_2O application upon the gel area.

To determine if a compound had an effect on cHASM cell contraction, AUC analyses were performed for each condition and compared to the H_2O control. This allowed all time-points to be considered, and prevented any potential bias of data by selecting one single time-point, such as the endpoint, for analysis. Therefore, the data presented in this section are given as the AUC of each condition in AU.

5.2.1.2.1 The effect of extracellular nucleotides on the contraction of cHASM cells from a healthy donor

H_20 (0.2%), bradykinin (1 nM) and ATP, UTP, ADP and UDP (nucleotides all at 100 μM) were applied to cHASM cells from one healthy donor and contraction was measured by collagen gel-contraction assay. When analysed using AUC analyses, the contraction curves for H_20 and bradykinin had areas of 1078.0 and 1006.0, respectively (n=1). The curves for ATP, UTP, ADP and UDP had areas of 1078.0, 1077.0, 1068.0 and 1079.0, respectively (n=1) (figure 5.10).

Bradykinin (1 nM) was also applied to the cells with and without pre-treatment of the cells with each extracellular nucleotide at 100 μM for 10 min. AUC analysis of the contraction curves measured the areas at 984.0, 996.5, 940.2 and 911.4 for bradykinin-induced contraction of cells pre-treated with ATP, UTP, ADP and UDP, respectively (n=1 for all) (figure 5.11). Conclusions cannot be drawn at this time

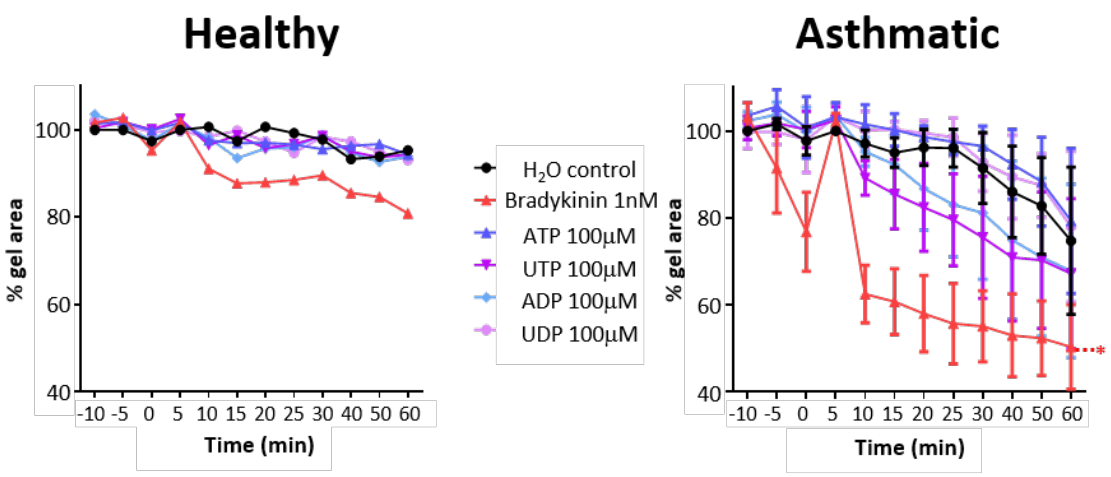

Figure 5.10. Extracellular nucleotides do not induce significant contractions of cHASM cells from healthy or asthmatic donors.

*The contraction of cHASM cells (passages 2-5) from both healthy (n=1 for all) and asthmatic (n=3 for all) donors was measured by collagen gel-contraction assay. The compounds tested include H_2O (0.2%) which acted as a diluent control, bradykinin (1nM) which acted as a positive control, and ATP, UTP, ADP and UDP (each at 100µM). Data are expressed as a % gel area compared to the H_2O control averaged at -10 and -5 min. Bars represent mean data ± SEM. Statistical significance was determined by one-way ANOVA (P=0.034) followed by Dunnett's multiple comparison test, comparing the average AUC for each compound to that of the H_2O control (*P=0.021).*

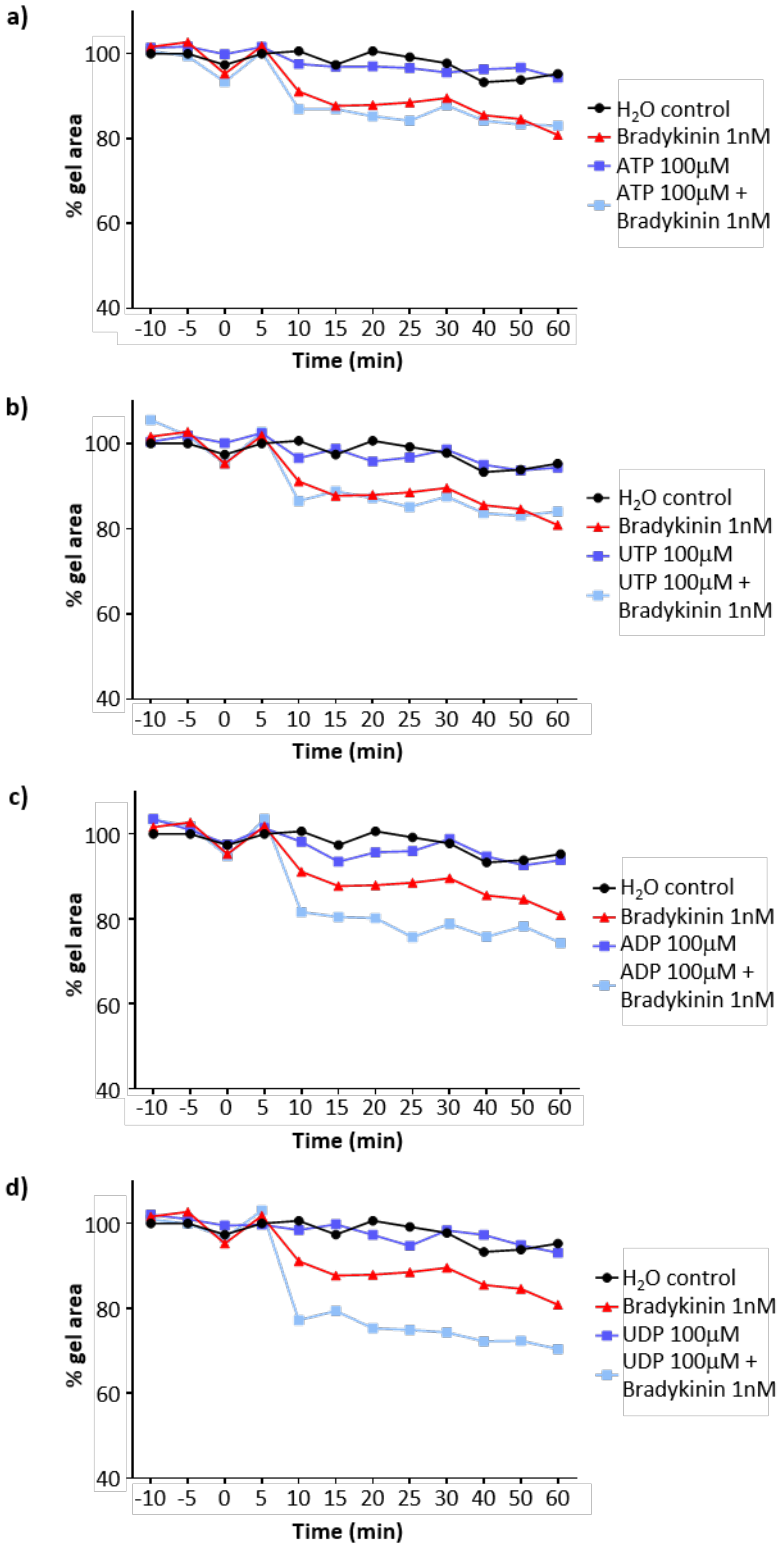

Figure 5.11. The effect of extracellular nucleotide pre-treatment of cHASM cells from a healthy donor on bradykinin-induced contraction.

The contraction of cHASM cells (passages 2-5) from a healthy donor was measured by collagen gel-contraction assay. A proportion of cells were pre-treated with either ATP, UTP, ADP and UDP (n=1 for all) (each at 100µM) for 10 min before bradykinin (1nM) addition in the presence of the respective nucleotide. Data are expressed as a % gel area compared to the H_2O control averaged at -10 and -5 min.

regarding the effect of these compounds on the contraction of cHASM cells from healthy donors due to low sample sizes.

5.2.1.2.2 The effect of extracellular nucleotides on the contraction of cHASM cells from asthmatic donors

H_20 (0.2%), bradykinin (1 nM) and ATP, UTP, ADP and UDP (at 100 μM each) were applied to cHASM cells from asthmatic individuals and contraction was measured by collagen gel-contraction assay. When analysed using AUC analyses, the averaged contraction curves for H_20 and bradykinin had areas of 1031.0±51.9 and 744.4±78.3, respectively (n=3). The curves for ATP, UTP, ADP and UDP had areas of 1076.0±21.5, 941.9±74.0, 976.3±85.5 and 1058.0±40.0, respectively (n=3 for all) (figure 5.10). These data suggest that bradykinin (P=0.021) but not ATP (P=0.676), UTP (P=0.229), ADP (P=0.518), or UDP (P=0.683), significantly induced contraction of cHASM from asthmatic donors.

Bradykinin (1 nM) was also applied to the cells with and without pre-treatment of the cells with each extracellular nucleotide at 100 μM for 10 min. AUC analysis of the contraction curves measured the areas at 772.7±85.6, 776.9±79.1, 814.7±65.4 and 860.0±87.6 for bradykinin-induced contraction of cells pre-treated with ATP, UTP, ADP and UDP, respectively (n=3 for all) (figure 5.12). Compared with the application of bradykinin (1 nM) without pre-treatment, these data suggest that pre-treatment of cHASM cells from asthmatic individuals with ATP (P=0.658), UTP (P=0.905), ADP (P=0.176) and UDP (P=0.252) does not potentiate bradykinin (1 nM)-induced contraction.

5.2.2 Preliminary study on the effect of extracellular nucleotides on cultured ASM cell proliferation

5.2.2.1 UTP (100 μM) induces cmT-ASM cell proliferation

cmT-ASM cells (passage 2) were counted after incubation of compounds in the presence of either 0.5% (n=5 for all) or 10% (n=3 for all) FBS. These data are summarised in figure 5.13 and table 5.1. When applied in the presence of 0.5 % FBS, none of the tested compounds appeared to have an effect on cell number. Furthermore, when the compounds were applied in the presence of 10% FBS, only histamine (P=0.038) which was used here as a positive control for ASM cell proliferation (Panettieri et al., 1990),

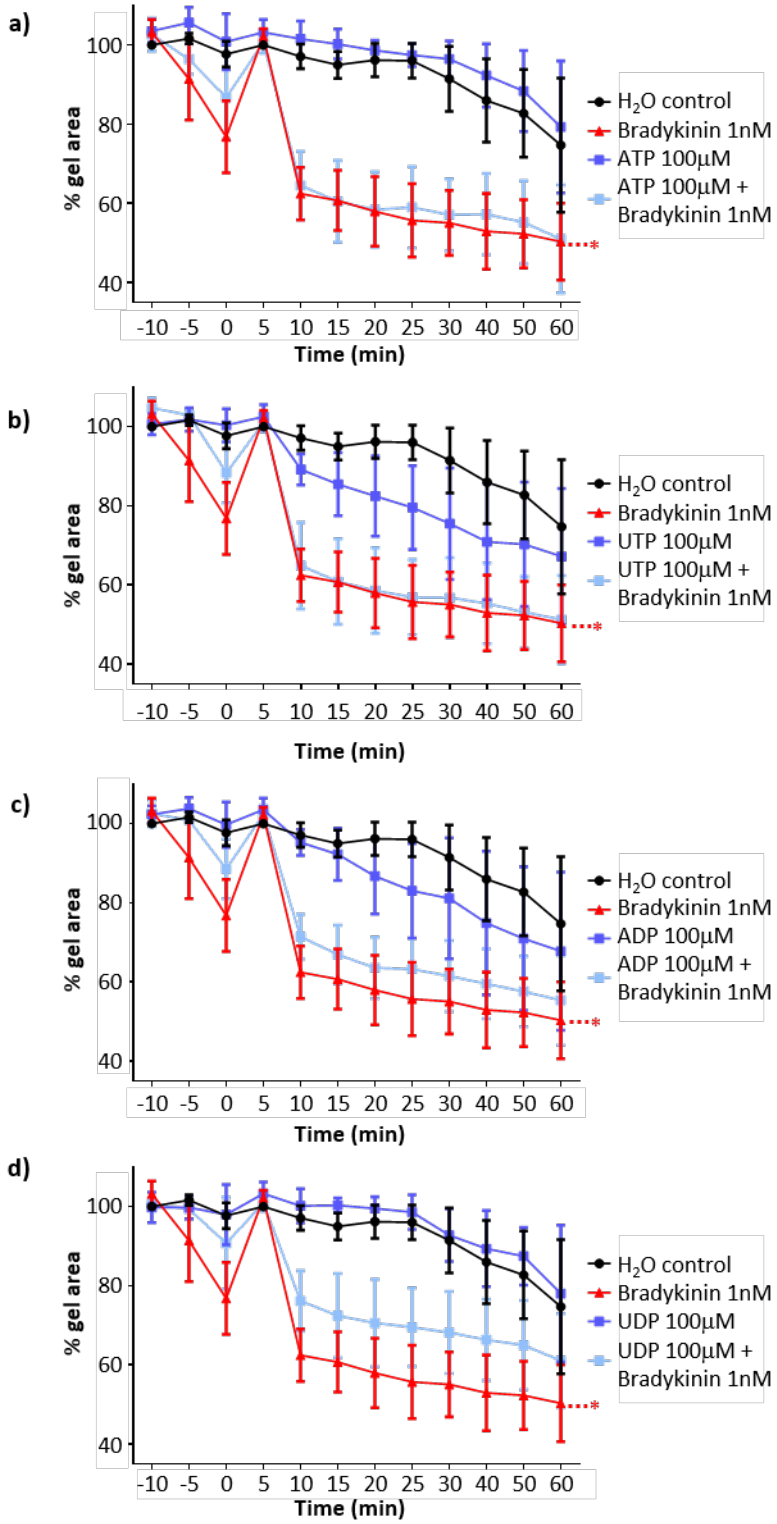

Figure 5.12. The effect of extracellular nucleotide pre-treatment of cHASM cells from asthmatic donors on bradykinin-induced contraction.

*The contraction of cHASM cells (passages 2-5) from a healthy donor was measured by collagen gel-contraction assay. A proportion of cells were pre-treated with either ATP, UTP, ADP and UDP (n=3 for all) (each at 100µM) for 10 min before bradykinin (1nM) addition in the presence of the respective nucleotide. Data are expressed as a % gel area compared to the H_2O control averaged at -10 and -5 min. Bars represent mean data ± SEM. Statistical significance was determined by one-way ANOVA (P=0.022, 0.025, 0.007 and 0.026 for panels a-d, respectively) followed by Sidak's multiple comparison test using the average AUC for each compound (*P=0.025 compared to H_2O control).*

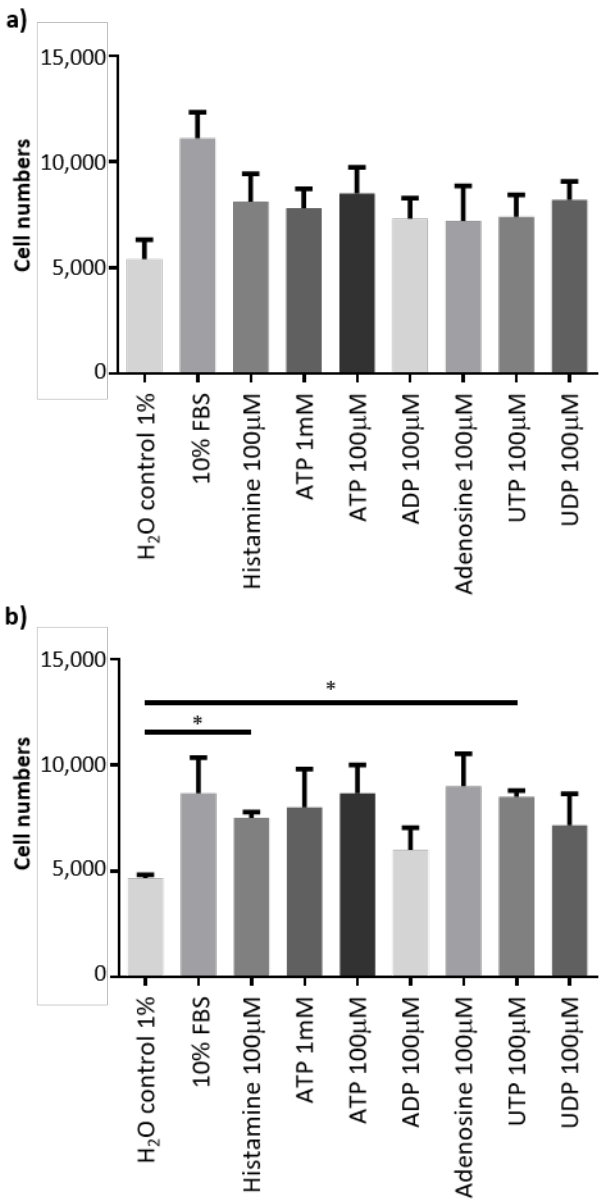

Figure 5.13. The effect of extracellular nucleotides on cmT-ASM cell proliferation.
cmT-ASM cells (passage 2) were serum-starved before treatment with one application of the tested compounds either in the presence of a) 0.5% (n=5 for all) or b) 10% FBS (n=3 for all). Data are expressed as cell numbers. Bars represent mean data ± SEM. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test, excluding the 10% FBS positive control for each group (P<0.05).*

Compound/treatment	Concentration	Number of cells in well when compounds applied in the presence of:	
		0.5% FBS	10% FBS
H_2O control	1%	5,400±914	4,667±167
FBS	10%	11,100±1,229	8,667±1,691
Histamine	100μM	8,100±1,336	7,500±289 *
ATP	1mM	7,800±930	8,000±1,803
ATP	100μM	8,500±1,235	8,667±1,333
ADP	100μM	7,300±982	6,000±1,041
Adenosine	100μM	7,200±1,655	9,000±1,528
UTP	100μM	7,400±1,042	8,500±289 *
UDP	100μM	8,200±875	7,167±1,481

Table 5.1. Summary of the effect of extracellular nucleotides on cmT-ASM cell proliferation. *cmT-ASM cells (passage 2) were serum-starved before treatment with one application of the tested compounds either in the presence of 0.5% (n=5 for all) or 10% FBS (n=3 for all). Data are expressed as cell numbers ± SEM. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test, excluding the 10% FBS positive control for each group (* P<0.05).*

and UTP (P=0.021) (both at 100 μM) showed significant differences in cell numbers compared to diluent controls of H_2O (1%). The 10% FBS positive control was removed from statistical analyses due to its large effect, so as to not mask any potential differences induced by other compounds. However, the more physiologically-relevant positive control of histamine was still included in these analyses.

None of the compounds appeared to have an effect on the number of dead cells present, as measured by trypan-blue exclusion (figure 5.14).

5.2.2.2 Extracellular nucleotides do not regulate the proliferation of cHASM cells from either healthy or asthmatic donors

cHASM cells (passages 2-5) from healthy and asthmatic donors were counted after incubation with compounds in the presence of either 0.5% (n=3 for all) or 10% (n=4 for all) FBS. These data are summarised in figure 5.15 and table 5.2. None of the compounds tested appeared to have an effect on the proliferation of cHASM cells from either healthy or asthmatic donors when incubated in the presence of either 0.5% or 10% FBS. The 10% FBS positive control was removed from statistical analyses due to its large effect, so as to not mask any potential differences induced by other compounds. However, the more physiologically-relevant positive control of histamine was still included in these analyses.

None of the compounds appeared to have an effect on the number of dead cells present, as measured by trypan-blue exclusion (figure 5.14).

5.3 Discussion

In this chapter, we investigated the contribution of P2 receptors to typical ASM physiological roles, including cell contraction and proliferation. The contractions of excised mouse trachea segments were measured using wire myography due to their relatively large size compared to smaller airways, making them easier to mount without damaging the segments. cHASM cells (passages 2-5) were used in collagen gel-contraction assays due to the lack of available fresh human airway samples, and as cHASM cells were readily available and had been used for previous experiments in this study. cmT-ASM (passage 2) and cHASM cells (passages 2-5) were used for proliferation assays. Although previous experiments in this study investigated the function of P2

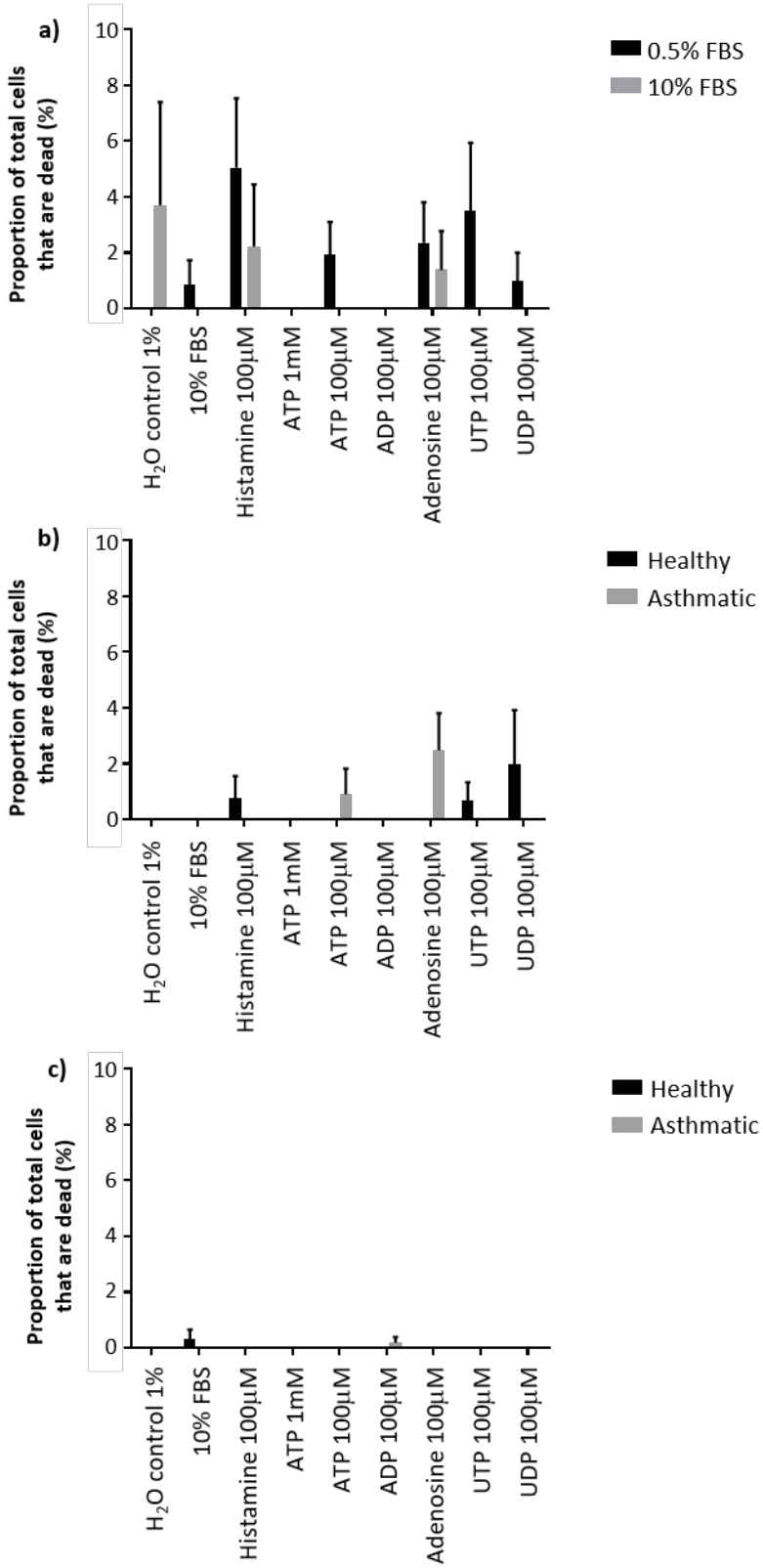

Figure 5.14. The effect of extracellular nucleotides on the number of dead cells counted in proliferation experiments.

The effect of compounds on the proportion of dead cells counted when compounds are applied to a) cmT-ASM cells (passage 2) in the presence of either 0.5% FBS (dark bars) (n=5 for all) or 10% FBS (light bars) (n=3 for all), and to cHASM cells from both healthy (dark bars) and asthmatic (light bars) donors in the presence of b) 0.5% FBS (n=3 for all) and c) 10% FBS (n=4 for all). Data are expressed as % of total cells counted that were dead. Bars represent mean data ± SEM.

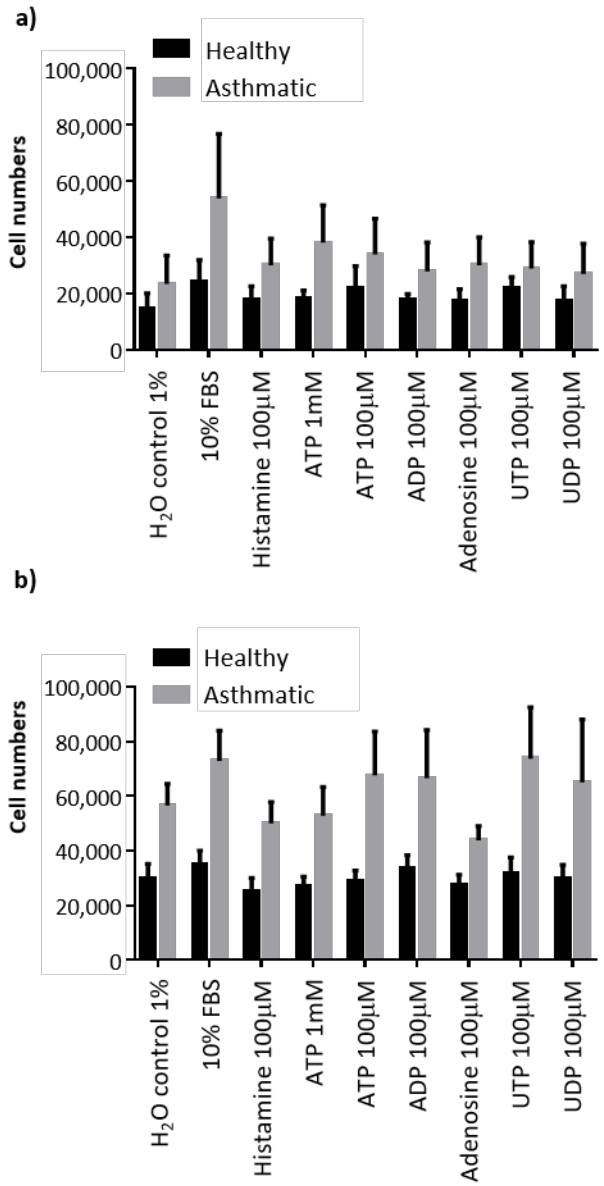

Figure 5.15. The effect of extracellular nucleotides on the proliferation of cHASM cells from healthy and asthmatic individuals.

cHASM cells (passages 2-5) from healthy (dark bars) and asthmatic (light bars) individuals were serum-starved before treatment with one application of the tested compounds either in the presence of a) 0.5% (n=3 for all) or b) 10% FBS (n=4 for all). Data are expressed as cell numbers. Bars represent mean data ± SEM. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test, excluding the 10% FBS positive control for each group.

Compound/treatment	Concentration	Number of cells in well when compounds applied in the presence of:			
		0.5% FBS		10% FBS	
		Healthy	Asthmatic	Healthy	Asthmatic
H_2O control	1%	14,833±5,183	23,333±10,105	30,000±5,264	41,500±7,896
FBS	10%	24,000±7,858	53,667±22,995	35,000±5,087	72,875±11,205
Histamine	100μM	18,000±4,537	30,000±9,438	25,375±4,719	50,000±7,850
ATP	1mM	18,333±2,744	38,000±13,326	27,000±3,571	52,875±10,491
ATP	100μM	22,000±7,697	33,833±12,729	29,000±3,780	67,625±16,113
ADP	100μM	18,000±1,893	27,833±10,305	33,500±4,873	66,500±17,707
Adenosine	100μM	17,500±4,041	30,000±9,929	27,750±3,544	43,875±5,238
UTP	100μM	21,833±3,941	28,833±9,387	31,875±5,778	73,750±18,813
UDP	100μM	17,500±5,058	26,833±10,856	29,875±4,964	65,000±23,158

Table 5.2. Summary of the effect of extracellular nucleotides on the proliferation of cHASM cells from healthy and asthmatic individuals.
cHASM cells (passages 2-5) from healthy and asthmatic individuals were serum-starved before treatment with one application of the tested compounds either in the presence of 0.5% (n=3 for all) or 10% FBS (n=4 for all). Data are expressed as cell numbers ± SEM. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test, excluding the 10% FBS positive control for each group.

receptors in cmT-ASM cells at passage 1, cmT-ASM cells were passaged an additional time in order to give sufficient cells for use with this experiment.

The normalization of mouse trachea segments was important to not only ensure that data can be compared between experiments, but that data is physiologically relevant. To our knowledge, the tension that mouse trachea is under in physiological conditions is unknown. The contraction of excised mouse trachea has been investigated using a number of approaches in previous studies, including myography using wires or pins, and other isometric force contraction assays where a weight is attached to the sample for normalization [from 0.4 g (Li et al., 1998) and 0.5 g (Moffatt et al., 2009) to 2g (Stengel et al., 2000)]. Normalization forces applied to trachea segments also range from 0.8 mN (Adner et al., 2002, Zhang et al., 2005, Zhang et al., 2007) and 1 mN (Srivastava et al., 2013) to even 10 mN (van Heuven-Nolsen et al., 1997, Semenov et al., 2012). The rationale behind the use of these tensions or forces were not discussed in these studies. Therefore, for our experiments, a previously published approach was taken (Adner et al., 2002) whereby 60KPSS was applied to mouse trachea segments over a range of tensions. The high [K^+] of 60 mM in this buffer depolarises the smooth muscle cell membrane, opening VDCCs and inducing contraction (Karaki et al., 1984). The resting tension that gave the biggest 60KPSS-induced contraction, 0.8 mN, was chosen for use in our myography experiments. This value agrees with published data using the same approach to normalize mouse trachea segments (Adner et al., 2002, Zhang et al., 2005, Zhang et al., 2007). Due to its contractile effect, 60KPSS application was used as a positive control in these experiments. Carbachol, which is a synthetic derivative of choline and therefore is related to acetylcholine, was also used as a more physiologically relevant positive control in myography experiments (Fryer and Jacoby, 1998, Stamper et al., 2009).

The application of the extracellular nucleotides ATP, UTP, ADP and UDP (each at 100 μM) did not appear to induce contraction of mouse trachea segments. In the first instance, these findings do not agree with previously published data. ATP and UTP have been shown to contract isolated guinea-pig trachea at concentrations up to 100 μM, after which a relaxation effect is seen (Fedan et al., 1993), and P2X1 receptor specifically has been implicated in the contraction of isolated guinea-pig trachea (Candenas et al., 1992). In addition, P2X4 receptor activation has been associated with the contraction of

porcine ASM (Nagaoka et al., 2009). However, these differences in observations could be due to species differences. Another study implicated P2Y2/4 receptors in the contraction of murine ASM specifically (Bergner and Sanderson, 2002), yet these findings were obtained using agarose-embedded lung slices from the BALB/C strain of mouse whilst this study has used trachea from C57BL6 mice. Therefore, differences in mouse strain and the location of the tested tissue along the respiratory tree could explain these differences. There is also the possibility that the nucleotides are degrading before they act upon the ASM, or that the high concentrations of nucleotides used in these experiments are desensitizing the functional P2 receptors before a physiological response can be initiated. To eliminate this possibility, non-hydrolysable nucleotide analogues could be used in future experiments in addition to nucleotides over a wider range of concentrations. However, it is also plausible that the activation of the functional P2 receptors in murine ASM simply does not increase $[Ca^{2+}]_i$ to the threshold required to induce contraction. To investigate this, the $[Ca^{2+}]_i$ changes induce by the nucleotides could be compared with those of a contractile agonist, such as carbachol or bradykinin. As described, Ca^{2+} imaging experiments performed using cmT-ASM cells in chapter 4 used bradykinin as a positive control and as a reference against which $[Ca^{2+}]_i$ could be normalised. It would have been ideal to compare any potential contractile responses induced by the nucleotides to that by bradykinin, but bradykinin induces relaxation of mouse trachea and not contraction (van Heuven-Nolsen et al., 1997, Li et al., 1998). Therefore, future experiments could measure the $[Ca^{2+}]_i$ induced by carbachol to determine if the application of nucleotides induces a change in $[Ca^{2+}]_i$ sufficient to trigger contraction.

Inflammatory cells such as mast cells are known to release bronchoconstrictors like histamine (Bradding et al., 2006, Wang and Lau, 2006) in addition to nucleotides, including ATP (Wang et al., 2013). As extracellular nucleotides (Kitazawa et al., 1991a, Kubota et al., 1992) and P2 receptors (Oguma et al., 2007, Yu et al., 2013) have been implicated in the sensitization of ASM contraction (mechanisms outlined in section 1.2.4.4), nucleotides released as part of the inflammatory response could result in potentiated ASM contraction. This could have implications for inflammatory airway diseases such as asthma, as the concentration of ATP in the airways of asthmatics appears to be increased (Idzko et al., 2007). Therefore, to test whether P2 receptor

activation sensitizes ASM contraction, cHASM cells (see later in this section) and mouse trachea segments were pre-treated with extracellular nucleotides before the induction of contraction. Here, mouse trachea segments were pre-treated with extracellular nucleotides (100 μM) for 5 min before the application of carbachol, a known ASM bronchoconstrictor (Fryer and Jacoby, 1998). Pre-treatment was performed at 5 min as a standard starting point for these experiments, which also maintained similar conditions across experiments. However, it is important to remember for the interpretation of these data that any functional P2 receptors present are likely to desensitize during this time. Furthermore, nucleotide application, in addition to pre-treatment of the trachea segments with nucleotides, was performed at 100 μM. This concentration allowed the comparison of these physiological measurements with those data obtained from Ca^{2+} imaging experiments in chapter 4. However, it is plausible that these high concentrations could facilitate the desensitization of P2 receptors and, in fact, mask any observable responses. Future experiments could make use of a wider range of concentrations. The EC_{50} of carbachol in mouse trachea was first determined for use in these experiments to allow the observation of extracellular nucleotide-mediated potentiation and inhibition of mouse trachea contraction. Our data suggests that the EC_{50} of carbachol in mouse trachea is ~0.3 μM, which appears to agree with current literature (Sausbier et al., 2006, Bonvin et al., 2008, Semenov et al., 2012).

Pre-treatment of mouse trachea segments with ADP (100 μM) significantly increased carbachol (0.3 μM)-induced contractions. It could be argued that pre-treatment with ATP, UTP and UDP (each at 100 μM) had the same effect, although these data were limited by small sample sizes and so conclusions regarding the effects of these 3 nucleotides cannot be made. The reapplication of carbachol induced a contraction whose peak amplitude was significantly reduced compared to that of the ADP pre-treated contraction, although data suggested that the final carbachol-induced contraction was increased slightly compared to the first. Though performed in different conditions, the reapplication of carbachol did not affect the amplitude of contractile responses (figure 5.9). Although only two control carbachol applications were performed here and so these data cannot provide information regarding the second carbachol applications in figure 5.5, these data suggest that the increase in contraction

amplitude observed following ADP pre-treatment are due to the effect of the nucleotides themselves and so that pre-treatment of mouse trachea segments with ADP (100 μM) potentiates carbachol-induced contraction. However, in addition to increasing n numbers for these experiments, further control experiments should be performed in the future to clarify this point, including repeated applications (minimum of 3 total applications) of carbachol according to the initial myography schematic as indicated in figure 5.4.

It must be noted that the carbachol-induced contraction response profiles appeared to change when pre-treated with each nucleotide to become slower, albeit reaching a higher magnitude. Our data in chapter 5 suggested that the functional P2 receptor subtype expressed in cmT-ASM cells which responds to all 4 of the tested nucleotides is P2Y2 receptor. As the activation of epithelial P2Y2 receptors is associated with prostaglandin release (Marcet et al., 2007) and the relaxation of ASM it could be argued that, whilst pre-treatment of the trachea segments potentiates the carbachol-induced contraction, the activation of P2Y2 receptors on the epithelium or the ASM itself releases PGE_2 which counteracts the contractile response, slowing the contraction and increasing the time taken for the potentiated contraction to reach its peak amplitude. Further experiments were performed using the COX pathway inhibitor, indomethacin (1 μM), to test this hypothesis. The pre-treatment of mouse trachea segments with indomethacin had no significant effect on the peak amplitudes of the contractions induced by carbachol when potentiated by ADP. Although a trend suggests that indomethacin increases the peak amplitudes of these contractions, additional experiments must be performed to clarify this. Furthermore, although there was a significant reduction in the time to peak amplitude of contraction when trachea segments were pre-treated with indomethacin followed by ADP compared to controls without indomethacin, there was no significant difference when the response was compared to the appropriate vehicle control. Therefore, these data suggest that indomethacin does not significantly modify ADP-potentiated carbachol-induced contractions, although the n numbers in this data set should be increased in future. In addition, more control experiments are required, including i) 4 successive applications of carbachol, and ii) the sequence of the applications 'carbachol followed by ADP pre-treatment' should be performed twice.

In the above experiments, applications were made until contraction responses plateaued, and 45 min was left between applications. However, as discussed above, final carbachol contractions did not return to their original amplitude. This suggested that responses could be affected by previous applications. Therefore, the time left between applications was increased to 2 hours. Published data suggests that repeating carbachol (1 μM) applications, with 1 hour between each application, does not induce statistically different contractions (Trevisani et al., 1999). Therefore, waiting for 2 hours between applications should ensure that contractions are not affected by previous applications. Pre-treatment of mouse trachea with extracellular nucleotides also increased the time taken for contractions to reach their peak amplitudes. To ensure that the additional time during applications did not itself result in larger contractions, all applications in future experiments were made for the same time (~30 min). Using these conditions, the reapplication of carbachol (0.3 μM) resulted in contractions whose peak amplitudes were not significantly different from each other, suggesting that any potentiation would be due to the nucleotide pre-treatment itself. Additional applications should be implemented here to provide essential control data for myography experiments presented in this chapter.

The data presented in chapter 4 suggested that the functional P2 receptor subtypes expressed in cmT-ASM cells from mice were P2X7, P2Y1 and P2Y2 receptors, and that P2Y2 receptor was activated by ATP, UTP, ADP and UDP. ADP pre-treatment significantly potentiated carbachol-induced contractions of mouse trachea segments as indicated previously. Although ATP, UTP and UDP pre-treatment did not have a significant effect on carbachol-induced contractions due to small sample sizes, the data could suggest that they had a similar effect to ADP. If all 4 nucleotides were activating P2Y2 receptor as our previous data indicated, this potentiation could be due to P2Y2 receptor. Therefore, experiments were performed to determine if P2Y2 receptor was involved in the observed potentiation effect. However, experiments were first performed to clarify if ATP affected carbachol-induced contractions of mouse trachea and, if an effect was observed, to determine if it was a result of P2Y2 receptor activation. ATP was chosen as it is a widely recognised agonist of P2Y2 receptor (von Kügelgen, 2006), unlike ADP. Using the same conditions as outlined in figure 5.7, the pre-treatment of mouse trachea segments with ATP (100 μM) significantly increased carbachol (0.3

μM)-induced contractions. Therefore, mouse trachea segments were pre-treated with the P2Y2-selective antagonist, AR-C 118925XX (10 μM) prior to pre-treatment with ATP. Our data demonstrates that, at this sample size, AR-C 118925XX had no significant effect on the peak amplitudes of contraction compared to controls performed without AR-C 118925XX. However, a trend suggests that AR-C 118925XX reduced the amplitude of ATP-potentiated carbachol-induced contractions. In future experiments, these experiments should be repeated to increase sample sizes. Furthermore, although DMSO did not have a significant effect on ADP-potentiated contractions (figure 5.6), appropriate vehicle controls should be performed here to clarify that DMSO does not affect ATP-potentiated contractions. Additional control experiments concerning the repeated application of carbachol should also be performed, with at least 4 total applications, to determine if the reapplication of carbachol itself affects peak contraction amplitudes. Although P2Y2 receptor itself has not been implicated in the sensitization of ASM contraction, other P2 receptors have been, including P2X1 and P2Y6 receptor (Oguma et al., 2007, Yu et al., 2013).

When the contraction of cHASM cells was investigated indirectly using collagen gel-contraction assays, AUC analyses were performed for each condition tested. A decrease in the area of the gel over time is reflected by a decrease in the AUC. This approach was taken so that all recorded time points could be included in the analysis. Single applications of ATP, UTP, ADP and UDP (100 μM) were made to cHASM cells from asthmatic donors and a single healthy donor. Due to the small sample size, conclusions cannot be drawn from the data for the healthy donor. By comparing the AUC, bradykinin (1 nM) induced a significant contraction in cHASM cells from asthmatic donors, although none of the tested nucleotides tested did so. This finding is not in line with current literature as P2X receptors are thought to contribute to the contraction of human intra-pulmonary bronchi (Mounkaila et al., 2005), and ATP and UTP (100 μM) have been shown to contract cHASM cells from healthy individuals (Govindaraju et al., 2005). Increasing the number of donors would clarify this point further. Furthermore, it is possible that the nucleotides could be broken down throughout the experiment and so opposing effects due to the activation of several P2 receptors, or even adenosine

receptors, could be complicating observable results. In future experiments, non-hydrolysable nucleotide analogues, such as ATPγS, could be used to prevent this issue.

To our knowledge, P2 receptors have not been shown to regulate the sensitization of HASM cell contraction. However, preliminary experiments were performed here to investigate the effect of cHASM cell pre-treatment with extracellular nucleotides on bradykinin (1 nM)-induced contractions. Pre-treatment was performed for 10 min to ensure that compounds could diffuse through the gel and exact any effects before bradykinin addition. Again, donor numbers were limited for these experiments and so conclusions cannot be drawn concerning the data from the healthy donor tested. By comparing the AUC measurements of extracellular nucleotide pre-treated bradykinin-induced contractions to bradykinin controls in cHASM cells from asthmatic donors, the pre-treatment of cells with extracellular nucleotides did not significantly affect the induced contractions. Our data could suggest that the pre-treatment of cells with ADP and UDP (100 μM) reduces bradykinin-induced contraction. Experiments must be repeated using more donors to allow appropriate statistical tests and conclusions to be drawn from these experiments. Furthermore, experiments should be performed to determine the EC_{50} of bradykinin in these cells using this approach as bradykinin (1 nM) could be inducing such a strong contraction that any effect of extracellular nucleotides on bradykinin-induced contraction cannot be discerned.

Preliminary experiments regarding the effect of extracellular nucleotides on ASM proliferation were performed using a manual counting approach adapted from Michoud *et al*. (2002). Compounds were added in single applications in the presence of either 0.5% or 10% FBS in order to investigate the potential of each compound to induce or inhibit proliferation, respectively. 10% FBS was used as a positive control for these experiments, but this condition was not included in statistical analyses to allow the smaller effects of the other tested compounds to be discerned. Instead, histamine (100 μM) was used as a physiologically-relevant positive control due to its reported effect of stimulating the proliferation of ASM cells from canines (Panettieri et al., 1990) and humans (Maruno et al., 1995).

In cmT-ASM and cHASM cells, no discernible differences were observed when cells were incubated with any compound in the presence of 0.5% FBS. This could be due to a lack of nutrients and growth factors in the media due to the reduced FBS content.

In cmT-ASM cells, only histamine and UTP (100 μM) significantly increased cell numbers compared to the diluent control of H_2O (1%) when compounds were incubated in the presence of 10% FBS. Error bars appear to be smaller for these two conditions compared to the others, reflecting more consistent and less variable data. To confirm that the other compounds do not affect cell proliferation, the sample size should be increased. In cHASM cells from both healthy and asthmatic donors, none of the compounds had any significant effect on cell numbers, including the positive control histamine. It could be that conditions for these experiments require further optimisation, and particularly so for use with cHASM cells as Michoud *et al*. (2002) used ASM cells cultured from rat trachea. The cell density that is initially plated could be titrated to ensure that cells are not overconfluent, resulting in cell death. Although compounds did not appear to increase the number of dead cells present in the well when cells were counted, it could be that when supernatants were removed and discarded before trypsin was added to each well, the dead cells were discarded too. Therefore, if the same approach were to be used for future experiments, any live or dead cells in the supernatants should also be counted. Furthermore, some studies investigating the effects of extracellular nucleotides on cell proliferation use more than one application in order to maximise the effect of the nucleotide, particularly as nucleotides are prone to degradation (Dixon et al., 1997, Roedersheimer et al., 2011). This approach could be adopted for future experiments. In addition, non-hydrolysable nucleotide analogues could also be used to prevent degradation of compounds. There are limited published data regarding the effects of extracellular nucleotides on ASM cell proliferation. In cultured rat ASM cells, UTP increases cell proliferation in a concentration dose-dependent manner up to 100 μM (Michoud et al., 2002), which shows similarities with our finding that UTP application to cmT-ASM cells in the presence of 10% FBS significantly increased cell numbers compared to diluent controls. However, in the same study, ATP increased cell proliferation only up to concentrations of 10 μM and inhibited cell proliferation at 100 μM.

There are several limitations to the experiments performed in this chapter. The main limitation was that sample sizes were not increased to acceptable levels. For example, only one healthy donor was tested during experiments regarding cHASM cell

contraction. Furthermore, the sample sizes for myography experiments must be increased to allow conclusions to be drawn for the effect of all nucleotides tested. In addition, the experimental protocol should be fully optimised and used for all experiments, and further control experiments should be performed. It would also be ideal to perform myography experiments using small human airways so that contraction data regarding HASM cells was not limited to cHASM cell use. This would allow a more direct measurement of HASM cell contraction without any potential effects of cell culture.

In the future, experiments need to be completed by increasing sample sizes. Furthermore, cell proliferation experiments need to be optimised and used in conjunction with other assay types, such as those that measure the rate of DNA replication like ^{3}H-thymidine-based assays, in order to corroborate any findings derived from manual counting assays.

6 Discussion

Asthma is defined as a heterogeneous disease that is typically characterized by chronic airway inflammation (GINA, 2017) and has several hallmark pathophysiological features including mucous hypersecretion, airway oedema and AHR. AHR is a classic sign of the dysfunctional ASM that is present in asthmatic airways, yet its underlying causes are not fully understood. P2 receptors, receptors for extracellular nucleotides, regulate key functions of several types of smooth muscle, including contraction (Mulryan et al., 2000, Vial and Evans, 2000a, Vial and Evans, 2002, Yamamoto et al., 2006, Yu et al., 2014) and proliferation (Coutinho-Silva et al., 2005). In ASM specifically, several studies have indicated that these receptors can regulate contraction (Fedan et al., 1993, Bergner and Sanderson, 2002, Govindaraju et al., 2005, Mounkaila et al., 2005, Nagaoka et al., 2009) and proliferation (Michoud et al., 1997, Michoud et al., 1999, Michoud et al., 2002, Abbracchio et al., 2006). Interestingly, the concentration of the nucleotide ATP (a natural agonist for many subtypes of P2 receptors) is elevated in the airways of asthmatics compared to healthy controls (Idzko et al., 2007). Therefore, we hypothesised that P2 receptors are functionally expressed in ASM where they regulate physiological functions including cell contraction and proliferation, and that they contribute to the dysfunction of ASM observed in asthma. This thesis aimed to perform a comprehensive study of P2 receptor function in ASM from mice and human and, by comparing P2 receptor function in cHASM cells from both healthy and asthmatic individuals, to determine if P2 receptor dysfunction could contribute to asthma pathogenesis.

Our data from chapter 3 established the P2 receptor transcript expression profiles in mouse lung, ASM from mouse trachea and cHASM cells from healthy and asthmatic donors using qPCR. These data confirmed that P2 receptors are expressed in ASM. Key data from these experiments showed that, in the above samples, the relative expression of P2X1 receptor transcripts was low compared to other P2 receptor subtypes whilst that of P2X4 and P2X7 receptors was high.

Comparing data generated from mouse and human samples highlighted differences in P2 receptor expression between the two species. The main difference in P2 receptor expression between mouse and human ASM samples were that P2Y4 receptor transcripts are expressed in cHASM cells but could not be confidently detected

in murine airways. Comparing data generated from cHASM cells from both healthy and asthmatic individuals could reflect differences due to the presence of an asthmatic phenotype. However, there were no significant differences in P2 receptor transcript expression between those cells cultured from healthy and asthmatic HASM. These data could suggest that differences in P2 receptor expression are not at the core of ASM dysfunction in asthma. However, these data were generated using cultured cells and not native tissues, and so further experimentation would be required to clarify this.

This study also made use of ASM cells cultured from mouse trachea. Our data demonstrated that these cells can be phenotypically characterised as ASM cells, and that culturing these cells to passage 1 generally decreased the relative P2 receptor expression transcript levels compared to those in fresh T-ASM strips, but that the expression profiles were very similar despite this. Protein expression investigations in chapter 3 demonstrated that P2X1, P2X4 and P2X7 receptor subtypes are expressed in airway samples from both mouse and human without any observable differences in expression levels between mouse and human samples, or between cHASM cells from healthy and asthmatic donors. Furthermore, the expression of P2X1, P2X4 and P2X7 receptors co-localised to the ASM in mouse airways (trachea and lungs). Although the expression of select P2 receptors in ASM has been previously determined (Michoud et al., 2002, Govindaraju et al., 2005, Nagaoka et al., 2009, Gui et al., 2011), an investigation into the expression of all P2 receptor subtypes has not previously been performed in ASM from any species in a single study. Therefore, these data from WT mice and cHASM cells from healthy and asthmatic individuals are all novel. Furthermore, P2 receptor expression has not previously been compared between samples from healthy and asthmatic HASM, and our data is the first to indicate that P2 receptor expression is similar in both healthy and asthmatic HASM.

Data regarding P2 receptor function in chapter 4 were generated using cmT-ASM cells (passage 1) and cHASM cells from both healthy and asthmatic donors (passages 2-5) and investigated the functional expression of P2 receptor subtypes which regulate $[Ca^{2+}]_i$ in these cells. Key data suggested that P2X7, P2Y1 and P2Y2 receptors were functionally expressed in cmT-ASM cells. Functional P2Y2 receptor was identified in cHASM cells from both healthy and asthmatic donors, whilst functional P2X4 receptor was identified in cHASM cells from asthmatic but not healthy individuals. These data

suggest that cmT-ASM cells express more functional P2 receptor subtypes than cHASM cells, which could be due to species differences or to the lower passage numbers used in experiments. A significant difference in the UDP-induced $[Ca^{2+}]_i$ rises was observed between cHASM cells from healthy and asthmatic individuals, yet in both cells types these responses were attributed to P2Y2 receptor. Therefore, these data could suggest that differences in functional P2 receptor expression do not contribute to ASM dysfunction in asthma, although experiments with freshly isolated ASM cells from both healthy and asthmatic individuals should be performed to confirm this. Extracellular nucleotides, and so P2 receptors, have been previously implicated in the regulation of $[Ca^{2+}]_i$ in ASM (Michoud et al., 1997, Bergner and Sanderson, 2002, Govindaraju et al., 2005, Nagaoka et al., 2009). Yet, to our knowledge, no single study has previously investigated and compared the functional expression of P2 receptors in ASM from both mice and humans. Therefore, the identification of the functional P2 receptor subtypes in murine and human ASM, and the differences in functional expression between these tissues, is novel and could have implications for those studies which use murine models to inform P2 receptor function in HASM. Furthermore, the comparison of the functional P2 receptor subtypes in cHASM cells from both healthy and asthmatic donors has not previously been performed, and so our conclusion that P2 receptors are functionally similar between healthy and asthmatic HASM is novel.

Investigations into the roles of P2 receptors in ASM contraction used wire myography in conjunction with mouse trachea segments and collagen gel-contraction assays with cHASM cells from both healthy and asthmatic individuals. Early data suggested that whilst the application of extracellular nucleotides to mouse trachea segments did not induce contractions *per se*, the pre-treatment of mouse trachea with extracellular nucleotides could potentiate carbachol-induced contractions by mechanisms as outlined in section 1.2.4.4, and that this effect may be mediated by P2Y2 receptor. Furthermore, the application of nucleotides to mouse trachea may have activated a COX-mediated relaxation pathway which worked against the contractions induced by carbachol. This pathway could be induced following the activation of P2Y2 receptors on either the ASM or the epithelium (Marcet et al., 2007). Our data also suggested that the application of extracellular nucleotides did not induce contraction of cHASM cells *per se*, and that extracellular nucleotides do not regulate ASM contraction.

However, further experiments (including key control experiments) are required in order to confirm these findings in HASM. It could be concluded at this early stage of our investigation that P2 receptor activation does not induce ASM contraction, and that P2 receptors could be involved in the sensitization of ASM contraction in mice but not humans. Although P2X1 receptor has previously been implicated in the sensitization of guinea-pig ASM contraction (Oguma et al., 2007), the implication of P2Y2 receptor in the sensitization of murine ASM contraction is a novel finding.

Our data also suggested that UTP, but none of the other tested nucleotides, may increase cmT-ASM cells proliferation (passage 2) whilst extracellular nucleotides do not regulate the proliferation of cHASM cells from either healthy or asthmatic individuals. However, further experimental optimisation is necessary to confirm these findings.

Therefore, data from this thesis demonstrate that P2 receptors are functionally expressed in ASM. P2X7, P2Y1 and P2Y2 receptors are functional in cmT-ASM cells. P2Y2 receptors are functionally expressed in cHASM cells from both healthy and asthmatic donors whilst P2X4 receptor is functional in cHASM cells from asthmatic, but not healthy, donors. Furthermore, extracellular nucleotides do not induce the contraction of ASM *per se*, although P2 receptors (namely P2Y2 receptors) may regulate the sensitization of murine ASM contraction, but not that of HASM. Additionally, the application of UTP regulates ASM proliferation in mice but not humans.

These data indicate that care must be taken when relating data regarding the roles of P2 receptors generated from murine models to studies using human samples. In addition, these data suggest that there may be no functional difference between P2 receptors in HASM from healthy or asthmatic individuals. It seems more likely that, although data presented here did not demonstrate an effect of extracellular nucleotides upon cHASM cell contraction or proliferation, potentially due to small sample sizes and the approaches used, a change in the local environment surrounding the ASM of asthmatics could explain ASM dysfunction in asthma pathogenesis. Namely, an increased concentration of extracellular nucleotides surrounding asthmatic ASM, as suggested by Idzko *et al*. (2007) (Idzko et al., 2007), could regulate ASM functions, possibly increasing ASM cell number and mass and even sensitizing the ASM so that, upon contact with a contractile stimulus, asthmatic ASM contracts with greater force.

As suggested throughout this thesis, each study that has investigated the expression, function and physiological roles of P2 receptors in ASM has focused on select receptors instead of performing a full, systematic study of P2 receptors in this tissue. As one nucleotide can activate several receptors, this approach is not optimal. This present study has provided the first full investigation into the functional P2 receptor subtypes expressed in cmT-ASM cells, and in cHASM cells from healthy and asthmatic donors. Although the data presented here suggests that there are no observable differences in the functional P2 receptor expression profiles between healthy and asthmatic donors, the data does indicate a role for P2 receptors in the sensitization of ASM contraction. Further studies could capitalize on this, perhaps performing a systematic investigation into the roles of P2 receptors in asthma pathogenesis using a mouse model of asthma. Furthermore, it would be ideal to repeat aspects of this study using freshly isolated human samples, including investigating the roles of P2 receptors in the regulation of small airway contraction in humans from healthy and asthmatic donors using myography. This work could potentially lead to a novel approach to reduce ASM hypercontractility in susceptible individuals, such as those with asthma, by antagonizing those P2 receptors involved in this process.

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