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Mechanisms of bronchoconstriction in the early allergic response

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Table of contents

1 Introduction 1

1.1 Asthma bronchiale 1

1.1.1 Epidemiology of asthma 1

1.1.2 Definition of asthma 1

1.1.3 Types of asthmatic responses after allergen exposure 1

1.1.3.1 The early allergic response (EAR) 2

1.1.3.2 The late phase response (LPR) 2

1.1.3.3 Airway hyperresponsiveness (AHR) 2

1.1.3.4 Airway remodeling 3

1.2 Inflammatory cells and mediators in asthma 3

1.2.1 Dendritic cells 3

1.2.2 Macrophages 3

1.2.3 T lymphocytes 4

1.2.4 B lymphocytes 5

1.2.5 Mast cells and its mediators 5

1.2.5.1 Preformed mediators 6

1.2.5.2 Newly synthesized mediators 6

1.2.5.3 Cytokines 7

1.2.6 Eosinophils 9

1.2.7 Neutrophils 9

1.2.8 Basophils 9

1.3 Signal transduction pathways of airway smooth muscle contraction 10

1.3.1 Ca^{2+}-dependent signal transduction pathway 11

1.3.1.1 Ca^{2+} channels in the plasma membrane 11

1.3.2 Ca^{2+} sensitization mechanism 12

1.4 Mechanism of airway relaxation 12

1.5 Models of asthma 13

1.5.1 Limitations to study asthma in humans 13
1.5.2 Animal models 14
1.5.2.1 Animals with naturally occurring recurrent airway obstruction 14
1.5.2.2 Animals showing AHR without provocation 14
1.5.2.3 Models of airway obstruction and AHR after allergen challenge 15
1.5.2.4 *In vitro* models in small mammalian species 18
1.5.2.5 The model of precision-cut lung slices (PCLS) 18
2 Aim of the study 20
3 Material and Methods 21
3.1 Animals 21
3.1.1 Rats 21
3.1.2 Mice 21
3.1.3 Guinea pigs 22
3.1.4 Monkeys 22
3.1.5 Pentobarbital solution 22
3.2 Human lung donors 22
3.3 Chemicals 24
3.4 Equipment 25
3.5 Methods 26
3.5.1 Precision -cut lung slices (PCLS) 26
3.5.1.1 Preparation of rat lung slices 26
3.5.1.2 Preparation of mouse lung slices 26
3.5.1.3 Preparation of guinea pig lung slices 26
3.5.1.4 Preparation of human lung slices 27
3.5.2 Culture medium 28
3.5.2.1 Incubation medium 28
3.5.2.2 Agarose solution 28
3.5.2.3 Slicing medium 28
3.5.3 Viability of the PCLS 29

3.5.3.1 Lactate dehydrogenase (LDH) release 29
3.5.3.2 Two-photon microscopy 29
3.5.4 Measurement and Imaging of broncho - or vasoconstriction 30
3.5.5 Passive-sensitization as a tool to study the EAR 30
3.5.5.1 Rat serum 30
3.5.5.2 Mouse serum 30
3.5.5.3 Guinea pig serum 31
3.5.5.4 Human serum 31
3.5.6 Pharmacological intervention studies 31
3.5.6.1 Mediators/ agonists and antagonists 31
3.5.6.2 Inhibitors of the signal transduction pathways 32
3.5.7 Airway relaxation in the guinea pig 32
3.5.8 Calcium imaging with two-photon microscopy 32
3.6 Statistics 33
4 Results 34
4.1 Part Ia: Establishment of guinea pig (GP) PCLS 34
4.1.1 Viability of GP PCLS 34
4.1.1.1 Lactate dehydrogenase-release 34
4.1.1.2 Two-photon microscopy 35
4.1.2 Mediator-induced bronchoconstriction in GP PCLS 35
4.1.3 Relaxation of precontracted airways 36
4.1.4 Airway and vascular responses within one slice 37
4.1.5 Induction of the EAR in GP PCLS 38
4.2 Part Ib: Data from monkey PCLS 38
4.2.1 Viability of monkey PCLS 38
4.2.2 Airway responses of monkey PCLS 39
4.3 Part II: The EAR in different species 40
4.3.1 C48/80 40

4.3.1.1 C48/80-induced bronchoconstriction 40
4.3.1.2 Viability of C48/80-treated PCLS 41
4.3.1.3 Mediators of the EAR induced by C48/80 42
4.3.2.1 Mediators of the allergen-induced EAR in rat PCLS 46
4.3.2.2 Mediators of the allergen-induced EAR in human PCLS 47
4.3.2.3 Mediators in the allergen-induced EAR in GP PCLS 48
4.3.3 Active sensitization 50
4.3.3.1 Airway hyperresponsiveness 51
4.3.3.1.1 Mice: Airway hyperresponsiveness 51
4.3.3.1.2 Rats: Airway hyperresponsiveness 52
4.4 Part III: Mediator signal cascades during the EAR 53
4.4.1 Signaling in human PCLS 53
4.4.2 Signaling in GP PCLS 55
4.4.3 Mediator-induced bronchoconstriction in murine PCLS 56
4.4.3.1 Airway responses in Maxi-K-channel knockout mice 57
4.4.4 Signaling in rat PCLS 58
5 Discussion 63
5.1 The model of guinea pig PCLS 63
5.2 The early allergic response in PCLS 65
5.3 PCLS from non-human primates 67
5.4 Mast cell degranulation 68
5.5 Airway hyperresponsiveness in PCLS 69
5.6 Mechanisms of bronchoconstriction 70
5.7 Role of the Maxi-K channel in bronchoconstriction 73
6 Summary 75
7 Deutsche Zusammenfassung 77
8 Reference List 79

Abbreviations

AHR	airway hyperresponsiveness
BAL	bronchoalveolar lavage
C48/80	Compound 48/80
COX	cyclooxygenase
CTMC	connective tissue-type mast cell
DAG	diacylglycerol
ELISA	enzyme-linked immunosorbant assay
EAR	early allergic response
EC_{50}	concentration leading to half maximal effect
EP-receptor	prostaglandin E-receptor
ET-1	endothelin-1
GM-CSF	granulocyte monocyte colony stimulating factor
GP	guinea pig
5-HT_2-receptor	5-hydroxytryptamine$_2$ (serotonin)-receptor
IP_3	inositol 1,4,5 trisphosphate
LDH	lactate dehydrogenase
LPR	late phase response
LTD_4	leukotriene D_4
MLC_{20}	myosin light chain 20 kDA
MLCK	myosin light chain kinase
MLCP	Myosin light chain phosphatase
MC_T	mast cell containing only tryptase
MC_{TC}	mast cell containing both: tryptase and chymase
MMC	mucosal-type mast cell
Ova	ovalbumin
PAF	platlet-activating factor
PCLS	precision-cut lung slices
PDE	phosphodiesterase
PE	pollen extract
PGD_2	prostaglandin D_2
PI-PLC	phosphatidylinositol specific phospholipase C
ROCC	receptor-operated Ca2+-channel
ROCK	Rho-kinase
SOCC	store-operated Ca2+-channel
TP-receptor	thromboxane-prostanoid receptor
VOCC	voltage-operated Ca2+-channel

1 Introduction

1.1 Asthma bronchiale

1.1.1 Epidemiology of asthma

Asthma bronchiale is one of the most important chronic diseases which occurs in 10% of the children and 5% of the adult population in Germany [1]. It is the most common chronic disease in infancy. 64% of german asthmatic individuals suffer from mild, 34% from moderate asthma, and 2% are affected by severe asthma. As most of the individuals receive medication, the costs increase exponentially with the degree of asthma. About 2 billion € are spent every year for the treatment and medication of the approximately 4 million asthmatics in Germany [2]. In central Europe one person out of 100 000 dies because of asthma [2]. Genetic predispositions are an important factor for the development of atopy, and several genes have now been identified [3]. In addition, environmental factors appear to be important in determining whether asthma develops in an atopic individual [2].

1.1.2 Definition of asthma

Asthma is defined on three levels: physiologically, pathologically, and clinically. Physiologically, asthma is an airway disease, characterized by airway obstructions with airflow limitations that are variable and reversible either spontaneously or with treatment, accompanied with an unspecific airway hyperresponsiveness [4]. Central to its development is a chronic inflammation of the airways. Pathologically, asthma is defined by multiple abnormalities in airway epithelium, lamina propria and submucosa [5]. Many different cell types play a role in this inflammatory disorder of the airways, in particular mast cells, eosinophils and T lymphocytes. Clinically, this inflammation causes recurrent episodes of wheezing, breathlessness, chest thightness, and cough, particularly at night or in the early morning in susceptible individuals. Various forms of asthma exist, e.g. extrinsic (allergic) asthma, intrinsic asthma, exercise-induced asthma, aspirin-sensitive asthma and occupational asthma, demonstrating that asthma is a very heterogeneous lung disease.

1.1.3 Types of asthmatic responses after allergen exposure

Apart from various forms of asthma, there are cardinal symptoms that are: the early allergic response, the late phase response, airway hyperresponsiveness, and airway remodeling.

1.1.3.1 The early allergic response (EAR)

In the lungs, the immediate response to allergens occurs within minutes, is triggered by an exposure to inhaled antigens or irritants and results in bronchoconstriction [6]. It is caused by specific allergens that cross-link IgE-molecules bound to the surface of mast cells. After this activation the mast cells degranulate and release a number of inflammatory mediators. Some mediators are preformed and stored in mast cells` cytoplasmatic granules, i.e. histamine, proteoglycans and serine proteases. Other mediators, e.g. certain lipid-derived substances including prostaglandins, cysteinyl leukotrienes, thromboxane and platelet-activating factor (PAF) are synthesized *de novo* after mast cell activation [7]. Histamine, prostaglandins, thromboxane, cysteinyl leukotrienes and PAF then cause an airway smooth muscle contraction, thereby narrowing the airways.

1.1.3.2 The late phase response (LPR)

The early allergic response is followed 4 - 6 hours later by a late phase response, which is defined by a second contraction of the airways and by infiltration of eosinophils, activated neutrophils, mast cells, T lymphocytes, and leukocytes into the tissue [6,8]. There is evidence from both clinical and animal studies that mast cell-derived mediators, in particular TNFα but also other cytokines, are responsible for the leukocyte recruitment [9-11]. The interaction of complementary adhesion molecules, expressed on the surface of leukocytes (e.g. VLA-4, CD11/18) and on vascular endothelial cells (e.g. VCAM-1, ICAM-1 and E-selectin (ELAM)), as well as the action of chemokines, contribute to leukocyte emigration into the lung during the late phase response [12-14]. These molecules are part of a complex mechanistic interplay *in vivo* and products from activated mast cells and many other cell types [13,14].

1.1.3.3 Airway hyperresponsiveness (AHR)

Airway hyperresponsiveness is an exaggerated airway narrowing in response to a variety of unspecific stimuli and an important characteristic of bronchial asthma. AHR can be measured, for example, by a bronchial provocation with histamine or methacholine. Compared to a non-asthmatic individual, the airways of an asthmatic patient respond stronger, indicated by a left shift of the dose-response curve [15-17].

1.1.3.4 Airway remodeling

The term "airway remodeling" refers to structural changes in the airways and is thought to occur as a consequence of chronic airway inflammation. These structural changes are characterized by a thickening of the airway wall which has been shown in pathologic as well as in radiographic studies [18,19]. This airway wall thickening may explain the incomplete reversibility of airway narrowing in asthmatic patients [20]. Hyperplasia of goblet cells coupled with hyperthrophy of submucosal glands and increased vascularity of the airway wall amplify the mucus secretion and plasma protein leakage, which are responsible for the formation of the characteristic mucus plugs that obstruct the airways [21,22]. Furthermore, smooth muscle hypertrophy and hyperplasia are characteristic features in airway remodeling as well as subepithelial fibrosis [23-25].

1.2 Inflammatory cells and mediators in asthma

In asthma, pulmonary immunity plays an important role with a variety of inflammatory cell types involved.

1.2.1 Dendritic cells

In the respiratory tract, dendritic cells are localized in the epithelium and act as antigen-presenting cells [26]. They have the capacity to bind allergens, process them into peptides and present them via the major histocompatibility complex II (MHC II) molecules on the cell surface to undifferentiated T lymphocytes [27]. The number of dendritic cells is increased in asthmatic patients [28], and they may initiate and sustain airway inflammation through enhanced expression of costimulatory molecules that facilitate T-cell activation and differentiation [29,30].

1.2.2 Macrophages

Macrophages are derived from blood monocytes. They perform several functions in the immune response. In asthma, they function in uptake, processing and presenting of antigens, and in the secretion of a variety of cytokines, arachidonic acid metabolites and proteases [31-33]. Macrophages may both enhance and diminish inflammation. Alveolar macrophages normally suppress inflammatory lymphocyte function, but this may be impaired in asthma after allergen exposure [34]. It has been shown that IL-10, which is one of the anti-inflammatory cytokines secreted by macrophages, is reduced in asthmatics [33]. The alveolar macrophages from these individuals are functionally and phenotypically activated [35,36], but up to now it is only poorly understood

what this activation means for the airway inflammation in asthma. The role of proinflammatory cytokines is described in 1.2.5.3.

1.2.3 T lymphocytes

T lymphocytes are classified by the molecules they express on their surface and by the cytokines they release. The surface molecules and cytokines mediate highly specified functions. T lymphocytes express either CD4 or CD8 surface markers in addition to the T cell receptor, which serve as co-receptors for antigen peptides presented by the MHC molecules on dendritic cells. The immune response in asthma is defined by $CD4^+$ T cells in the airway epithelium and the submucosa [37,38]. T lymphocytes of the Th2 phenotype dominate in asthmatic individuals. The differentiation of either Th1 or Th2 cells from a $CD4^+$ precursor T-helper cell (Th0) depends on the locally released cytokines during the process of antigen presentation from a dendritic cell to the Th0 cell. Infection with bacteria and viruses may promote the release of Interleukin-12 (IL-12) from dendritic cells and macrophages, which leads to the development of Th1 cells. Typical cytokines released from Th1 cells are interferon (IFNγ) and IL-12. IL-4, synthesized and released from mast cells, basophils, eosinophils and Th2 cells themselves, promote Th2 cell development. The cytokines (IL-4, IL-5, IL-9, IL-13) secreted by Th2 cells [39] are responsible for the recruitment and survival of eosinophils (IL-5), and for the maintenance of mast cells and stimulation of B Lymphocytes (IL-4, IL-13) in the airways. In atopic individuals an increase in activated T cells and Th2 cytokines after allergen challenge has been observed. An overview of the Th1/Th2 cell concept is given in figure 1.1.

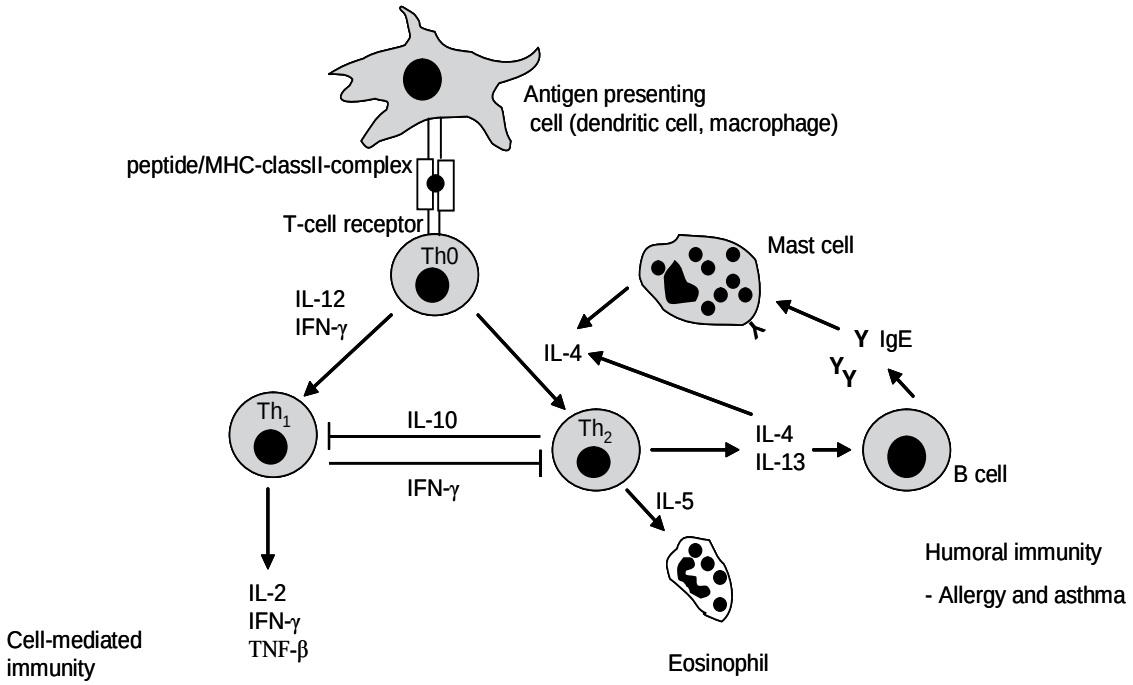

Figure 1.1 A schematic model for the asthmatic immune response: activation of T lymphocytes through antigen presenting cells and subsequent predominant development of Th2 cells over Th1 cells in the context of certain cytokines during antigen presentation.

1.2.4 B lymphocytes

In allergic diseases, B lymphocytes are responsible for the secretion of IgE molecules [40]. An important player in stimulating B lymphocytes to produce and secrete IgE is IL-4. Immature B-cells express surface IgM molecules, which serves as an antigen receptor. B lymphocytes travel to lung draining lymphnodes, and act as efficient antigen presenting cells. Peptides of a captured antigen are presented via the MHC II complex to T lymphocytes of the same antigen specificity. T helper cells then start upregulation of the accessory molecule CD40, which interacts with the CD40 ligand on B cells. Then B-cells progress from a quiescent state to cell cycle and proliferation [41]. Furthermore T helper cells support the switch from IgM isotype molecules on the surface to IgE or IgG_1 isotype.

1.2.5 Mast cells and its mediators

In the human body mast cells are located in proximity to blood and lymphatic vessels in tissues that interface with the environment, like the gastrointestinal tract, the respiratory system or the skin [42]. Increased numbers of mast cells in the lung and enhanced levels of specific mast cell-derived mediators in bronchoalveolar lavage (BAL) fluid have been found in asthmatics [43]. Human mast cells are derived from a $CD34^+$ bone marrow progenitor [44], circulate in the blood and lymphatics and migrate into different tissues. There they start differentiation and maturation with differential expression of the secretory granule proteases, like chymase, tryptase, carboxypeptidase, and cathepsin-G. The heterogeneity of mature mast cells in humans is reflected by their expression of these different proteases. Mast cells expressing only tryptase (MC_T) are found in the lung tissue and in the intestinal mucosa. Those containing both tryptase and chymase (MC_{TC}) are found in the skin, lymphnodes and intestinal submucosa [45,46]. Next to these, mast cells lacking tryptase are found [47]. In rodents (rats and mice) two classes of mast cells can be distinguished: connective tissue-type mast cells (CTMC) and mucosal-type mast cells (MMC) [48,49]. The quantity of rat or mouse MMC expand clearly during T cell dependent immune responses to certain intestinal parasites. In contrast CTMC are T cell independent [48-51].
Activation of mast cells occurs *via* high affinity receptors for IgE (FcεRI), a tetrameric complex, which consists of one α, one β and two identical disulfide-linked γ-chains expressed on the surface of mature mast cells [7]. When the α-chains of adjacent receptors are cross-linked by a multivalent antigen, the β and the γ chain are phosphorylated, activating membrane associated serine proteases. This leads to the activation of several signal transduction pathways [43], which in turn leads to solubilization of granule contents, swelling of the granules, ruffling of the membrane, and fusion of the perigranular and plasma membranes for exocytosis of the granule contents [52] (Fig. 1.2).

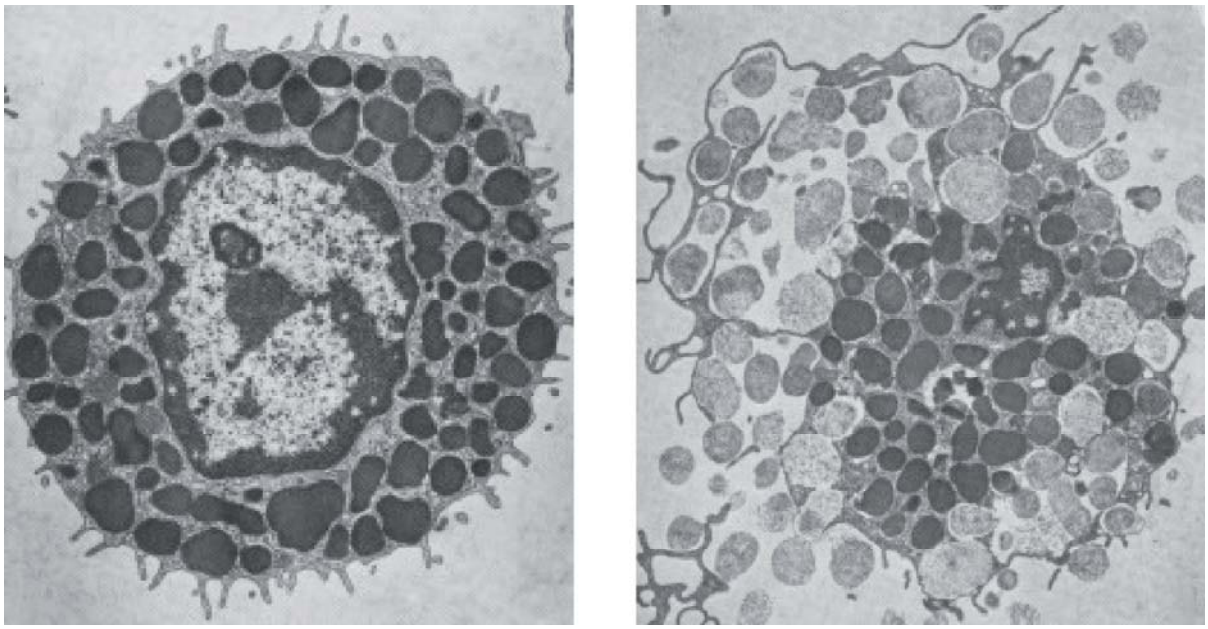

Figure 1.2 Electronic micrography of a peritoneal rat mast cell before (left side) and after (right side) degranulation. From reference 10.

1.2.5.1 Preformed mediators

Preformed in granulae are the biogenic amines histamine and 5-HT, as well as the serine endopeptidases tryptase, chymase, cathepsin G, and the carboxypeptidase A. Histamine causes bronchoconstriction, enhances airway mucus production and increases vascular permeability [53]. Tryptase levels are increased in the BAL fluid from allergen-challenged patients with asthma and are used as a marker for mast cell activation. Tryptase has several functions *in vitro*, including inactivation of fibrinogen, inhibition of fibrinogenesis, activation of tissue matrix metalloproteinases, inactivation of bronchodilatory neuropeptides, stimulation of fibroblast proliferation, collagen synthesis, eosinophil chemotaxis, and upregulation of the adhesion molecule ICAM-1 expression by bronchial endothelial cells. Therefore mast cell tryptase may be important in the pathogenesis of asthmatic inflammation [54-57]. Chymase is also an activator of matrix metalloproteinases. It converts angiotensin I to angiotensin II and inactivates bronchodilatory neuropeptides [58,59]. Cathepsin G leads to endothelial cell injury and stimulates mucus gland secretion [60].

1.2.5.2 Newly synthesized mediators

Activation of the FcεRI leads through the trimeric G-protein Ras to the activation of the MAPK pathway [61], which results in translocation of the cytosolic phospholipase A_2 ($cPLA_2$) to the membrane compartment. Within the compartment the stimulated $cPLA_2$ mobilizes arachidonic acid from membrane phospholipids to form lipid mediators, such as leukotrienes, prostaglandins, and thromboxane within 2 minutes [62]. The synthesis of these lipid mediators is shown in figure 1.3.

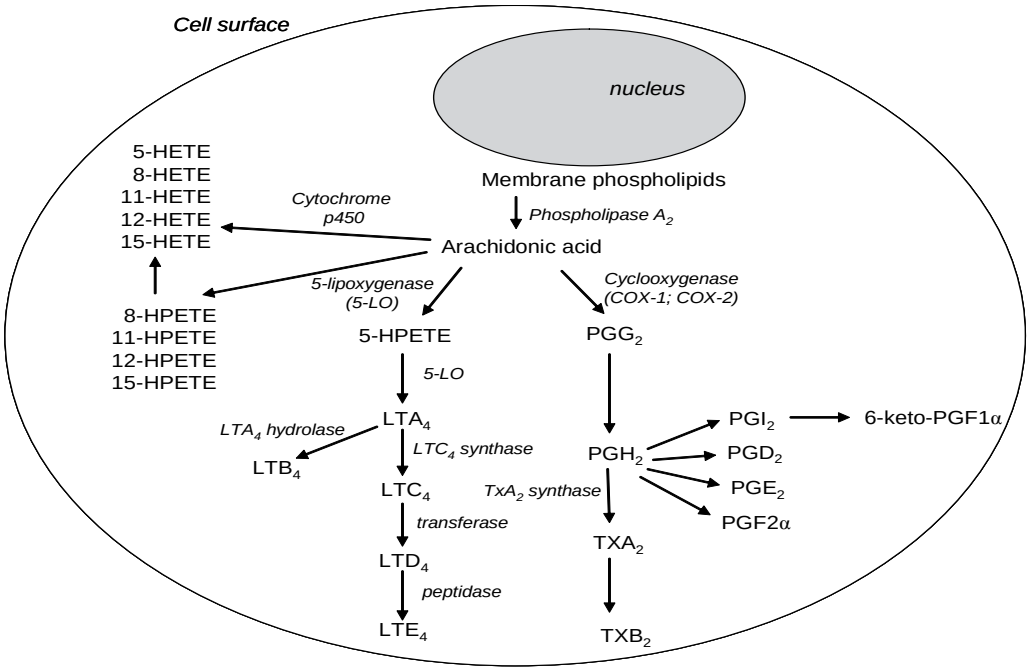

Figure 1.3 Arachidonic acid metabolism. Archidonic acid can be metabolized by lipoxygenase to 5-HPETEs, leukotrienes, and HETEs, by cyclooxygenase to prostaglandins and thromboxane, and by cytochrome p450 to HETEs.

In addition platelet-activating factor (PAF) is synthesized, which may induce AHR [63]. All these mediators act as potent bronchoconstrictors in the allergen-induced mast cell degranulation mediated EAR.

1.2.5.3 Cytokines

Mast cells also generate a variety of cytokines, which may take part in the development of asthma [64]. In *in vivo* and *in vitro* studies of human mast cells the secretion of Th2 cytokines, including IL-4, IL-5, IL-13 and GM-CSF is well described [65-67]. IL-4 and IL-13 favor the upregulation of Th2 cells, stimulate fibroblasts, promote eosinophil growth, and raise the production of IgE molecules by B lymphocytes [68,69]. IL-5 promotes the recruitment, growth and activation of eosinophils, whereas IL-8 recruits neutrophils. Mast cells also contain growth factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factors (BFGF), promoting vessel-, endothelial cell- and fibroblast growth, all important in airway remodeling.

Table 1.1 Overview on mast cell mediators from reference [70]

BIOGENIC AMINES	
Histamine	increases vascular and endothelial permeability; bronchoconstriction (BC) [71]
Serotonin	little or none in human mast cells; main mediator of immediate hypersensitivity reactions in rat and mouse lung [72,73]
GLYCOSAMINOGLYCANS AND PROTEOGLYCANS	
Heparin	acts as local anticoagulant; stabilizes mast cell tryptase; allows packing of cationic mediators [74,75]
Chondroitin sulfate	structural role similar to heparin ? [76]
NEUTRAL PROTEASES	
Tryptase	bronchoconstriction (induces AHR) [60,77]; cell growth (promotes fibroblast growth via PAR-2 [78] and smooth muscle growth) [60]; anticoagulation (hydrolyses fibrinogen and activates stromelysin (MMP-3) [60]; connective tissue degradation (hydrolyzes fibrinogen and high molecular weight kiniogen) [60]); Activates pro-urokinase [79]; control of neurogenic inflammation (inactivates calcitonin gene related peptide [60]).
Chymase	stimulates mucus glands secretion [60]; connective tissue degradation (cleaves type IV collagen, laminin, fibronectin, proteoglycan, glycocalyx [60]); converting enzyme activity (generates extravascular angiotensin II [60]); neurogenic inflammation (inactivates Substance P [60]; augments histamine-induced vessel leak [60]; activates progelatinase B; liberates latent TGF-ß from extracellular matrix
Cathepsin G	degrades extracellular matrix [60]; stimulates mucus glands secretion [60]; endothelial cell injury [60]; antimicrobial defence (kills bacteria noncatalytically) [60]
Carboxypeptidase A	degrades peptides in tandem with chymase and cathepsin G
TPA	activates plasminogen, leading to fibrinolysis [80]
Gelatinase A, B	degrade extracellular matrix [81]
ACID HYDROLASES	
Aryl sulfatase	lysosomal enzymes, extracellular roles? [60,82-84]
Glucuronidase	
Hexosaminidase	
Cathepsin C (Dipeptidylpeptidase I)	actives protryptase and prochymase intracellularly [85,86]
PURINES	
Adenosine	causes weak bronchocondtriction; vasodilation; augments mast cell mediator release [87]
Cytokines, Chemokines and Growth Factors	
TNF-α	enhances eosinophil cytotoxicity; induces expression of endothelial adhesion molecules [88]; recruits neutrophils, monocytes and basophils; enhances microvascular permeability; increases bronchial responsiveness
IL-1	enhances IL-9 production in eosinophils [89]
IL-4, IL-13	promote IgE production; stimulate fibroblasts; promote eosinophil growth [68,69,90]
IL-5	promotes eosinophil recruitment, growth and activation [69]
IL-8	recruits neutrophils [91]
MIP-1α	recruits leukocytes [91]; induces mediator release from inflammatory cells; regulates T-cell binding to adhesion molecules
BFGF	promotes growth of fibroblasts, vessels and other cells [92]
VEGF	promotes endothelial cell and vessel growth [90,93]
Tryptase	promotes fibroblast and airway smooth muscle growth [60]
LIPID MEDIATORS	
PGD_2	bronchoconstriction; dilates vascular smooth muscle [94,95]
LTB_4	attracts neutrophils [94,95]
LTC_4, LTD_4, LTE_4	bronchoconstriction; increases vascular permeability [94,95]
PAF	Bronchoconstriction; increases vascular permeability; induces airway hyperreactivity [94,95]

1.2.6 Eosinophils

Eosinophil infiltration is a characteristic feature in asthmatic inflammation. Before 1916, asthma was termed "chronic eosinophilic bronchitis". Asthmatics underlying a LPR show an increased number of eosinophils in the BAL, compared to those individuals who experienced only an EAR. This demonstrates clearly the accumulation of eosinophils in the airways as part of the LPR in asthma. Eosinophil recruitment to the airways initially involves adhesion of eosinophils to vascular endothelial cells, followed by migration into the submucosa and their subsequent activation. Activated eosinophils release some toxic granule products. Eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN) are the four major granule proteins. In parallel, Th2-like cytokines, oxygen-free radicals, eicosanoids and growth factors are released. These products are able to contract airway smooth muscle [96], induce airway hyperreactivity [97], or increase vascular permeability [98]. Activated eosinophils have been associated with tissue destruction [99].

1.2.7 Neutrophils

The general role of neutrophils in asthma is still poorly defined. Neutrophils have been found in airways of patients with acute severe asthma [21,100], but are not prominent in patients with mild to moderate asthma [101,102]. In patients who died suddenly because of asthma, large amounts of neutrophils have been found in the airways [103]. Neutrophil elastase, cathepsin G, and proteinase 3 are secreted by neutrophils and are important mediators of goblet cell and submucosal gland cell degranulation [104,105]. Therefore, neutrophils may play an important role in acute exacerbations by inducing mucin hypersecretion.

1.2.8 Basophils

Basophils can be considered as the circulating form of mast cells, and they can play a role in inflammatory reactions through recruitment to different tissues sites [106]. Like mast cells, basophils express the high affinity receptors for IgE (FcεRI). They are also activated by cross-linking of these through an antigen. Secreted mediators of a basophil are histamine (preformd) and leukotriene C4 (newly synthesized), as well as a variety of cytokines, including IL-4, IL-5, IL-6 and granulocyte-monocyte-colony-stimulating-factor (GM-CSF) [106]. The number of basophils is also increased in sputum of asthmatics after allergen inhalation challenge [107].

1.3 Signal transduction pathways of airway smooth muscle contraction

Some of the mediators released by inflammatory cells during the EAR and LPR, are strong bronchoconstrictors. The signaling pathways that lead to smooth muscle cell contraction, are still not completely defined. In general, smooth muscle cell contraction is regulated by receptor or mechanical (stretch) activation of the contractile proteins myosin and actin [108]. Also, a change in membrane potential can trigger a contraction [108]. To enable the molecular interaction of myosin with actin, the 20 kDa regulatory light chain (MLC_{20}) of myosin has to be phosphorylated. The increased myosin ATPase activity then enhances the velocity and force of the actomyosin crossbridging cycle [109,110]. Thus contractile activity in smooth muscle is determined primarily by the phosphorylation state of the MLC_{20}, which is dually regulated. On the one hand by myosin light chain kinase (MLCK), which enhance phosphorylation, and on the other hand by myosin light chain phosphatase (MLCP), which decreases phosphorylation (Fig. 1.4). Traditionally a calcium-dependent signal transduction pathway has been assumed to phosphorylate the MLC_{20}, but more recently calcium-sensitizing pathways have been discovered [111].

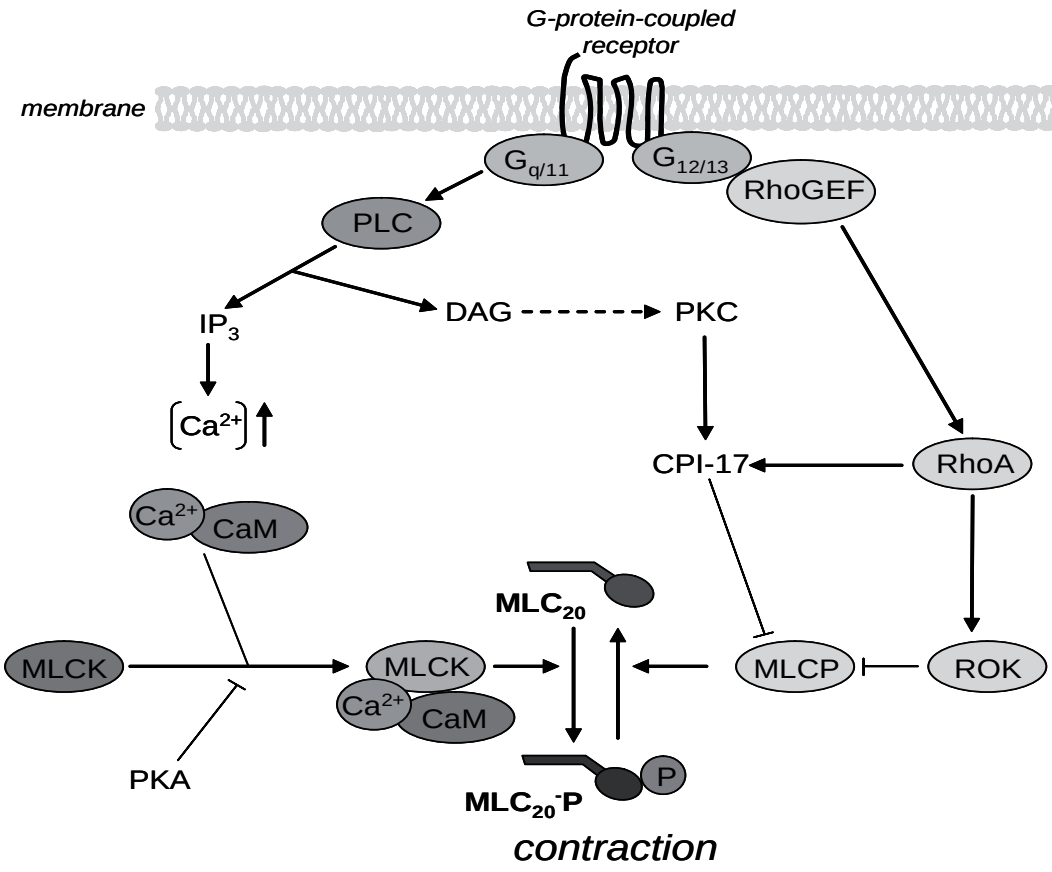

Figure 1.4 Simplified scheme of different signal transduction pathways leading to phosphorylation of 20 kDA myosin light chain, and thereby to smooth muscle contraction. Modified from reference 111.

1.3.1 Ca^{2+}-dependent signal transduction pathway

The initial step in the contractile activation of smooth muscle is an increase in the cytosolic Ca^{2+} provided by a Ca^{2+} flux into the cytoplasm [109]. Ca^{2+} can be released from intracellular stores, in particular the sarcoplasmatic reticulum or enter from the extracellular space through Ca^{2+} channels into the cytoplasm. Agonists binding to receptors, coupled to a heterotrimeric G protein (Gq/11), stimulate phospholipase C activity. This enzyme is specified to catalyze the formation of two potent second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG) from the membrane lipid phosphatidylinositol 4,5 bisphosphate. The binding of IP_3 to IP_3 receptors on the sarcoplasmatic reticulum results in Ca^{2+} release into the cytoplasm [112].
Ryanodine also promotes the release of Ca^{2+} from intracellular stores via ryanodine receptors. Binding of ryanodine to its receptor results either in opening (at low concentrations 1-10 μM) or in irreversibly inhibition (at high concentrations ~ 100μM) of their Ca^{2+} channels [113]. Increased cytosolic free Ca^{2+} binds to calmodulin, and this complex activates the MLCK, resulting in phosphorylation of MLC_{20}. MLCK is activated at cytosolic calcium levels higher than 10^{-6} M. It has been recognized, that the increase in intracellular calcium does not always correlate with the degree in MLCK activity and that the degree of MLC_{20} phosphorylation is higher than expected [114]. This has been referred to as the Ca^{2+} sensitization mechanism [109].

1.3.1.1 Ca^{2+} channels in the plasma membrane

The calcium flow from extracellular space into the cytoplasm can be regulated through voltage-dependent (VOCC), or voltage-independent Ca^{2+} channels. Voltage gated Ca^{2+} channels can be distinguished into four subtypes, the L-type (long lasting), T-type (transient), N-type (neuronal), and P-type (purkinje), depending on their characteristics in voltage activation or inactivation [115]. In smooth muscle cells only L-type and T-type Ca^{2+} channels have been identified [116]. T-type Ca^{2+} channels are activated at more negative potentials, their inactivation is much more rapid and they are insensitive to dihydropyridines, whereas the opposite is true for L-type Ca^{2+} channels [116]. Futhermore, both receptor operated (ROCC) and store operated Ca^{2+} channels (SOCC) are known to regulate voltage-independent Ca^{2+} entry. ROCCs are activated by a ligand, which is binding to a receptor-Ca^{2+} channel complex. Examples for such complexes are the P2x purinoreceptor [117] and the nicotinic acetylcholine receptor [118]. Depletion of intracellular Ca^{2+} stores trigger the activation of Ca^{2+} entry from the extracellular space *via* so called store operated Ca^{2+} channels, to refill again the stores (i.e. sarcoplasmatic reticulum) [119]. Re-uptake of Ca^{2+} ions in the intracellular stores is mediated by Ca^{2+}-ATPases.

1.3.2 Ca^{2+} sensitization mechanism

The Ca^{2+} mediated pathway has long been regarded as the main mechanism by which phosphorylation of MLC_{20} is regulated. Recently, by analyzing the phenomenon of "Ca^{2+} sensitization" in smooth muscle cells, a second pathway regulating the phosphorylation state of MLC_{20} has been found. Studies revealed that this regulation occurs through the inhibition of myosin phosphatase and involves the monomeric GTP-binding protein RhoA [109,120]. In unstimulated cells, Rho A is maintained in the cytoplasm through binding the guanine nucleotide dissociation inhibitor (GDI) [110]. Activation of heterotrimeric G proteins ($G_{12/13}$) leads to stimulation of guanine nucleotide exchange factors (GEFs), exchange of bound GDP to GTP, followed by dissociation of RhoAGTP from the complex and translocation to the plasma membrane [121,122]. One target protein of the now activated RhoA is the Rho-kinase, a serine/threonine-kinase that contains a Rho-binding domain. It is activated upon interaction with RhoA via a specific region in the C-terminal coil-coil domain [123]. This interaction occurs upon recruitment of both proteins to the plasma membrane [122,124]. Activated Rho-kinase phosphorylates the regulatory subunit MYPT1 of MLCP thereby inhibiting the myosin light chain phosphatase activity [125]. Besides inhibition of MLCP, activated Rho-kinase can also directly phosphorylate MLC_{20} [126]. Recently the presence of two other kinases, the Zip-like kinase and CPI-17, that can also regulate the MLCP have been reported, but their functional significance is still unclear. CPI-17 is activated via phosphorylation through proteinkinase C (PKC), an enzyme activated by DAG [127], but recent evidence has shown that CPI-17 is also phosphorylated by Rho-kinase [128].

1.4 Mechanism of airway relaxation

A rise in intracellular Ca^{2+} results in contraction of airway smooth muscle. To relax airway smooth muscle there must be efficient intracellular mechanisms for lowering again the Ca^{2+} level. Bronchodilator action might be due to this calcium efflux mechanism. Adenosine 3`5-cyclic monophosphate (cAMP)-elevating agents such as β-adrenergic agonists and phosphodiesterase (PDE) inhibitors are most widely used clinically to relax airway smooth muscle [129]. An increased cAMP level in the cytoplasm may stimulate calcium efflux to the extracellular space via Na^{+}/Ca^{2+} exchange mechanism or via Ca^{2+}-ATPases into the sarcoplasmatic reticulum. Also proteinkinase A (PKA) is a target of cAMP, its activation leads to phosphorylation of the MLCK, thereby reducing its activity, leading to decreased basal MLC_{20} phosphorylation, as shown by Garcia and coworkers for endothelial cells [130]. PKA activity also attenuates RhoA activation via RhoA phosphorylation at Ser 188 [131] , which decreases Rho association with its downstream target Rho kinase [132].

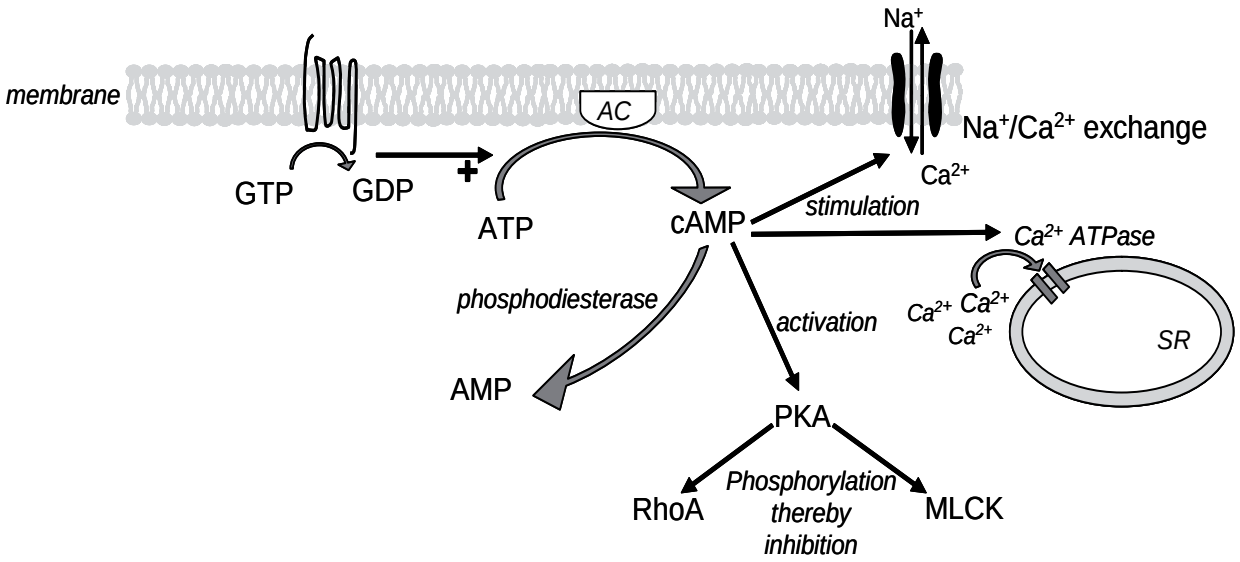

Figure 1.5 Mechanisms of airway relaxation via increase of cytosolic adenosine monophosphate (cAMP). Stimulation of a β_2-receptor leading to activation of adenylate cyclase (AC), which in turn lead to formation of cAMP from adenosine triphosphate (ATP). An increase of cAMP results in activation of proteinkinase A (PKA), re-uptake of free cytosolic Ca^{2+} into the sarcoplasmatic reticulum (SR), and stimulation of Na^{+}/Ca^{2+} exchanger, leading to relaxation of the cell. Degradation of cAMP to AMP was perfomed by phosphodiesterases.

1.5 Models of asthma

Different animal models are used to study several aspects of asthma. In general, the primary focus is to understand the pathogenesis of asthma in humans to develop new strategies in the treatment of asthma.

1.5.1 Limitations to study asthma in humans

In clinical settings, it is very difficult to determine certain parts of asthma. For example, the function of IgE in the LPR is not easily characterized, because immunization of humans to produce specific IgE molecules or transfusion of humans with antigen specific antibodies is impossible. Also the transfer of inflammatory cells from the circulation into the lung tissue cannot be performed within a clinical setting. In addition, it is difficult to completely characterize airway responses to allergens, such as bronchoconstriction, which is the main effect during EAR. Such a provocation is unpleasant and risky. Although the use of BAL and bronchial biopsy in humans has given new insights into cells and mediators playing a role in asthma [133,134] also these procedures are not without risk for the patients. Additionally, there is limitation in the availability of human lung tissue from atopic patients, for use in *in vitro* studies. Another limitation of clinical investigations is that some potentially useful compounds are not available to use in humans. To gain more insights into asthma, animal models that mimic asthma have been developed.

1.5.2 Animal models

Three main groups of animal models for asthma can be distinguished: (i) animals having naturally occurring recurrent airway obstruction, (ii) animals having an AHR before any provocation in the laboratory occurs, and (iii) animals developing reversible airway obstruction and /or increased airway responsiveness only after challenge.

1.5.2.1 Animals with naturally occurring recurrent airway obstruction

This group of animals is represented by cats, which express so called feline asthma. The feline asthma is characterized by recurrent attacks of dyspnea, wheezing, and cough [135]. Enhanced levels of eosinophils are found in the blood and sputum during an attack, and BAL fluids from cats with bronchial disease revealed that eosinophils may be the predominant cell type within the fluid [136]. Pathological aspects of the feline asthma include hyperplasia of submucosal glands, proliferation of goblet cells, and smooth muscle hypertrophy [135]. Norris Reinero *et al.* showed allergen-specific IgE production, airway hyperreactivity, airway eosinophilia and an acute Th2 cell cytokine profile in BAL fluid cells in cats, sensitized with house dust mite or Bermuda grass allergen [137]. However, the cat has not become a widely distributed asthma model, because feline asthma is not commonly recognized, and the generation and identification of asthmatic cats in a laboratory would be very cost-intensive. Horses and Ponies represent the second group of animals, in which naturally recurrent airway obstruction occurs. The exposure of sensitive animals to hay containing mold spores leads to a response of their airways. In pathologic studies of such animals Thurlbeck and Lowell have found a bronchiolitis, including leukocytes, in particular eosinophils in the bronchioles, bronchoconstriction, smooth muscle hyperthrophy, and mucus hypersecretion [138]. The number of neutrophils in the BAL is enhanced during exposure to hay in the barn compared to animals removed from that environment [139]. Airway hyperresponsiveness has been shown during the exposure to hay, and removal from that environment leads to reduction of responsiveness to a normal level [140-142]. Airway obstruction in horses and ponies resembles some forms of industrial asthma in humans in which symptoms are only present during exposure and decrease with removal from that environment. But there are some differences nevertheless. Increased neutrophils have been found in ponies instead of eosinophils in the BAL fluid, and ponies do not show an AHR during remission, whereas most human individuals with allergic asthma persistently exhibit an AHR.

1.5.2.2 Animals showing AHR without provocation

There is a strain of dogs, based on a basenji-greyhound cross, showing a non-specific AHR to various stimuli, without a previous allergen challenge before [143,144]. This persistent AHR is comparable with moderate to severe human asthma, but in contrast to asthma, the dogs do not show a naturally occurring recurrent airway obstruction, which might be induced in laboratories [143].

1.5.2.3 Models of airway obstruction and AHR after allergen challenge

Several animal models have been developed to investigate different aspects of asthma. Therefore, different species have been used in the laboratory, and various models *in vivo* and *in vitro* are created. However, such studies must be performed with awareness of the strength and weakness of the currently available animal models. Table 1.2 summarizes the characteristic features of asthma that can be investigated in the different animal models. Canine models of allergic asthma have been primarily employed to achieve a better characterization of the EAR [145]. In other studies neutrophilia during LPR in dogs, accompanied by increasing vascular permeability and submucosal thickness have been shown [146,147]. Airway remodeling has been studied in a canine model of hyperpnea, with cold, dry air, to get more information about an increased incidence of asthma in winter athletes [148]. Pigs have been used to investigate the role of eosinophils and neutrophils in allergen-induced LPR [149]. The sheep model of allergic airway diseases represents many pathophysiological properties of human allergic airway diseases. Abraham *et al.* demonstrated 1983 that sheep with a natural sensitivity to ascaris suum challenged with this antigen, showed an immediate EAR, and 6 to 8 hours later a LPR [150-153]. Furthermore AHR has been observed in sheep, only if an EAR and LPR was present before [154]. A study from Bischof *et al.* [155] describes, for the first time, the ability of house dust mites to induce allergic responses in sheep lungs. Using human relevant allergens in the sheep model of allergic lung inflammation will be a useful tool to study the immunological and physiological mechanisms of allergic asthma. A model to study airway remodeling in sheep has also been developed [156]. Primate models have been used to study LPR [157], AHR and eosinophilia [158]. Also the importance of adhesion molecules in terms of changes in airway function has been investigated in monkeys [159,160]. An asthma model in rabbits has been developed to investigate the immunopathogenesis of the LPR. As in humans, the LPR leads to stronger airway obstruction than the EAR [161], and the importance of antigen-specific IgE and IgG to this pattern of airway obstruction has been investigated within this model [162]. Furthermore the LPR in rabbits is accompanied by subsequent airway hyperresponsiveness [163]. The rat has been extensively used to study allergen-induced bronchoconstriction, eosinophilic inflammation, as well as LPR and AHR. Furthermore airway remodeling has been observed after repeated allergen exposure [164,165]. Mice, another species of small mammals have

also become important to study antigen-induced airway responsiveness. They are interesting and attractive for these studies, because their immune system is very well known, specific knockout or transgenic mice are available, and studying the global transcriptional changes after allergen challenge is possible [166]. Although mouse models rarely completely reproduce all features of human asthma, murine strains have also been used to investigate AHR. Furthermore mice develop after sensitization clinical syndromes that resemble allergic asthma, including eosinophilia, AHR, increased IgE levels, mucus hypersecretion and sometimes airway remodeling [167], but there are no published descriptions of antigen-induced LPRs [168-171]. There are some known differences between mouse and human physiology of asthma. First, methacholine-induced AHR in mice is only transient after allergen exposure in contrast to humans, which show an increased AHR even when they are symptom free [167]. Second, repeated exposure of allergen to sensitized mice results in suppression of the disease, whereas in human patients it leads to chronic allergic asthma [167]. Third, mast cells and IgE molecules seem to play no role in the development of allergic asthma in mice [167]. These differences limit the value of mouse models for allergic asthma. Taken together, these observations demonstrate that no animal model allows investigation of all questions.

Guinea pigs, which are no rodents [172], are widely used in pulmonary pharmacology, because their airways responsiveness to mediators and drugs is thought to resemble human airway more closely than do those of mice or rats [173,174]. Most of the previous studies have been focussed on *in vivo* experiments, where EAR, LPR, AHR, and eosinophilia have been investigated [175-177]. To study airway pharmacology there is a great need for experimental *in vitro* models, which are relevant with regard to human main components in allergen-induced airway obstruction. Until now, used models dealing with tissue from small mammals (rats and mice) show that the mediators of bronchoconstriction and airway inflammation are very different from those in humans. The bronchoconstriction during EAR in humans is mainly mediated through leukotrienes, thromboxane, and partly by histamine [178,179], whereas mouse and rat airways do not respond with significant bronchoconstriction to challenge with histamine or leukotrienes [180-184]. The guinea pig shows responsiveness to those mediators [185], making it a valuable model to study airway pharmacology relevant with regard to humans.

Table 1.2 Aspects of asthma and the use of different animal models

Aspects of asthma	cat	horse	dog	pig	sheep	monkey	rabbit	rat	mouse	guinea pig
EAR	-	-	+ [145]	-	+ [150]	-	-	+ [164]	-	+ [175,177]
LPR	-		+ [146]	+ [149]	+ [150-153]	+ [157]	+ [161,162]	+ [164]	+[168-171]	+ [175,177]
AHR	+ [135]	+ [138]	+ [143,144]	-	+ [154]	+ [158]	+ [163]	+ [164]	+[167]	-
Eosinophilia	+ [135-137]	-	-	+ [149]	+ [151]	+ [158]	-	+ [164]	-	+ [175,177]
Neutrophilia	-	+ [139]	+ [147]	+ [149]	-	-	-	-	-	+
Airway remodeling	+ [135]	-	+ [148]	-	+ [156]	-	-	+ [165]	+[167]	-
Mucus over production	-	+ [138]	-	-	-	-	-	-	-	-
Smooth muscle hyperthrophy	-	+ [138]								
Bronchoconstriction	-	+	-	-	-	-	-	+ [164]		+ [178]
genetics	-	-	-	-	-	-	-	-	+ [166]	-

+ = animal model exist [Literature]; - = no animal model

1.5.2.4 *In vitro* models in small mammalian species

Apart from the *in vivo* asthma models in several species, there are also a variety of *in vitro* models, to investigate single physiological parameters of asthma. These *in vitro* models are best approached within small mammalian species, like rats, mice and guinea pigs, for which the costs for "living" in the animal facility are less prohibitive. To study broncho- or vasoconstriction tissue organ bath preparations have been widely applied [180,186,187]. These include parenchymal strips, as well as isolated tracheae, bronchi or vessels. Isolated bronchi or tracheae are well established standard methods for studying airway smooth muscle. However, they are best suited to monitor large (> 2 mm diameter) rather than peripheral airways that recently have received much attention [188]. Furthermore, in that kind of preparation interaction with the surrounding parenchymal tissue is missing, so a comparison with the *in vivo* situation remains difficult. Parenchymal strips include peripheral airways and are easy to prepare, but they do not allow to distinguish between vascular and airway responses. The isolated perfused lung (IPL) represents a very good *ex vivo* model to investigate lung parameters, crucial in asthma, including bronchoconstriction, vasoconstriction, edema, and gas exchange. Its disadvantages are: (i) one animal is needed for one experiment, and (ii) the amount of drugs or agonists needed may be relatively high.

1.5.2.5 The model of precision-cut lung slices (PCLS)

A recently developed alternative to the classical pharmacological models is the precision-cut lung slice model. In 1980 Krumdieck *et al.* published for the first time the establishment of precision-cut liver slices using a mechanical tissue slicer [189]. Organ slices have been used for a long time to investigate biochemical pathways, but the production of slices with reproducible properties was difficult, because only manual techniques have been applied. Only the development of the mechanical slicing procedure allowed the production of slices of almost identical thickness, which is important for the comparability of spatial relationship of gas and nutrient exchange by diffusion. Liver slices, which long have been used for toxicological studies, were followed by production of kidney, heart and lung slices. In 1992 Stefaniak *et al.* published the first paper describing the use of agar-filled precision-cut lung slices [190]. Siminski *et al.* [191] studied the long term maintenance of lung slices cultured in defined media, followed later by toxicological studies [192]. The use of precision-cut lung slices in pharmacological studies was introduced by Martin *et al.* [193]. They produced rat lung slices of 250 µm ± 20 µm thickness with a Krumdieck tissue slicer. Contraction of single airways were induced with methacholine (a stable analogue of acetylcholine), and via videomicroscopy and digital imaging the proportion of contraction were visualized and quantified. Further, pharmacological investigations followed this initial study, characterizing the response of mouse airways and pulmonary vessels to several endogenous mediators, and comparing these responses to those in the isolated perfused and ventilated mouse lung [181]. Utilization of PCLS were applied for further studies of peripheral airway pharmacology [194,195]. Beside the use of tissue from laboratory animals (rats and mice), human lung tissue from patients undergoing

surgery for cancer can also be used to produce PCLS, providing insight into human responses to various endogenous and exogenous stimuli [178,196].

In PCLS smooth muscle contraction in airways and vessels is auxotonic, i.e. stress and length change simultaneously, which may resemble *in vivo* airway contraction more appropriately than other *in vitro* methods. Another advantage of this model is the amount of slices, prepared from one lung (up to 50 slices), which does save animals, and can also help to reduce experimental error by internal controls and statistical pairing. PCLS provide the opportunity to examine physiological responses in different species by the same experimental model. This seems of particular importance at a time when differences between various asthma models compared to human asthma are increasingly being recognized as a major impediment for drug development [197,198]. With regard to the guinea pig, as a small mammalian animal that shows many similarities to human airway responses, PCLS from the guinea pig would be a valuable tool in airway pharmacology.

2 Aim of the study

One characteristic feature of human asthma is the early allergic response (EAR), occurring within minutes after allergen contact and resulting in a strong contraction of the airways. For induction of an EAR in PCLS, two methods can be applied, on the one hand passive sensitization of the slices, on the other had the use of PCLS from actively sensitized animals. Another characteristic feature is airway hyperresponsiveness (AHR), an increased reactivity of inflamed and remodeled airways in response to unspecific stimuli. Various animal models are used to investigate characteristic features of asthma, but important species differences limit the portability of these models to human asthma.

The model of precision-cut lung slices (PCLS) offers the opportunity to investigate airway responses in different animal species, as well as in human lung tissue. The access to human tissue provides an almost unique possibility to explore lung functions in human airways with an intact microanatomy. However, because of the limited availability of human tissue, and the fact that the tissue comes from patients undergoing surgery for lung cancer, that are treated with diverse pharmaceuticals, there is a strong need of animal models resembling the human responses. Previous studies had indicated that guinea pig lungs resemble human airway responses more closely than do rats or mice.

Therefore, a major aim of the present study was to establish the model of PCLS from guinea pigs. A further aim was, to identify the mediators inducing bronchoconstriction during EAR and the activated signal transduction pathways in PCLS of the different species, i.e. guinea pigs, monkeys, rats, humans and mice. Finally, the results should be compared among these species.
Additionally, we wanted to investigate EAR and AHR in PCLS from actively sensitized mice and rats.

3 Material and Methods

3.1 Animals

The animal experiments were approved by the local ethic committee.

3.1.1 Rats

Female Wistar rats (220 ± 20 g) obtained from Charles River (Sulzfeld, Germany) were kept under controlled conditions (22° C, 55% humidity and 12 hours day/night rhythm) on a standard laboratory chow and ozon water and were used as lung donors for the preparation of the precision-cut lung slices (PCLS).
Actively sensitized, female Brown Norway rats, underlying a standard 21 day sensitization protocol (Fig. 3.1), were obtained from our collaboration partner A. Braun (ITEM Hannover, Germany).

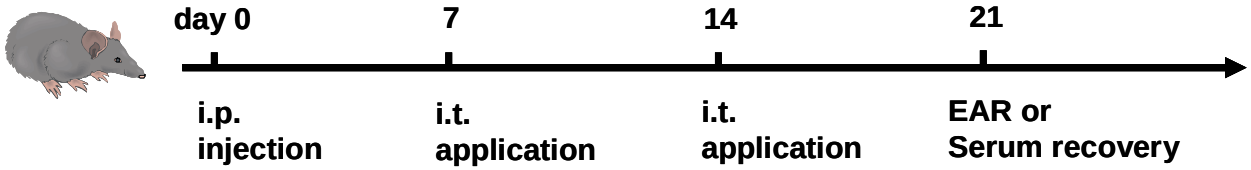

Figure 3.1 Sensitization protocol for actively sensitized rats. i.p. = intraperitoneal injection of ovalbumin; i.t. = intra-tracheal application of ovalbumin. On day 21 performance of the early allergic response (EAR) and serum sampling , which was used for passive sensitization of PCLS from unsensitized rats.

3.1.2 Mice

Lungs were taken from female Balb c mice (21-28 g) obtained from Charles River (Sulzfeld, Germany). The mice were kept under controlled conditions (22° C, 55% humidity and 12 hours day/night rhythm) on a standard laboratory chow.
Actively sensitized mice, underlying a standard 35 day sensitization protocol (Fig. 3.2) were obtained from A. Braun (ITEM, Hannover, Germany). Mast cell-deficient mice were obtained from M. Maurer (Charité Berlin, Berlin, Germany), and Maxi-K knockout mice from Dr. P Ruth and Dr. M. Sausbier (Pharmaceutical Institute, University Tübingen, Tübingen, Germany).

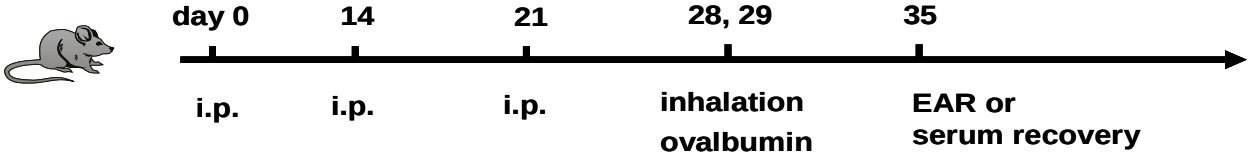

Figure 3.2 Sensitization protocol for actively sensitized mice. i.p. = intraperitoneal injection of ovalbumin. On day 35 performance of the early allergic response (EAR) and serum sampling , which was used for passive sensitization of PCLS from unsensitized mice.

3.1.3 Guinea pigs

Female Dunken Hartley guinea pigs (350 g ± 30 g) obtained from Charles River (Sulzfeld, Germany) were kept under controlled conditions (22° C, 55% humidity and 12 hours day/night rhythm) on standard laboratory chow and were used as lung donors.

3.1.4 Monkeys

Lungs from cynomolgus monkeys, baboons, and rhesus monkey were obtained from K. Mätz-Rensing (Center of Primate reasearch, Göttingen, Germany) in collaboration with A. Braun (ITEM, Hannover, Germany).

3.1.5 Pentobarbital solution

Pentobarbital solution (Narcoren) was purchased from the Wirtschaftsgenossenschaft deutscher Tierärzte (Hannover, Germany), and was used to anesthesize the animals.

3.2 Human lung donors

The human PCLS were prepared from healthy parts of a lung from patients undergoing surgery for lung cancer. In table 3.1 the patient characteristics are shown.

Table 3.1 Patient characteristics

patient	sex	age [years]	smoking habits [P/Y]	histological finding	atopic/ asthmatic	Gold stage	Forced vital capacity L (%)	FEV1 L (%)
1	M	59	50 (ex since min. 1 month)	Renal cell cancer	allergic rhinitis	0	5.22 (107)	4.14 (108)
2	F	66	20	Ca	n	II	3.29 (98)	1.67 (57)
3	F	68	50	Ca	n	II	2.49 (85)	1.4 (56)
4	M	62	140	Ca	n	0	3.96 (86)	3.03 (83)
5	M	72	35 (ex since min. 1 month)	Ca	n	I	4.74 (112)	3.26 (100)
6	M	53	35	Ca	n	I	4.9 (104)	2.95 (78)
7	M	70	40	Ca	n	I	3.82 (104)	2.58 (91)
8	M	51	40 (ex since min. 1 month)	Ca	n	0	3.6 (93)	2.71 (85)
9	M	77	60 (ex since min. 1 month)	Ca	n	I	3.56 (120)	2.43 (108)
10	M	59	45	Ca	n	II	4.19 (105)	2.02 (63)
11	F	76	25 (ex since min. 1 month)	Ca	n	II	2.16 (99)	1.04 (58)
12	F	54	50	Ca	allergic rhinitis	0	3.45 (134)	2.61 (120)
13	F	57	30	Ca	n	I	3.5 (114)	2.27 (87)
14	M	64	30 (ex since min. 1 month)	Ca	n	I	3.82 (91)	2.55 (78)
15	F	62	40	Ca	n	0	2.43 (84)	1.95 (79)
16	M	67	50	Ca	n	0	2.85 (72)	2.0 (65)
17	M	67	Pipesmoker	Ca	n	0	4.55 (106)	3.66 (110)
18	F	70	-	Colon cancer	n	n.d.	3.31 (117)	2.65 (112)
19	M	69	50	Ca	n	I	4.94 (112)	3.26 (106)
20	M	71	26 (ex since min. 1 month)	Ca	n	0	4.48 (117)	3.66 (124)
21	M	68	40	Ca	n	I	3.26 (94)	2.16 (80)
22	M	44	20	Ca	n	0	4.0 (95)	3.26 (92)
23	M	63	10 (ex since min. 1 month)	Ca	n	0	4.0 (86)	3.53 (95)
24	F	78	20 (ex since min. 1 month)	Ca	n	I	3.23 (100)	1.61 (84)
25	M	71	40 (ex since min. 1 month)	Ca	n	n.d. after neck dissection	n.d.	n.d.
26	M	64	40 (ex since min. 1 month)	Ca	n	II	2.79 (71)	1.55 (50)
27	M	70	110 (ex since min. 1 month)	Ca	n	I	3.85 (95)	2.6 (83)
28	M	69	50 (ex since min. 1 month)	Ca	n	0	3.5 (109)	2.5 (99)
29	M	66	14 (ex since min. 1 month)	Ca	n	I	5.73 (130)	4.0 (117)
30	M	64	70	Ca	n	III	3.6 (85)	1.6 (45)
31	F	56	40	Ca	n	II	2.51 (76)	1.62 (58)
32	M	66	55 (ex since min. 1 month)	Ca	n	0	3.76 (86)	2.85 (79)
33	M	60	60 (ex since min. 1 month)	Ca	n	0	4.7 (97)	3.62 (95)

3.3 Chemicals

Table 3.2 Chemicals

Substance	Producer
AA861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadinyl)-1,4-benzochinone)	Biomol (Hamburg, Germany)
Adenosine	Sigma (Deisenhofen, Germany)
Agarose Type VII Low Gelling	Sigma (Deisenhofen, Germany)
Albumin chicken egg (ovalbumin) Grade V	Sigma (Deisenhofen, Germany)
Amino acids	PAA Laboratories (
2-aminoethoxydiphenyl borate (2APB)	Sigma (Deisenhofen, Germany)
Atropine	Sigma (Deisenhofen, Germany)
Bosentan	Dr. Clozel (Actelion, Allschwil, Switzerland)
$CaCl_2$	Sigma (Deisenhofen, Germany)
Compound 48/80	Sigma (Deisenhofen, Germany)
Cromolyn	Sigma (Deisenhofen, Germany)
Endothelin-1 (ET-1)	Bachem (Weil, Germany)
Glucose	Sigma (Deisenhofen, Germany)
Glutamine	PAA Laboratories
HEPES (N'-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid)	Sigma (Deisenhofen, Germany)
Histamine	Sigma (Deisenhofen, Germany)
5-Hydroxytryptamin (Serotonin)	Sigma (Deisenhofen, Germany)
IBMX (isobutylmethylxanthine)	Sigma (Deisenhofen, Germany)
Indomethacin	Fluka, (Deisenhofen, Germany)
Isoproterenol	Sigma (Deisenhofen, Germany)
KCl	Sigma (Deisenhofen, Germany)
Ketanserin	Sigma (Deisenhofen, Germany
L108 (1-O-Octadecyl-2-O-methyl- sn-glycero-3-phosphorycholine)	Biomol (Hamburg, Germany)
Leukotriene D_4	Biomol (Hamburg, Germany)
Methacholine	Sigma (Deisenhofen, Germany)
$MgSO_4$	Sigma (Deisenhofen, Germany)
ML-7 (1-(5-Iodonophthalene-1- sulfonyl)-1H-hexhydro-1,4-diazepine-HCl)	Biomol (Hamburg, Germany)
Montelukast [R-(E)]-1-[[[1-[3-[2-(7-chloro-2-quinolinyl) ethenyl] phenyl]-3-[2-(hydroxy-1-methylethyl) phenyl] propyl] thio] methyl] cyclopropaneacetic acid monosodium salt	MSD SHARP & DOME GMBH, Haar, Germany
NaCl	Sigma (Deisenhofen, Germany)
$NaHCO_3$	Sigma (Deisenhofen, Germany)
Natrium pyruvate	PAA Laboratories
Oregon green BAPTA-1 AM	Molecular Probes distributed by MoBiTec (Göttingen, Germany)
Phleum pratense extract (pollenextract)	Dr. Gabriele Schramm (Research Center Borstel, Borstel, Germany)
Pluronic F-127	Molecular Probes distributed by MoBiTec (Göttingen, Germany)
Salbutamol	Sigma (Deisenhofen, Germany)

SQ29548 (5-heptenoic acid, 7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo-[2.2.1]hept-2-yl]-, [1S-[1α,2α(Z),3α,4α]]-)	RBI (Deisenhofen, Germany)
Substance P	Sigma (Deisenhofen, Germany)
Sulfobromophthalein	Sigma (Deisenhofen, Germany)
Triprolidine	Sigma (Deisenhofen, Germany)
Triton X-100 Detergenz	Roche (Mannheim)
U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid)	Cayman (Ann Arbor, Michigan, USA)
vitamins	PAA Laboratories
WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thienol[3,2-f][1,2,4]triazolo-[4,3-a][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanon)	Dr. Heuer (Boehringer Mannheim, Mannheim, Germany)
Xestospongin C	Calbiochem (Bad Soden, Germany)
Y27623 (trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide)	Tocris distributed by Biotrend (Cologne, Germany)

3.4 Equipment

Table 3.3 Instruments and equipment

Instrument	**Producer**
Beam splitter	Trim Scope, La Vision, BioTec, Bielefeld, Germany
Coring tool (Black & Decker battery operated screwdriver + self-made coring device 0.9 mm	
Digital camera (2-Photon microscope)	Imager QE, La Vision BioTec, Bielefeld, Germany
ELISA (Histamine)	IBL, Hamburg, Germany
ELISA (Serotonin)	IBL, Hamburg, Germany
GraphPadPrism 4.02 software	GraphPad, San Diego, CA, USA
Imspector software	La Vision BioTec; Bielefeld, Germany
Incubator	Heraeus, Hannover, Germany
Inverted microscope Leica DMIRB and DMIL	Zeiss, Oberkochen, Germany
Fixed stage upright microscope BX51WI	Olympus, Hamburg, Germany
Krumdieck tissue slicer	Alabama research and development, Munford, USA
Optimas 6.1	Optimas Corporation, Bothell, USA
Optimas 6.5	Optimas Corporation, Bothell, USA
Polytron PT1200 (Ultrathorax)	Kinematica AG, Littau, Switzerland
Ti:Sa femtosecond laser	Coherent, Dieburg, Germany
Viability/cytotoxicity assay kit	Molecular Probes (Eugene, Oregon, USA)
Video camera JAI PROTEC	JAI20040, JAI Pulnix, Alzenau, Germany
Video camera Visicam 1300 and Visicam 640	Visitron Systems, Munich, Germany

3.5 Methods

3.5.1 Precision -cut lung slices (PCLS)

3.5.1.1 Preparation of rat lung slices

The rats were anesthesized through intraperitoneal injection of 60 mg/kg pentobarbital (Narcoren, Pharmazeutische Handelsgesellschaft mbH, Garbsen, Germany). After narcotization the trachea was cannulated and the animals were exsanguinated by cutting the vena cava inferior. The diaphragm was cut, to collapse the lungs, which were then subsequently filled with a low-melting point agarose solution (0.75%, final concentration) through the cannula. In order to solidify the agarose and harden them for cutting, the whole chest was covered with ice for 10 to 15 minutes. After that time, the lungs were removed from the thoracic cavity and placed on ice for another 10 minutes. The lobes were separated and tissue cores prepared with a rotating sharpened metal tube (diameter 8 mm). These cores were cut into 220 µm thick slices with a Krumdieck tissue slicer (Alabama Research and Development, Al, USA).

3.5.1.2 Preparation of mouse lung slices

Mice were anesthesized with 45 mg/ml pentobarbital. Preparation follows as described for the rats. After the lungs solidified on ice and were removed from the chest, they were separated into the single lobes and embedded in 3% agarose in a cryotube. The cryotubes were then put on ice again to solidify the agarose. From the embedded lung lobes mouse PCLS (220 µM) were prepared with the Krumdieck tissue slicer (Alabama Research and Development, Al, USA).

3.5.1.3 Preparation of guinea pig lung slices

Guinea pig PCLS were prepared as described for the other species (rat, mouse) with the following modifications. After injection of pentobarbital (95 mg/kg) the trachea was cannulated and the animals exsanguinated by cutting the vena cava inferior. Through the cannula the lung was filled with a low-melting point agarose solution (0.75%, final concentration) containing isoproterenol (1 µM). To allow the agarose to solidify, the whole chest was covered with ice. Subsequently the lungs were removed, lobes were separated and tissue cores prepared with a rotating sharpened metal tube (diameter 8 mm). These cores were cut into 220 µm thick slices with a Krumdieck tissue slicer (Alabama Research and Development, Al, USA). In figure 3.3 the preparation of GP PCLS was shown.

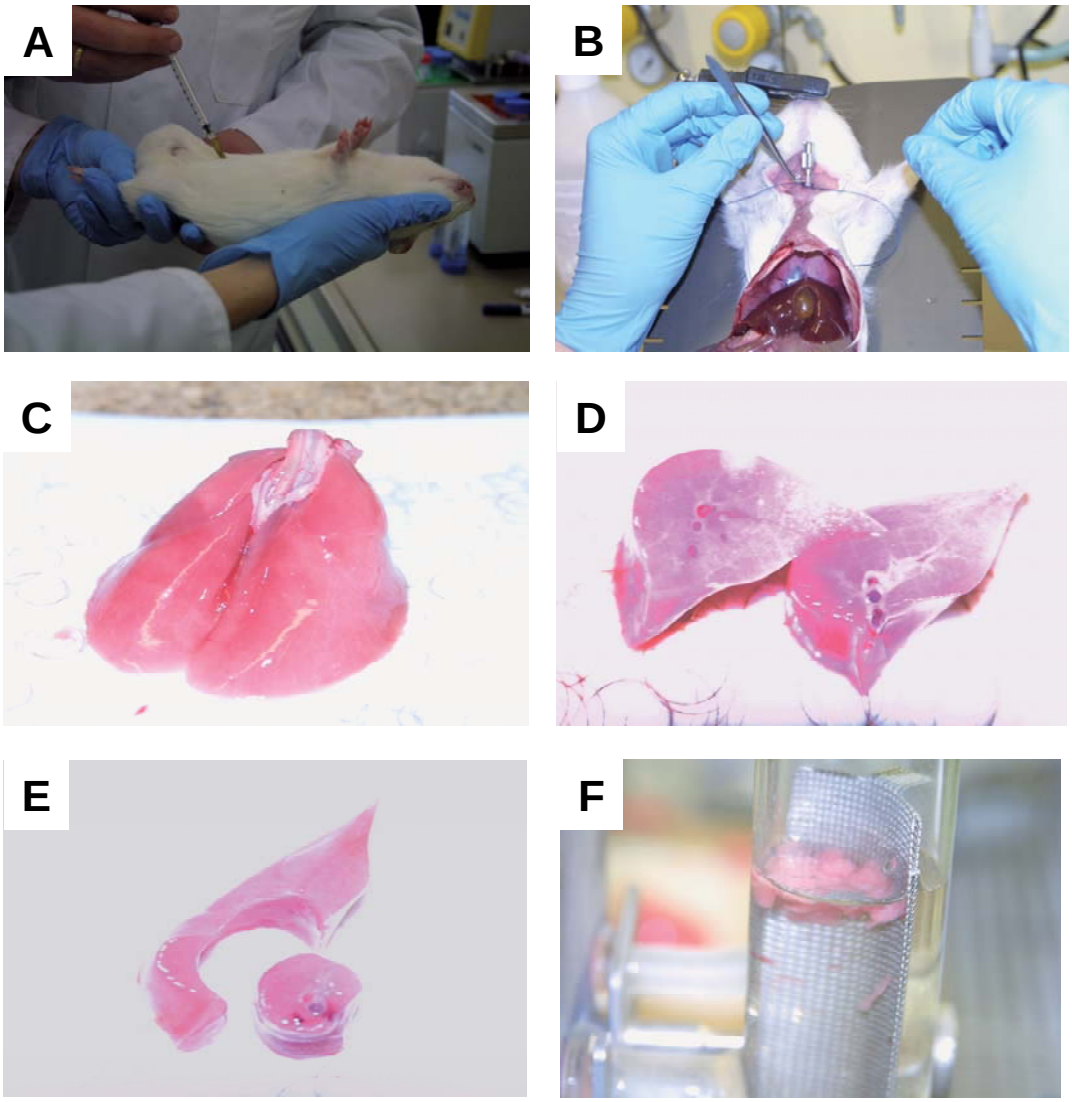

Figure 3.3 Preparation of guinea pig precision-cut lung slices (PCLS). (A) The guinea pig was anesthetized by intraperitoneal injection of 0.8 ml pentobarbital. (B) After exsanguination of the animal, the lungs were filled via the trachea with 0.75% agarose solution, prewarmed to 37° C, and supplemented with isoproterenol (1 µM). (C) The chest was covered with ice, until the instilled agarose get hardened, and then the whole lung was removed. (D) Single lobes were separated and cut into 1 cm thick parts, (E) from which tissue cores, containing the airways, were prepare. (F) From these tissue cores, PCLS were prepared, using the Krumdieck tissue slicer (Krumdieck, Alabama, USA), and collected in a reservoir filled with slicing medium. From here 250 µm thin slices were moved into Petri-dishes for incubation at 37° C and 5% CO_2.

3.5.1.4 Preparation of human lung slices

Human lung tissue was obtained from patients, undergoing surgery for cancer. Healthy regions were filled via the main bronchus with low melting point agarose solution (1.5% final concentration). Afterwards the lobe was put on ice until the agarose had solidified. The tumor part of the lobe was then separated from the rest, and remains in the pathology. The healthy regions were taken for further preparation. The lobe was cut into 1 cm thick plates, from which tissue cores around the airways were prepared. These cores were cut into 220 µm thick slices with a Krumdieck tissue slicer (Alabama Research and Development, Al, USA).

3.5.1.5 Preparation of monkey PCLS

Monkey PCLS were prepared in accordance to human PCLS. Complete lungs were filled via the trachea with agarose solution (1.5% final concentration), which was prewarmed to 37˚ C before. The trachea was closed with a threat and the lungs were put on ice for approximately 30 minutes until solidity. After that, the lungs were separated into the single lobes, which were then cut into 1 cm thick slabs. With a coring tool tissue cores were prepared containing airways, vessels or both. As in the other species, the tissue cores were cut into 250 ± 20 µm thin slices with a Krumdieck tissue slicer (Krumdieck, Alabama research, USA). After the slicing procedure the PCLS were incubated at 37° C and 5% CO_2.

3.5.2 Culture medium

3.5.2.1 Incubation medium

After the slicing procedure the lung slices were incubated at 37˚ C in a humid atmosphere in minimal essential medium (MEM), (pH 7.2) composed of CaCl2 (1.8 mM), MgSO4 (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), glucose (16.7 mM), NaHCO3 (26.1 mM), Hepes (25.17 mM), natrium pyruvate (10 ml/l), amino acids (20 ml/l), vitamins (10 ml/l), glutamine (10 ml/l). The pH-value was adjusted to 7.2. The incubation medium was changed every 30 minutes during the first two hours after slicing, followed by a change every hour for the next two hours, in order to remove the agarose and cell debris from the tissue. Subsequently, medium was further supplemented with penicillin and streptomycin (100 U and 100 µg/ml) and changed every 24 hours. In case of the guinea pig isoproterenol (1 µM) was added to the incubation medium during the first 3 hours after slicing.

3.5.2.2 Agarose solution

Double concentrated incubation medium was mixed with the same volume of low melting point agarose at 37° C, to obtain the final concentration of agarose solution for instillation into the lung.

3.5.2.3 Slicing medium

The slicing procedure was performed in approximately 500 ml incubation medium without natrium pyruvate, amino acids, vitamins, and glutamine.

3.5.3 Viability of the PCLS

3.5.3.1 Lactate dehydrogenase (LDH) release

The viability of the PCLS was assessed by measuring the relative amount of lactate dehydrogenase (LDH) released from the slices into the incubation medium. LDH was taken as a marker of cell death, because it was only released in great amounts, when cells start lyses. Three slices per well were placed into a 24-well-plate and covered with 1 ml incubation medium. At the indicated time points (24 hours, 48 hours, 72 hours), slices were removed from the incubation medium and lysed in 1 ml 0.2% Triton X-100 solution. The incubation supernatant was kept and stored on ice (4° C), until the end of the preparation. The slices in the 0.2% Triton X-100 solution were homogenized (Polytron; Kinematica AG, Littau, Switzerland) and centrifuged, 5 minutes at 13,000 x g (Eppendorf; Hamburg, Germany). The supernatant was taken and on ice. Then both solutions, the incubation supernatant and the supernatant from the homogenisation were analyzed by a commercially available LDH-assay (Dimension pan; Dade Behring, Schwalbach, Germany). Viability of the slices was expressed as the ratio of LDH in the incubation supernatant to the total LDH (sum of LDH in slices and the supernatatant).

3.5.3.2 Two-photon microscopy

To visualize also the viability of the guinea pig PCLS we used 2-photon microscopy in combination with the LIVE/DEAD® viability/cytotoxicity assay kit (Molecular Probes, Eugene, Oregon, USA). PCLS were incubated with 5 µM Calcein AM (acetomethylester of calcein, live staining) and 10 µM ethidium homodimer (EthD, dead staining) for 40 minutes and then washed to remove external dye. The fluorescent dyes were excited at 800 nm with a Ti:Sa femtosecond laser (Coherent, Dieburg, Germany). The laser beam was split up into 64 individual beams (beam splitter from Trim Scope, LaVision BioTec, Bielefeld, Germany) that simultaneously excited and scanned the object on the microscope (Olympus, Hamburg, Germany). The images were acquired using a digital camera (Imager QE, LaVision, Bielefeld, Germany). The emission of calcein AM (emission filter 500/50 nm) for the cytoplasm (live staining, green) and the EthD (emission filter 625/50) for the staining of nuclei (dead staining, blue) were recorded separately. Figure 4.2 in the result part shows overlay images of both dyes. Slices were analyzed 24 hours, 48 hours, and 72 hours after preparation. To visualize the total amount of dead cells, some PCLS were treated with 1% Triton X-100 for 20 minutes prior incubation with dyes.

3.5.4 Measurement and Imaging of broncho - or vasoconstriction

The airways and vessels were imaged and digitized with a digital and an analogue video camera (IRB640, Visitron Systems, Munich, Germany; JAI 2040, JAI PULNiX, Alzenau, Germany). The airway or vessel area before addition of the mediators was defined as 100%. Bronchoconstriction or vasoconstriction was determined as the percentage decrease in the airway/vessel area compared to the initial area. For the measurements, slices with comparable airway size were selected. These slices were put on 24-well-plates and fixed with a nylon thread attached to a platinum wire. The slices were continuously covered with 1 ml incubation medium.
The 24-well plate was positioned on the stage of an inverted microscope. Images were recorded by analogue (JAI 2040, JAI PULNiX, Alzenau, Germany) or digital camera (IRB640, Visitron Systems, Munich, Germany). A control image was taken before addition of the mediator, frames were recorded every 5 or 30 s for 5, 10 or 20 minutes depending on the study.

3.5.5 Passive-sensitization as a tool to study the EAR

Animal PCLS were treated and incubated overnight (16 hours) with 1% serum from animals that have been actively sensitized with ovalbumin by standard protocols. Human PCLS were incubated overnight with 1% serum from an atopic individual with sensitivity to grass pollen (phleum pratense). The next day, passively sensitized slices were transferred into a 24-well plate with fresh medium free of serum. Changes of airway /vessel area was measured as described in 3.5.4.

3.5.5.1 Rat serum

Rat serum was obtained from Dr. A. Braun from the ITEM (Hannover, Germany). Female Brown Norway rats were sensitized to ovalbumin underlying a 21 day sensitization protocol. On day 0 the rats were sensitized by an intraperitoneal injection (i.p.) of 10 µg ovalbumin plus 20 mg $Al(OH)_3$ gel. On day 7 and 14 the rats were sensitized intratracheal. On day 21 the animals were killed, and blood samples were taken from their hearts (Fig. 3.1). The total IgE amount was determined by ELISA.

3.5.5.2 Mouse serum

Mouse serum was also obtained from Dr. A. Braun from the ITEM (Hannover, Germany). Mice underlying a 35 day sensitization protocol (Fig. 3.2).

3.5.5.3 Guinea pig serum

The guinea pig serum was a gift from Prof. S.-E. Dahlén (Karolinska Institute, Stockholm, Sweden). Guinea pigs were sensitized within four weeks by one i.p and one s.c. injection of 10 mg/ml ovalbumin dissolved in 0.4 ml of saline-aluminium hydrogel (1:1). Animals were killed, and blood was collected from the hearts.

3.5.5.4 Human serum

Human serum was from an atopic person, who has allergic rhinitis and specific IgE levels to grass pollen (*Phleum pratense*) and velvet grass (*Holcus lanatus*). The serum contains a total IgE amount of 450 IU/ml.

3.5.6 Pharmacological intervention studies

3.5.6.1 Mediators/ agonists and antagonists

To determine the mediators that are involved in the early allergic response (EAR), PCLS were preincubated for at least 10 min with different receptor antagonists. The leukotriene ($CysLT_1$)-receptor antagonist montelukast (10 µM), the thromboxane prostanoid-receptor antagonist SQ29548 (10 µM), or the histamine (H_1)-receptor antagonist triprolidine (5 µM) were added alone or in combination.

Table 3.4 Overview of the receptor agonists and antagonists

Agonist	Receptor	Antagonist
Leukotriene D_4	$CysLT_1$-receptor	Montelukast (10 µM)
Thromboxane	TP-receptor	SQ29548 (10 µM)
Histamine	H_1-receptor	Triprolidine (5 µM)

3.5.6.2 Inhibitors of the signal transduction pathways

To investigate on which signal transduction pathway the bronchoconstriction during EAR was mediated, different signal transduction pathways were inhibited. PCLS were preincubated 20 minutes with the following different inhibitors. During measurement the inhibitor was also present next to the agonist in the incubation medium.

Table 3.5 Inhibitors of the signal transduction pathways

Inhibitor	final concentration	solved in
L108 (ihibitor of PI-PLC)	30 µM	EtOH
Mibefradil (inhibitor of T-type Ca^{2+}-channel)	25 µM	Milli Q H_2O
ML-7 (inhibitor of MLCK)	35 µM	EtOH
Nifedipine (inhibitor of L-type Ca^{2+}-channel)	5 µM	EtOH
Xestospongin C (inhibitor of IP_3-receptor)	10 µM	EtOH
Y27632 (inhibitor of Rho-kinase)	10 µM	Milli Q H_2O

The final concentration of EtOH was always < 0,1% and did not affect the responses to the mediators.

3.5.7 Airway relaxation in the guinea pig

For the relaxation experiments PCLS were contracted with Mch ($10^{-6.5}$ M) to 10 - 20% of their initial airway area. Subsequently, increasing concentrations of the β-agonist salbutamol (10 nM to 10 µM), the unspecific PDE-inhibitor IBMX (10 nM to 10 µM) and the combination of both were added in the presence of Mch. Measurements were performed as described in 3.5.4.

3.5.8 Calcium imaging with two-photon microscopy

Rat PCLS were prepared as already described above with one modification: the final concentration of the agarose solution to fill the lung is 3,5%. For the experiments with serotonin, the slices were selected immediately after the slicing procedure. One slice per well was placed on a 24-well plate. Every well was covered with 500 µl incubation medium. To visualize later intracellular Ca^{2+}, the slices were loaded for 2 hours at 37° C with Oregon green (5 µM), 0,2% pluronic (Pluronic-F127; 5 µM) and sulfobromophthalein (100 µM). For the measurements the slices were placed on a petri dish fixed with a nylon thread attached to a platinum wire, and covered by a 1ml drop of incubation medium without dye. Then the perti dish was put under the microscope, and the layer of airway smooth muscle cells was adjusted via microscopy. The fluorescent dye (Oregon green) was excited

at 800 nm with a Ti:Sa femtosecond laser (Coherent, Dieburg, Germany). The laser beam was split up into 64 individual beams (beam splitter from Trim Scope, LaVision BioTec, Bielefeld, Germany) that simultaneously excited and scanned the object on the microscope (Olympus, Hamburg, Germany). The images were acquired in the time lapse mode with an exposure time of 258 ms (including 2x2 binning) using a digital camera (Imager QE, LaVision, Bielefeld, Germany). The laser beam was adjusted to 64% of power and no emission filter was used. For a time period of 20 minutes, calcium signals of the airway smooth muscle cells were recorded. Ca^{2+} levels were analysed using the Imspector software, and further Excel program and Graph Pad Prism Software.

3.6 Statistics

Data were analyzed by unpaired students t-test, either one-sided or two-sided as indicated. In case of heteroscedsticity (percentage data), data were arcsin-transformed prior to analysis. P-values were corrected for multiple comparisons according to the false-discovery rate procedure using the "R" statistical package [199]. The concentration-response curves were analyzed as sigmoidal dose-response curves in Prism (Version 4.02, Graphpad Software, San Diego, CA, USA). The two-photon microscopy measurements were analyzed as area under the curve (AUC) by one-sided t-test in Prism (Version 4.02, Graphpad Software, San Diego, CA, USA).

4 Results

4.1 Part Ia: Establishment of guinea pig (GP) PCLS

The first aim of this study was to establish the model of PCLS from the guinea pig and characterize it in terms of viability, mediator-induced bronchoconstriction, the EAR, and relaxation of the airways. The standard protocols used to prepare rat and mouse PCLS [193,196] led in GP PCLS to almost completely closed airways within the first 10 minutes after the slicing procedure. Closed airways then were extremely difficult to re-open again. To prevent this *post mortem* bronchoconstriction, the slices were put into incubation medium containing the muscarinic-receptor antagonist atropine, the TP-receptor antagonist SQ29548, the $CysLT_1$-receptor antagonist montelukast or the H_1-receptor antagonist triprolidine, but none of the inhibitors either alone or in combination was effective (data not shown). In contrast, addition of the β_2-agonist isoproterenol (1 µM) already to the agarose solution used for instillation, to the slicing medium and also to the incubation medium completely prevented the *post mortem* bronchoconstriction. After the first three hours of washing with incubation medium containing isoprotenerol, the following washing steps were performed without isoproterenol, but the airways nevertheless remained open and responsive. With this procedure up to 30 slices with open airways were obtained from one guinea pig lung.

4.1.1 Viability of GP PCLS

4.1.1.1 Lactate dehydrogenase-release

Viability of the slices was first determined by measuring the release of lactate dehydrogenase (LDH), a cytosolic protein, into the incubation medium as a marker of cell death over a period of three days (Fig. 4.1). LDH was expressed as percentage LDH in the supernatant according to the following equation: LDH [%] = 100 * LDH [supernatant] / (LDH [tissue] + [supernatant]). LDH leakage remained below 6 % during the three days, when the medium was changed every 24 hours, and was 17 % when the medium remained the same for 72 hours.

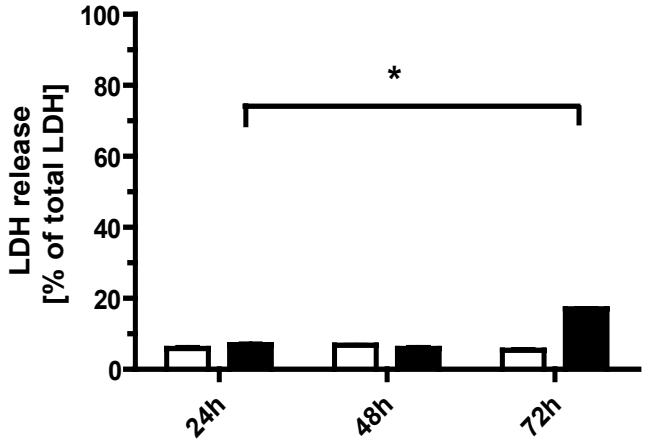

Figure 4.1 LDH-release in guinea pig precision-cut lung slices (PCLS). PCLS were cultured for 72 hours in incubation medium with (open bars, n = 4) or without (filled bars, n = 4) medium change every 24 hours. *, $p < 0.01$, two-sided t-test. Data are mean ± SE.

4.1.1.2 Two-photon microscopy

Second, the viability of the slices was determined by two photon microscopy. Viable slices showed less than 10% dead cells in the second or third cell layer of a slice, even after 72 hours (Fig. 4.2A-C). The first cell layer of a slice was partly disrupted by the slicing procedure. In figure 4.2D the number of all nuclei is visible in Triton-X 100 treated slices, as a positive control for dead cells in a slice. The low and stable number of dead cells over a period of three days indicated again that daily medium change is effective in keeping slices viable for at least three days.

4.1.2 Mediator-induced bronchoconstriction in GP PCLS

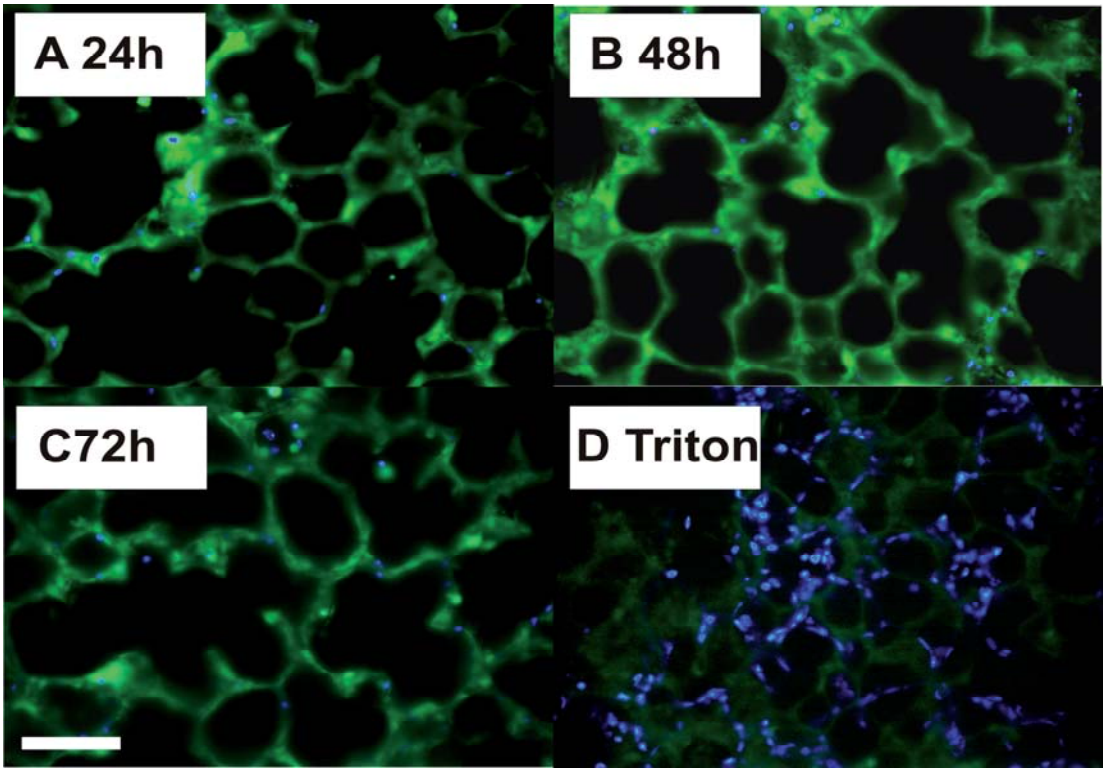

Figure 4.2 Two-photon microscopic image of guinea pig PCLS stained with calcein AM and ethidium homodimer. A 220 µm thin PCLS was loaded with 5 µM calcein/10 µM EthD-1 for 40 min and excited at 800 nm with a femtosecond laser. The images show overlay frames (cytoplasm (green) and nuclei (blue)). Emission was selected by the filters 500/50 and 625/50 for calcein and EthD-1, respectively. Images A to C show a viable PCLS at 24 hours (A), 48 hours (B), and 72 hours (C). Image D captures a PCLS pretreated with 1% Triton X-100 for 20 minutes followed by the LIVE/DEAD® staining. The percentage of nuclei in image A, B, or C compared to the dead slice (image D) was, 8.6%, 4.9%, or 5.5%, respectively. Bar corresponds to 80 µm.

The next step was to examine how the airways from GP PCLS respond to mediators that are relevant in asthma. Therefore cumulative concentration-response curves were performed for different mediators (Fig. 4.3A-E). The biogenic amines serotonin and histamine caused a bronchoconstriction

with EC_{50} values of 69 nM and 217 nM, respectively. Methacholine, the stable acetylcholine analogue, contracted the airways with an EC_{50} value of 231 nM. The stable TP-receptor antagonist U46619 and leukotriene D_4 as representatives for eicosanoids were the most potent bronchoconstrictors with EC_{50} values of 16 nM and 1.8 nM, respectively.

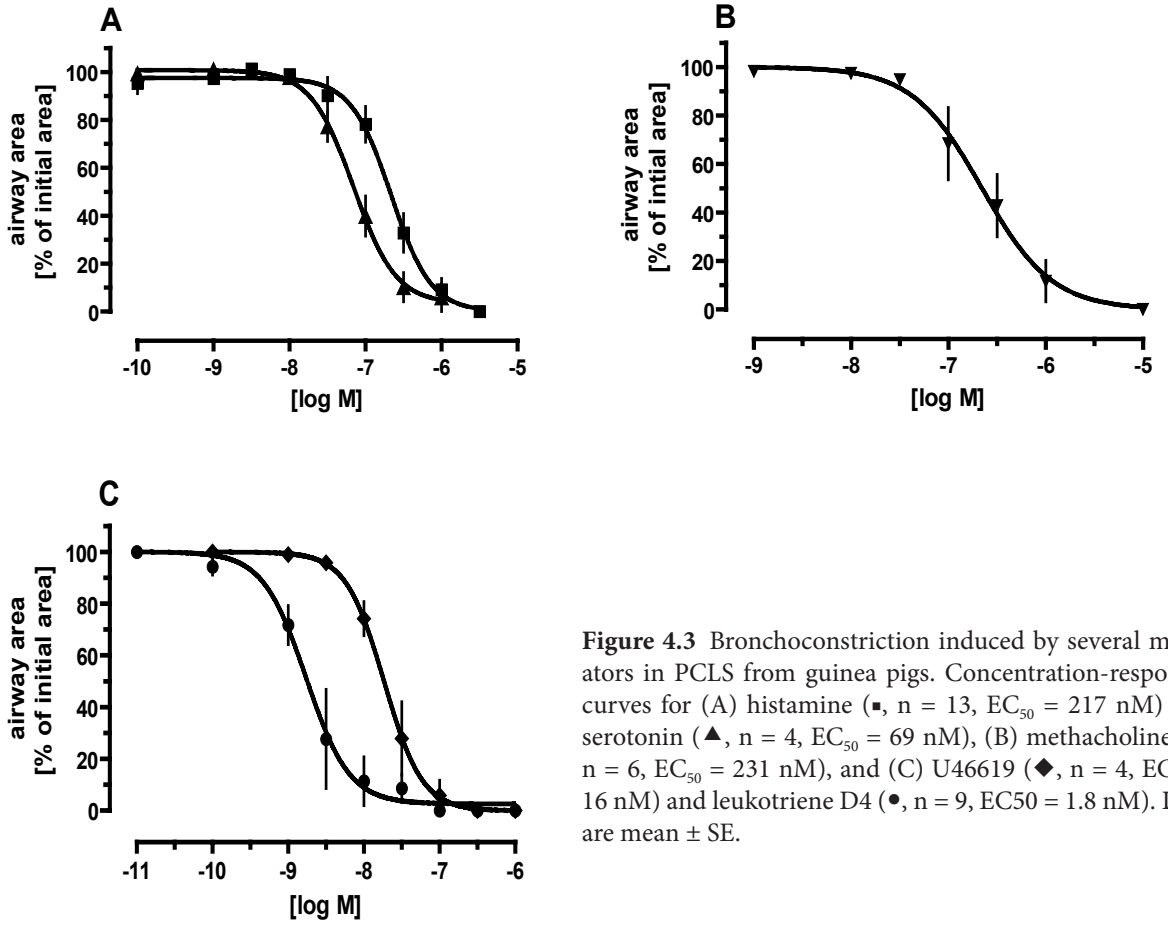

Figure 4.3 Bronchoconstriction induced by several mediators in PCLS from guinea pigs. Concentration-response-curves for (A) histamine (▪, n = 13, EC_{50} = 217 nM) and serotonin (▲, n = 4, EC_{50} = 69 nM), (B) methacholine (▼ n = 6, EC_{50} = 231 nM), and (C) U46619 (◆, n = 4, EC_{50} = 16 nM) and leukotriene D4 (●, n = 9, EC50 = 1.8 nM). Data are mean ± SE.

4.1.3 Relaxation of precontracted airways

β_2-agonists are widely used as spasmolytic agents in asthma therapy. Two commonly used bronchodilators, the β_2-agonist salbutamol and the unspecific phosphodiesterase (PDE)-inhibitor IBMX were used to study relaxation of precontracted airways in guinea pig PCLS. After precontracting airways with methacholine to 10% - 20% of their initial area, salbutamol, IBMX or a combination of both were added cumulatively. These interventions produced a concentration-dependent airway relaxation with significant effects of salbutamol above 100 nM and IBMX at 10 µM (Fig. 4.4A,B). The administration of both bronchodilators showed an additive effect, visible above concentrations of 100 nM each (Fig. 4.4C).

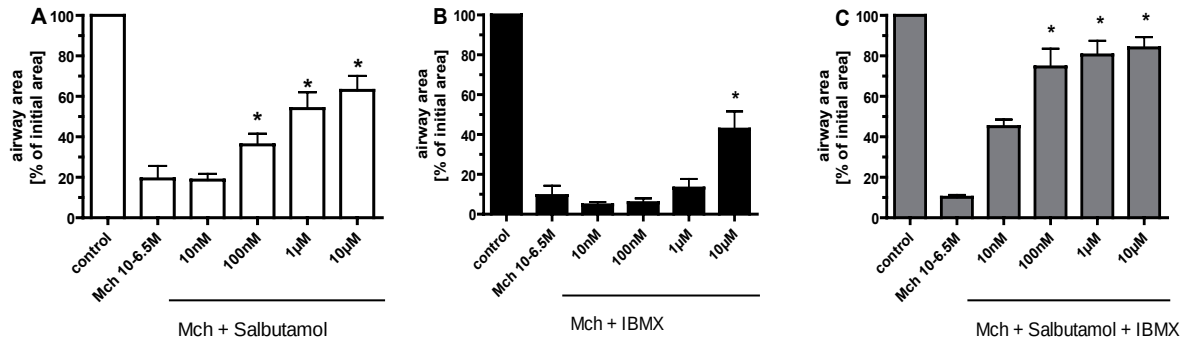

Figure 4.4 Relaxation of precontracted airways in guinea pig PCLS. Methacholine-induced ($10^{-6.5}$ M) airway contraction was reversed by (A) salbutamol (n = 6), (B) IBMX (n = 6) and (C) the combination of both (n = 4). Data are mean ± SE. *, $p<0.05$ vs. Mch alone

4.1.4 Airway and vascular responses within one slice

A remarkable feature of PCLS is the possibility to measure both airway and vascular responses within the same slice. Figure 4.5A and B show the response of an airway, a pulmonary artery and a pulmonary vein to endothelin-1 administrated cumulatively. Pulmonary arteries and veins can be distinguished by their relative position to the airway and by the amount of smooth muscle. The EC_{50} values for the endothelin-1-induced contraction of airway, pulmonary artery and pulmonary vein were 9.6 nM, 37 nM and 10 nM, repectively. As previously observed in slices also from other species [200] as well as in isolated perfused lung models [201] there was some perivascular edema around the pulmonary artery.

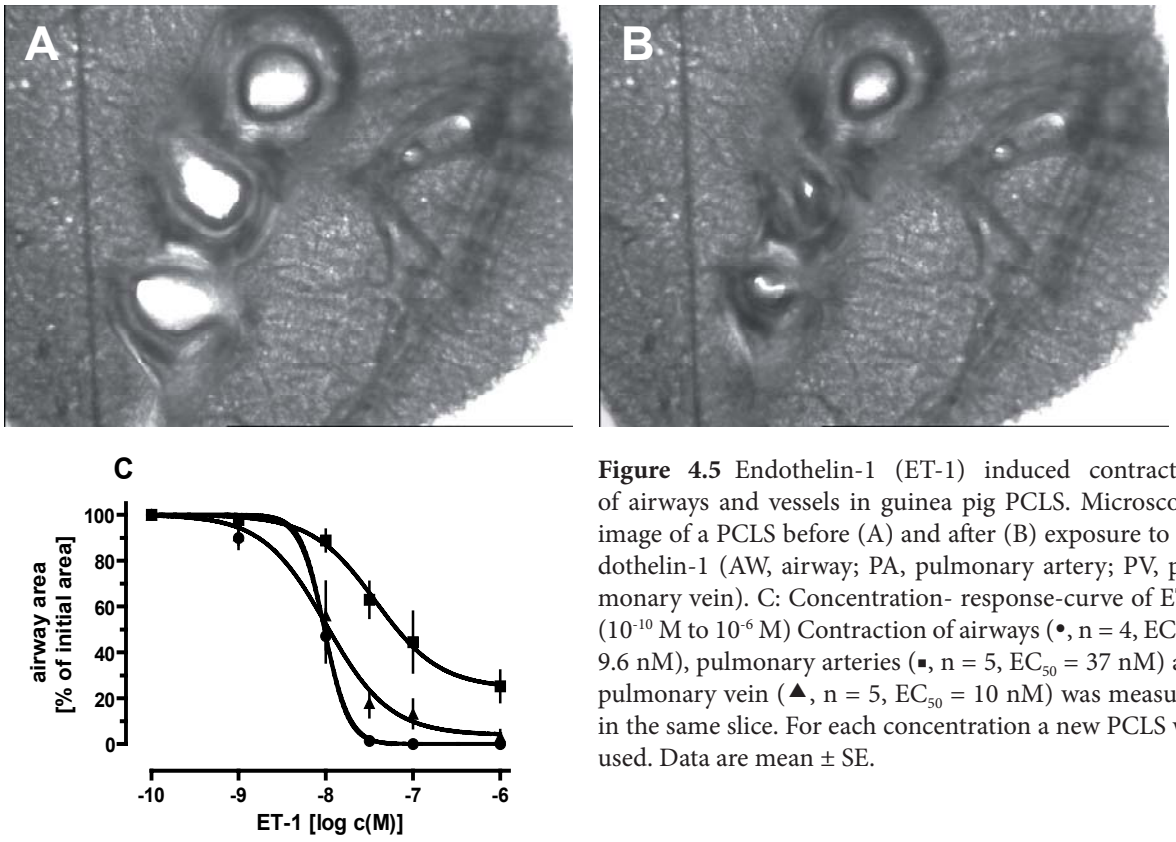

Figure 4.5 Endothelin-1 (ET-1) induced contraction of airways and vessels in guinea pig PCLS. Microscopic image of a PCLS before (A) and after (B) exposure to endothelin-1 (AW, airway; PA, pulmonary artery; PV, pulmonary vein). C: Concentration- response-curve of ET-1 (10^{-10} M to 10^{-6} M) Contraction of airways (•, n = 4, EC_{50} = 9.6 nM), pulmonary arteries (▪, n = 5, EC_{50} = 37 nM) and pulmonary vein (▲, n = 5, EC_{50} = 10 nM) was measured in the same slice. For each concentration a new PCLS was used. Data are mean ± SE.

4.1.5 Induction of the EAR in GP PCLS

The EAR is the initial event in asthma, accompanied by a strong bronchoconstriction. To assess wether an EAR can be evoked in guinea pig PCLS, slices were passively sensitized overnight (16 h) with serum from actively sensitized (ovalbumin) GPs. The subsequent exposure to allergen (ovalbumin) resulted in a concentration-dependent bronchoconstriction with an EC_{50} value for ovalbumin of 3.8 ng/ml (figure 4.6). In contrast to airways, vessels in passive sensitized PCLS were not affected by allergen challenge.

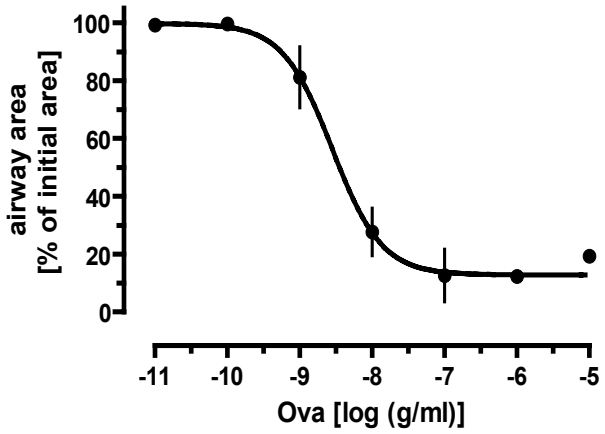

Figure 4.6 Ovalbumin-induced bronchoconstriction in passively sensitized PCLS. Cumulative concentration-response-curve; EC_{50} was 3.8 ng/mL. Similar results were obtained if each ovalbumin concentration was tested in a separate slice (data not shown).

4.2 Part Ib: Data from monkey PCLS

Primate models are used, because it is hoped that they reproduce pathological features of disease such as asthma, better than for instant rodent models. In this study, generation and responsiveness of monkey PCLS was for the first time studied. However, due to the limited supply the shown results are preliminary.

4.2.1 Viability of monkey PCLS

Viability of monkey PCLS was determined by measuring LDH-leakage from the slices into the incubation medium. After 24 hours LDH-leakage was about 7%, and did not exceed 17% within 72 hours, indicating that PCLS from monkeys are viable for at least three days (Fig. 4.7).

Figure 4.7 LDH-leakage in monkey PCLS. Slices were incubated in 24-well plates for at least 72 hours in incubation medium at 37° C and 5% CO_2. Data are mean ± SEM from n = 3 experiments. SEM was so small, that it is not obvious.

4.2.2 Airway responses of monkey PCLS

The airway responses to various stimuli were investigated in PCLS of three different monkey species, cynomolgus monkey (*macaca fascicularis*), baboon (*pavian*), rhesus monkey (*macaca mulatta*), by performing cumulative concentration-response curves for methacholine, serotonin, and histamine (Fig. 4.8A-C). Furthermore the airway reactivity to LTD_4 was investigated (Fig. 4.8D). All three species responded to methacholine, and histamine, but only cynomolgus monkeys and baboons respond also to serotonin, whereas the rhesus monkey shows no reaction. LTD_4 only led to bronchoconstriction in rhesus monkey and baboon, the cynomolgus monkey showed reaction.

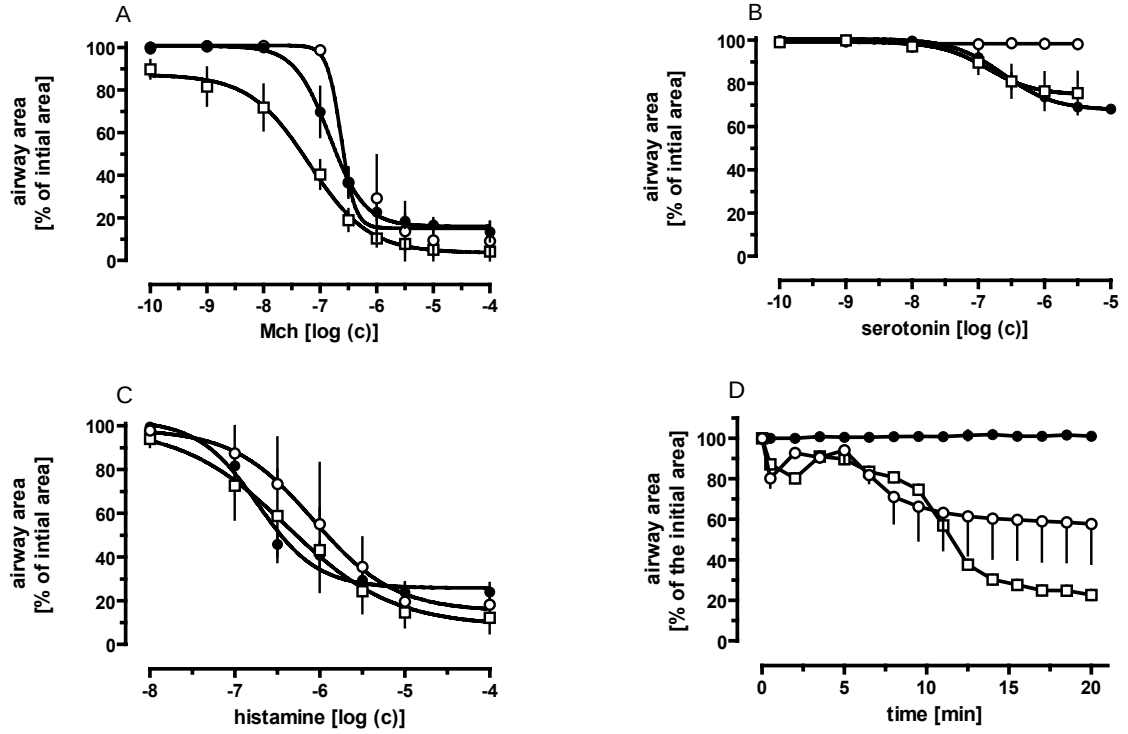

Figure 4.8 Concentration-response curves for methacholine (A), serotonin (B), histamine (C) and (D) leukotriene D_4 –induced bronchoconstriction in PCLS from different monkey species: cynomolgus monkey (•), baboon (□), and rhesus monkey (○). Data are mean ± SEM from n = 2 to 5. In case of rhesus monkey n = 2. The corresponding EC_{50} values are (A) 0.15 µM (cynomolgus monkey), 0.06 µM (baboon), and 0.24 µM (rhesus monkey); (B) 0.24 µM, 0.14 µM, and no reaction, respectively; (C) 0.19 µM, 0.41 µM, and 0.85 µM, respectively.

4.3 Part II: The EAR in different species

The early allergic response is a key event in asthma. This phase is characterized by an IgE-mediated mast cell degranulation combined with the release of mediators. Some of these mediators are strong bronchoconstrictors. To characterize and compare the mechanisms of bronchoconstriction during an EAR, experiments with slices of different species (GP, human, rat, and mouse) were performed. For induction of the EAR three different PCLS-based models were used:

1. A chemical stimulus leading to mast cell degranulation (Compound 48/80)
2. Passive sensitization and allergen challenge
3. Active sensitization and allergen challenge

To identify the mediators responsible for the bronchoconstriction, slices were preincubated with different receptor antagonists before administration of the stimulus (Compound 48/80 or allergen).

4.3.1 C48/80

Compound 48/80 is reported to induce mast cell exocytosis by different actions, such as direct activation of guanosine triphosphate binding proteins (G-proteins) [202], destabilization of the mast cell membrane, or action as calcium ionophore.

4.3.1.1 C48/80-induced bronchoconstriction

To see how airways of different species respond to C48/80, cumulative concentration-response curves were carried out in PCLS. The responses of GP, rat, human and murine PCLS to C48/80 are shown in figure 4.9.

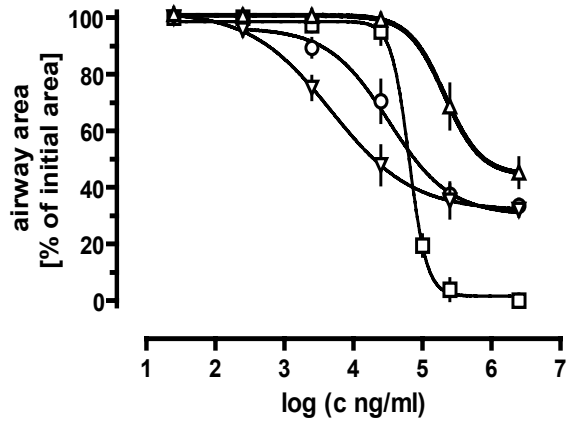

Figure 4.9 Concentration-response curves of C48/80-induced bronchoconstriction. A range of 25 ng/ml to 2.5 mg/ml was used. The effect of C48/80 was studied in 4 different species, mouse (△), rat (▽), guinea pig (□) and human lung slices (○). All data are mean ± SEM from 3 to 15 different lungs.

The four investigated species showed a different reactivity to C48/80. Rat and human PCLS responded already at lower concentrations (2.5 µg/ml), whereas a bronchoconstriction in guinea pig slices was obtained only beyond 100 µg/ml, and in murine PCLS beyond 2.5 mg/ml C48/80.

4.3.1.2 Viability of C48/80-treated PCLS

To examine, whether the concentrations of C48/80 are toxic for the PCLS, LDH leakage was measured. PCLS from guinea pigs, rats, humans, and mice were incubated overnight (16h) with C48/80 in concentrations ranging from 10 µg/ml to 2.5 mg/ml. The LDH-leakage increased in rat slices at 200 µg/ml, in GP and human slices at 50 µg/ml and in murine slices at 100µg/ml compared to untreated control slices (Fig. 4.10).

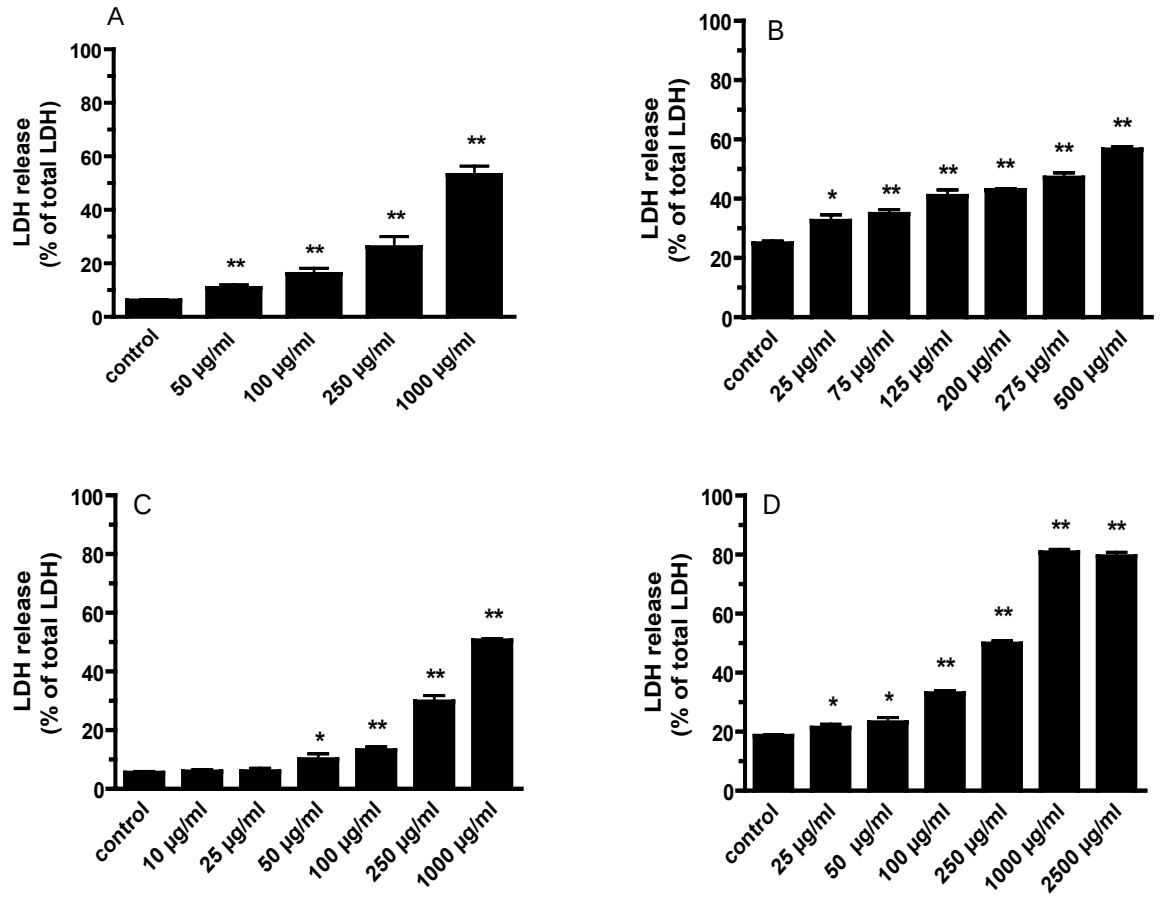

Figure 4.10 Influence of different concentrations of C48/80 on the viability of PCLS from guinea pigs (A, n = 6), rats (B, n = 3), humans (C, n =3), and mice (D, n =3). The slices were incubated overnight (16 h) with different concentrations of C48/80. The next day LDH-release was determined. Data are presented as mean ± SEM. **, $p < 0.01$ vs. solvent; *, $p < 0.05$ (one-sided t-test corrected by fdr procedure).

4.3.1.3 Mediators of the EAR induced by C48/80

To identify the mediators responsible for the bronchoconstriction triggered by C48/80 in the different species, PCLS from GP and humans were preincubated with receptor antagonists 20 minutes before C48/80 was added. Figure 4.11 shows the data obtained for these two species.

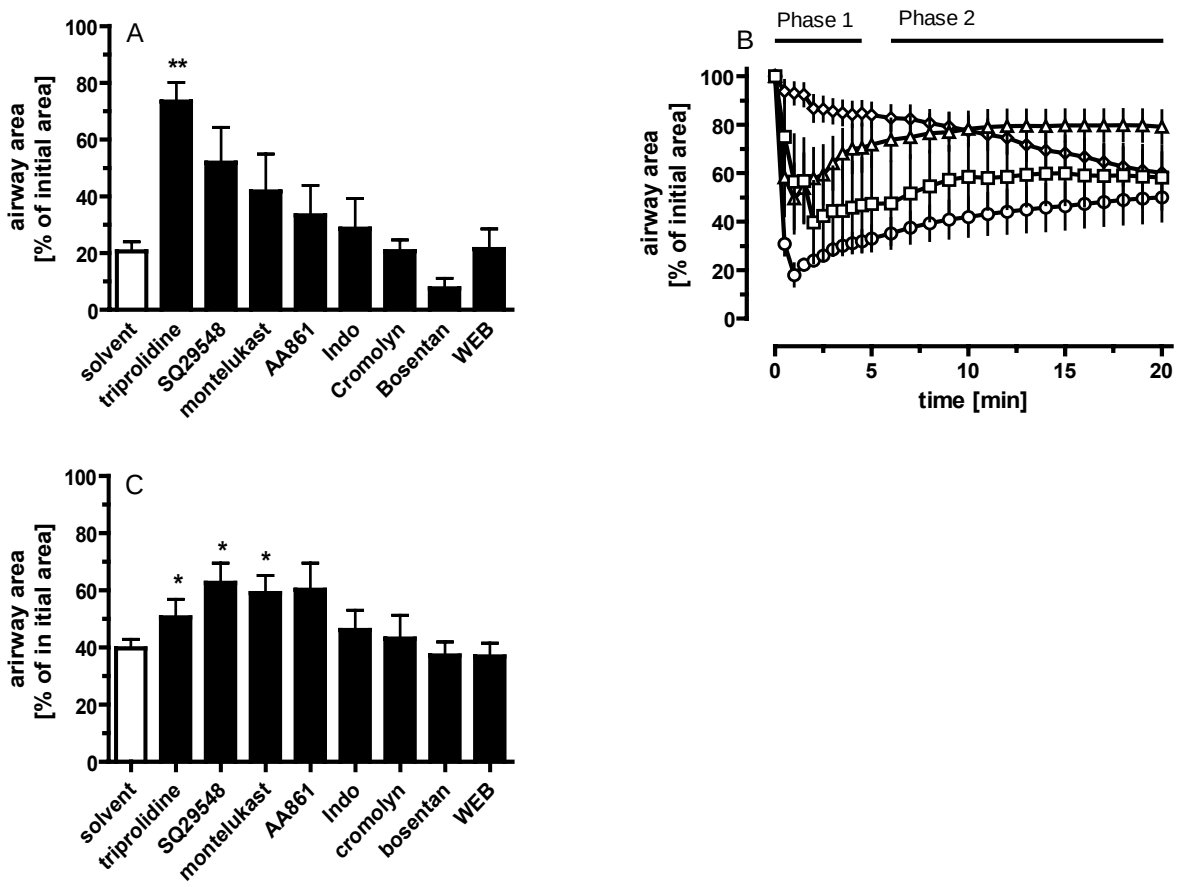

Figure 4.11 Influence of various inhibitors on C48/80-induced bronchoconstriction in guinea pig (A), and human (C) PCLS. Slices from guinea pig and human lungs were pretreated with various inhibitors 20 min before they were challenged with 100 µg/ml C48/80. Shown are the effects of the H_1-receptor antagonist triprolidine (5 µM), the TP-receptor antagonist SQ29548 (10 µM), the $CysLT_1$-receptor antagonist montelukast (10 µM), the 5-lipoxygenase inhibitor AA861 (10 µM), the cyclooxygenase inhibitor indomethacin (Indo, 10 µM), the mast cell stabilizing agent cromolyn (100 µM), the endothelin-receptor antagonist bosentan (100 µM), and the PAF-receptor antagonist WEB2086 (66 µM). (B) shows the kinetic effects of triprolidine (◇, 5 µM), SQ29548 (△, 10 µM), and montelukast (□, 10 µM) on the C48/80-induced bronchoconstriction compared to a control slice (○). Data are presented as mean ± SEM. **, $p< 0.01$ vs. solvent; *, $p< 0.05$ (one-sided t-test corrected by the fdr procedure).

For rats, it was already known, that the C48/80-induced bronchoconstriction is almost completely blocked by the 5-HT_2-receptor antagonist ketanserin [70], indicating that serotonin is the main mediator. C48/80-induced bronchoconstriction in GP slices was reduced by pre-incubation with the H_1-receptor antagonist triprolidine, and also with the thromboxane/prostanoid (TP)-receptor antagonist SQ29548, suggesting that both mediators are mainly responsible for bronchoconstriction elicited by C48/80 in this species (Fig. 4.11A). Other inhibitors, such as the $CysLT_1$-receptor antagonist montelukast, the 5-lipoxygenase inhibitor AA861, or the cyclooxygenase inhibitor indomethacin had no effect. Also the PAF-receptor antagonist WEB2086 and the endothelin-receptor antagonist

bosentan, as well as cromolyn, a substance stabilizing mast cells, failed to prevent C48/80-induced bronchoconstriction in the GP. These measurements were all done 20 minutes after administration of C48/80. As figure 4.11B shows, this can be misleading, because there appear to be two phases that are differently affected by the different inhibitors. The initial phase of bronchoconstriction was affected by triprolidine, whereas a second lasting phase of bronchoconstriction was affected by SQ29548 and montelukast (Fig. 4.11B).

In human PCLS pre-incubation with SQ29548, montelukast, AA861, or triprolidine reduced C48/80-induced bronchoconstriction, indicating a role for thromboxane, leukotrienes and partly histamine as mediators for that bronchoconstriction (Fig. 4.11C). The other inhibitors tested showed no effect.

Murine PCLS responded only at very high concentrations of C48/80 (Fig. 4.9), leading to the assumption that the bronchoconstriction following this stimulus occurs independent of mast cell degranulation. Therefore the airway responses to C48/80 were investigated in PCLS of mast cell-deficient mice compared to wild type mice (Fig. 4.12). No differences were detected between both mouse strains, indicating a mast cell-independent bronchoconstriction in murine PCLS by C48/80. These experiments were performed in collaboration with M. Maurer and his group (Charité, Berlin, Germany), who kindly provided theses mice.

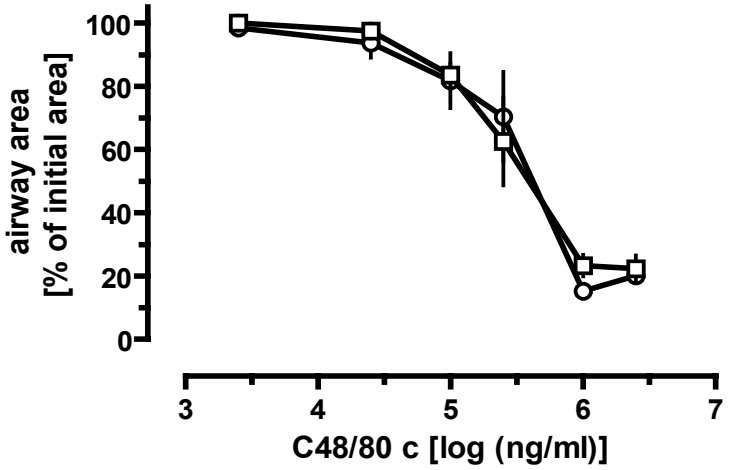

Figure 4.12 Concentration-response curves for C48/80-induced bronchoconstriction in wild type (○), and mast cell-deficient (□) mice. Slices were treated with different concentrations of C48/80 in a cumulative manner. Data are presented as mean ± SEM.

To further investigate whether C48/80 degranulates mast cells, PCLS from the different species were incubated for 5 minutes with C48/80 (25 µg/ml), before supernatant was collected and histamine and serotonin levels in the medium measured by ELISA (table 4.1). In mice, histamine levels were increased 2-fold compared to control values after addition of C48/80, whereas serotonin levels remained unchanged. In rat slices both mediators increased about 10-fold after C48/80 compared to unstimulated slices. GP slices also showed an enhancement of histamine and serotonin levels after C48/80, even though the increase was smaller than in rats. An increase of histamine and serotonin levels was also observed in human PCLS, whereas in monkeys no effect of C48/80 on histamine and serotonin release was observed.

Table 4.1 Histamine and Serotonin-release from precision-cut lung slices (PCLS)

Spezies		Serotonin				Histamin			
	Treatment	Mean	SD	N	P-value	Mean	SD	N	P-value
Mouse	Con	19.82	6.49	4		137.52	88.43	7	
	C48/80	25.96	8.18	4	0.28	262.25	109.63	7	0.037
Rat	Con	10.04	8.84	3		53.04	47.35	6	
	C48/80	283.05	57.45	3	0.012	641.19	212.93	6	0.0004
GP	Con	9.08	13.75	3		214.39	42.24	3	
	C48/80	33.55	3.76	3	0.041	291.85	18.46	3	0.043
Human NR	Con	17.60	15.65	3		55.50	42.42	4	
	C48/80	17.89	3.18	3	0.97	62.29	52.42	4	0.63
Human Responder	Con	7.07	1.51	3		14.13	2.79	3	
	C48/80	143.18	17.19	3	0.005	451.93	109.03	3	0.02
Monkey	Con	14.65	12.83	3		123.64	107.38	3	
	C48/80	15.03	13.49	3	0.97	217.48	12.29	3	0.26

Table 4.1 Histamine and serotonin release from PCLS from mice, rats, guinea pigs, humans (non-responder (NR), and responder), and monkeys measured by ELISA. Slices were incubated for 5 minutes with 100 µg/ml C48/80. Supernatant and tissue were collected and snap-frozen in liquid nitrogen and stored at -80°C until use. Data are shown as mean ± SD; $p < 0.05$ (one-sided t-test corrected by the fdr procedure) was considered significant.

4.3.2 Passive sensitization

Another possibility to study the mechanisms of the EAR is passive sensitization of PCLS. An EAR can principally be induced in passively sensitized rat and human PCLS as shown before [178,196]. An EAR can be also evoked in passively sensitized GP PCLS as described above (4.1.5). In murine PCLS, passive sensitization and subsequent challenge with allergen (ovalbumin 1mg/ml) next day failed to induce a bronchoconstriction, indicating that an EAR cannot be induced through passive sensitization of PCLS from mice (data not shown). These findings suggest that the early allergic bronchoconstriction in mice is independent of IgE and mast cells. Monkey PCLS were passively sensitized with serum from a human atopic patient, but no airway response was observed after allergen challenge the next day. Figure 4.13 shows images and time courses of passively sensitized lung slices of the different species before and after administration of allergen. In GP PCLS, the maximum airway contraction (20% of the initial area) was detected only one minute after allergen challenge, followed by a continuous relaxation to finally 40% of the initial area after 20 minutes. Airways in rat PCLS contracted also immediately within the first 2 minutes after allergen challenge; this contraction was stable for the next ten minutes, until the airway relaxes again on finally 80% of the initial area. In human PCLS a maximum airway contraction occured 5 minutes after allergen challenge and remained stable over a period of 20 minutes. In passively sensitized monkey PCLS no airway response to pollen extract was observed within 20 minutes after challenge.

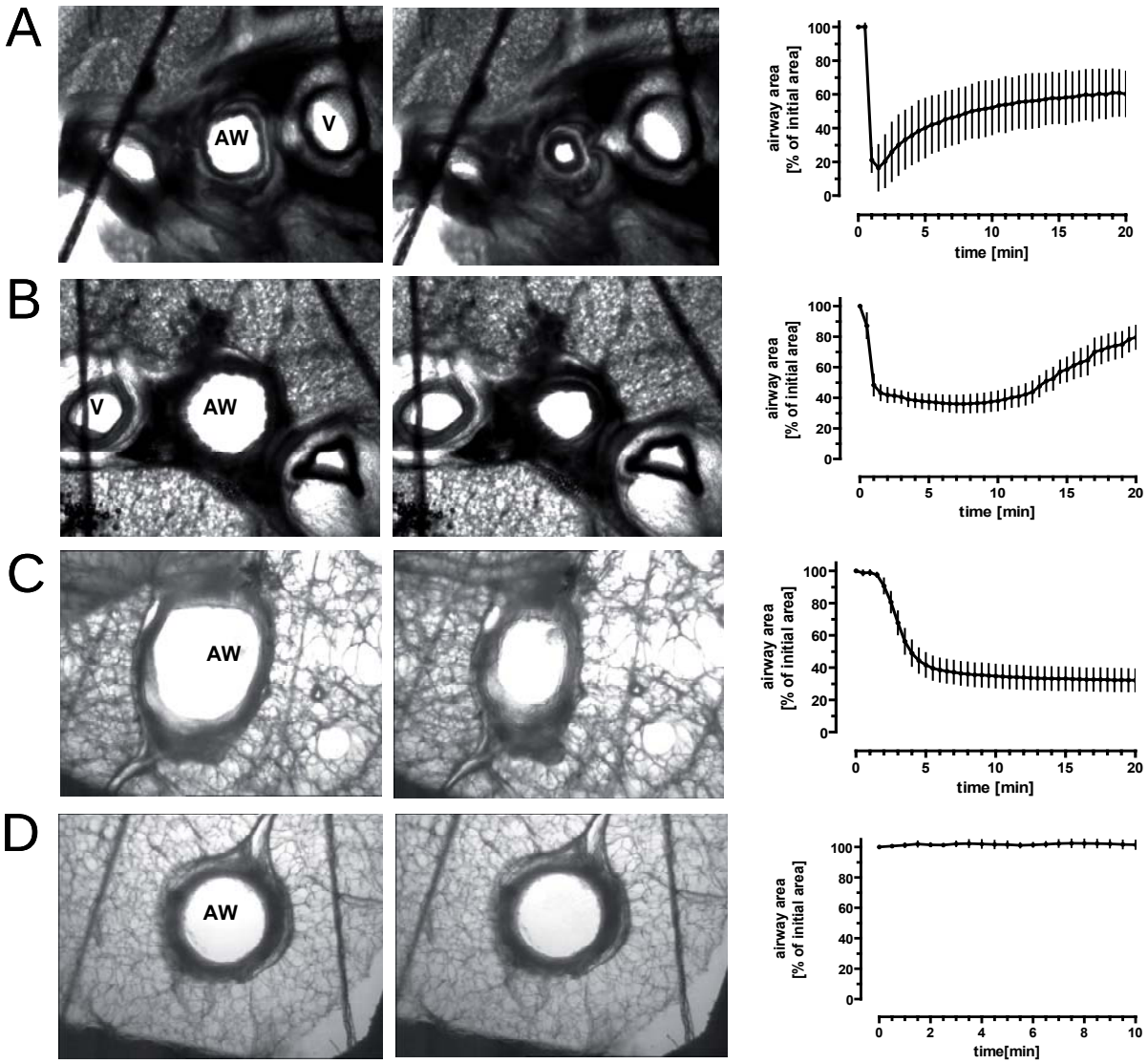

Figure 4.13 Video images and time courses of allergen-induced bronchoconstriction in passively sensitized PCLS from guinea pigs (A), rats (B), humans (C), and monkeys (D). Slices were sensitized overnight with 1% anti-Ova serum in case of guinea pigs and rats, and with 1% serum from an atopic donor in case of humans and monkeys. The following day slices were challenged with the specific antigen (ovalbumin 1 mg/ml or pollen extract 5 µg/ml), and bronchoconstriction was monitored for 20 minutes. The left row shows airways before, the middle row 3 minutes after allergen challenge. In the right graphs the corresponding time courses of bronchoconstriction are presented. AW = airway, V = vessel.

Mast cell degranulation during EAR leads to the release of preformed mediators and the *de novo* synthesis of lipid mediators. Our interest was now to investigate and compare which mediators are responsible for the bronchoconstriction during EAR in the different species.

4.3.2.1 Mediators of the allergen-induced EAR in rat PCLS

For the rat it was already known that serotonin is the main mediator causing bronchoconstriction during the EAR [196]. In line with this, allergen-induced bronchoconstriction was completely prevented with ketanserin or LY53857, two different 5-HT_2-receptor antagonists (Fig. 4.14). Triprolidine, the H_1-receptor antagonist, montelukast or AA861, the $CysLT_1$-receptor antagonist and leukotriene synthesis inhibitor alone had no effect. Pre-incubation with the TP-receptor antagonist, SQ29548 showed a small inhibitory effect at a low dose of allergen. These data further confirm that bronchoconstriction during the early allergic response in the rat is mainly mediated by serotonin, but thromboxane might play a small role as well. Further studies in cooperation with A. Larsson (on the leave from the Karolinska Institute, Stockholm, Sweden) showed that inhibition of the cyclooxygenase attenuates bronchoconstriction induced by allergen in passively sensitized rat PCLS. A reduction in bronchoconstriction was observed when the slices were preincubated with the EP3-receptor antagonist ONO8713, indicating that the bronchoconstriction during the EAR in rats is partly mediated by the EP3-receptor (data not shown, Manuscript in preparation).

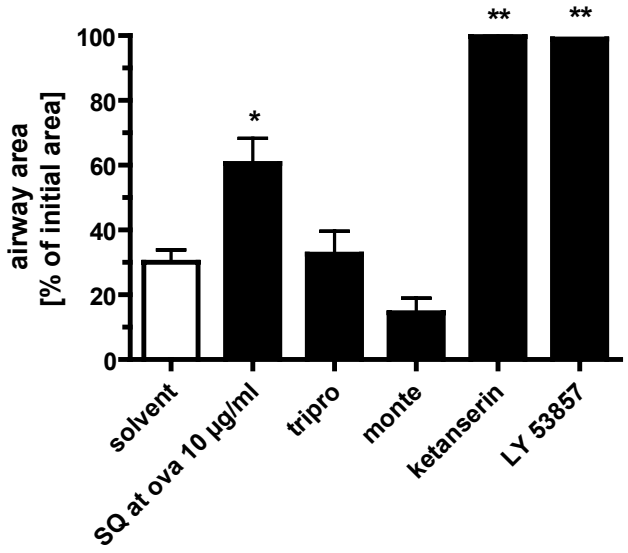

Figure 4.14 Influence of inhibitors on ovalbumin-induced airway contraction in rat PCLS. Slices were sensitized overnight with 1% anti-ova serum. Next day the slices were pre-incubated with SQ29548 (10 µM), triprolidine (5 µM), montelukast (10 µM), or two 5-HT_2-receptor antagonists, ketanserin (1 µM) or LY53857 (1 µM), before challenge with 1 mg/ml ovalbumin. Data are presented as mean ± SEM. **, $p < 0.01$ vs. solvent; *, $p < 0.05$ (one-sided t-test corrected by fdr procedure).

Serotonin levels in PCLS from rats showed a 10-fold lower increase of serotonin after allergen exposure compared to the strong increase after C48/80 (Table 4.1, Fig. 4.15). Histamine levels showed a profile after allergen or C48/80 challenge similar to serotonin levels (Fig. 4.15B).

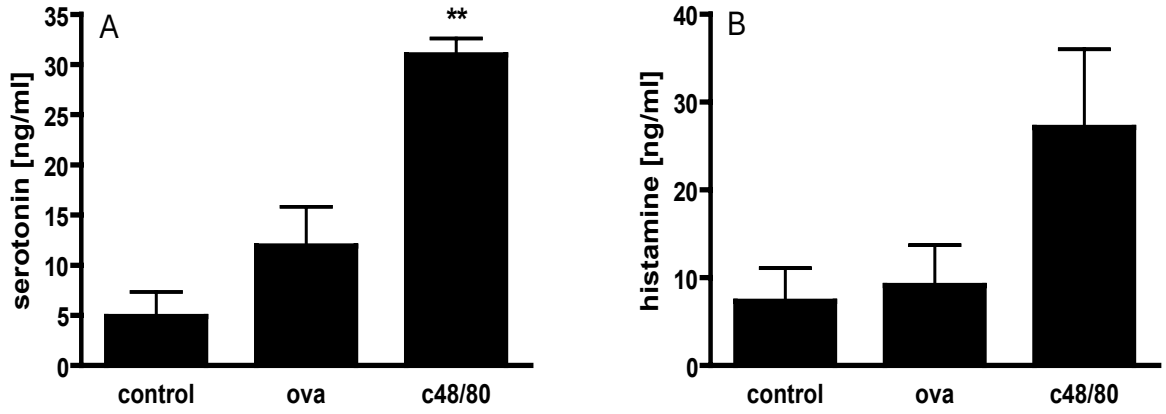

Figure 4.15 Increase of serotonin (A) and histamine (B) levels detected by ELISA in passively sensitized rat PCLS after allergen challenge (ovalbumin) for 5 minutes, and unsensitized PCLS challenged with 100 µg/ml C48/80 for 5 minutes. Supernatants were collected, snap-frozen in liquid nitrogen and stored at -80° C until use. Data are mean ± SEM. **, p< 0.01 vs. control; *, p< 0.05 (one-sided t-test corrected by fdr procedure)

4.3.2.2 Mediators of the allergen-induced EAR in human PCLS

For passive sensitization of human slices, PCLS were incubated overnight with serum from an atopic patient containing a high amount of IgE specific for timothy grass pollen (*phleum pretense,* 450 IU/ml). A challenge with pollen extract (5µg/ml) the next day always induced a bronchoconstriction within the first 5 minutes (Fig. 4.13).

A pre-incubation of human PCLS with the H_1-receptor antagonist triprolidine, the TP-receptor antagonist SQ29548, the $CysLT_1$-receptor antagonist montelukast, and the 5-HT_2-receptor antagonist ketanserin alone, did not influence the allergen-induced bronchoconstriction in passive sensitized PCLS. In contrast, pre-incubation with the combination of $CysLT_1$-receptor antagonist montelukast and TP-receptor antagonist SQ29548 almost completely inhibited bronchoconstriction (figure 4.16). These data are in line with the results from Wohlsen *et al.* [178], indicating that the main mediators for the bronchoconstriction in human PCLS are thromboxane and leukotrienes.

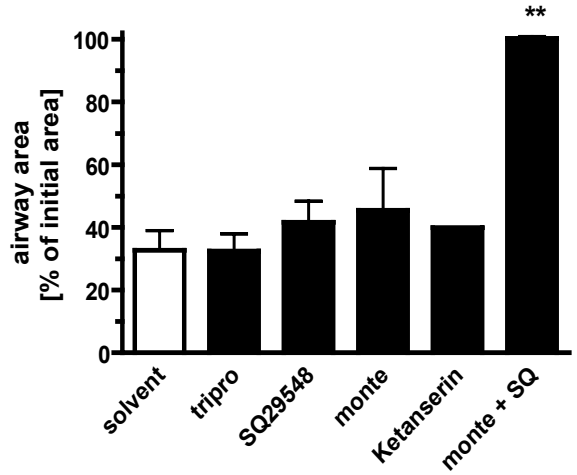

Figure 4.16 Influence of inhibitors on pollen-extract-induced bronchoconstriction in human PCLS. Slices were incubated overnight with 1% serum from an atopic patient (IgE 450 IU/ml). Next day the slices were pre-incubated with triprolidine (5 µM), SQ29548 (10 µM), montelukast (10 µM), ketanserin (1 µM), or the combination of SQ29548 and montelukast before challenge with 5 µg/ml pollen extract. Data are presented as mean ± SEM. **, p< 0.01 vs. solvent; *, p< 0.05 (one-sided t-test corrected by fdr procedure)

Nevertheless, serotonin and histamine levels were increased after allergen and C48/80 challenge, as shown in figure 4.17.

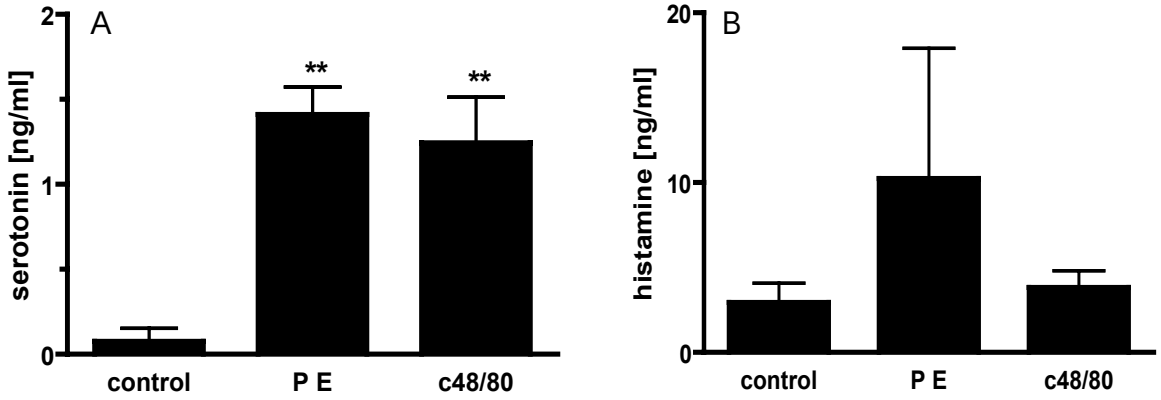

Figure 4.17 Increase of serotonin (A) and histamine (B) levels detected by ELISA in passively sensitized human PCLS after allergen challenge (pollen extract, PE) for 5 minutes, and unsensitized PCLS challenged with 100 µg/ml C48/80 for 5 minutes,. Supernatants were collected, snap-frozen in liquid nitrogen and stored at -80° C until use. Data are mean ± SEM. **, $p < 0.01$ vs. control; *, $p < 0.05$ (one-sided t-test corrected by fdr procedure)

4.3.2.3 Mediators in the allergen-induced EAR in GP PCLS

As already shown above (Fig 4.6A), passively sensitized GP PCLS show a bronchoconstriction after exposure to allergen (ovalbumin). In order to analyze the mediators responsible for inducing the bronchoconstriction during the EAR, passively sensitized GP PCLS were preincubated with three different inhibitors. The H_1-receptor antagonist triprolidine, the TP-receptor antagonist SQ29548, and the $CysLT_1$-receptor antagonist montelukast were used either alone or in all possible combinations (Fig. 4.18A). Preincubation of passively sensitized PCLS with triprolidine or SQ29548 alone before allergen challenge, attenuated the bronchoconstriction. The combination of these both antagonists led to an additive inhibitory effect. Montelukast alone did not influence the bronchoconstriction, but was effective in combination with triprolidine. All three antagonists together almost completely prevent the allergen-induced EAR in passive sensitized PCLS. The airway contraction by allergen showed a biphasic response. Triprolidine attenuated the initial phase of airway contraction as also shown for the C48/80-induced bronchoconstriction (Fig. 4.11B), whereas SQ29548 and montelukast affected a second lasting phase of contraction (Fig. 4.18B).

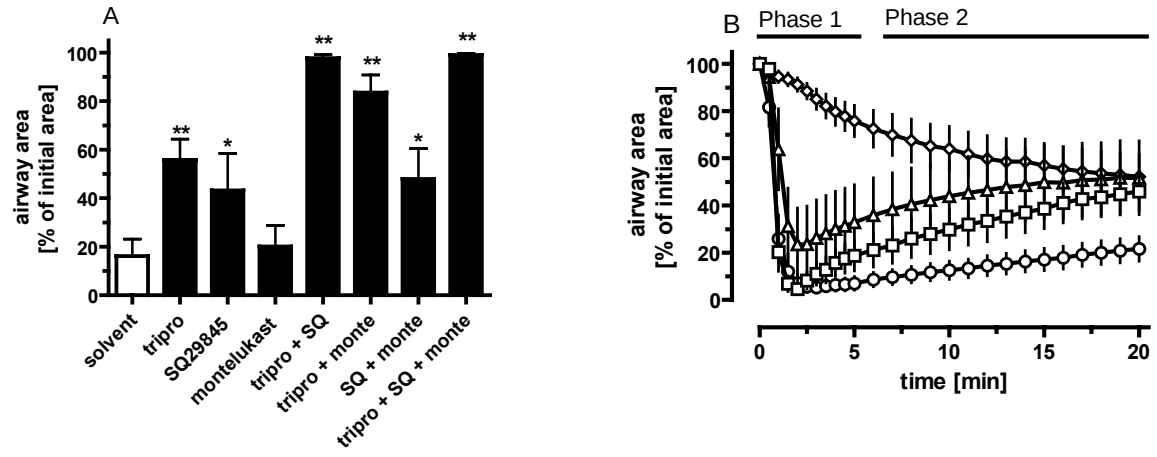

Figure 4.18 (A) Pharmacological inhibition of allergen-induced bronchoconstriction in guinea pig PCLS. Shown are the effects of the H_1-receptor antagonist triprolidine (5 µM, tripro), the thromboxane-prostanoid receptor antagonist SQ29548 (10 µM, SQ) and the $CysLT_1$-receptor antagonist montelukast (10µM, monte) alone or in combination. (B) shows the kinetic effects of triprolidine (◇, 5 µM), SQ29548 (△, 10 µM), and montelukast (□, 10 µM) on the allergen-induced bronchoconstriction compared to a control slice (○), thereby two phases of bronchoconstriction were identified. **, $p < 0.01$ vs. solvent; *, $p < 0.05$ (one-sided t-test corrected by fdr procedure). Data are mean ± SE from 4 to 13 independent experiments.

Also in GP PCLS the levels of serotonin and histamine after allergen exposure were determined, compared to the control levels and to those after an C48/80 stimulus. Figure 4.19 shows enhanced histamine levels after allergen challenge from 2.78 ± 1.31 ng/ml under control conditions to 5.88 ± 1.09 ng/ml. No increase was detected after administration of C48/80. Serotonin levels were unchanged after allergen or C48/80 challenge.

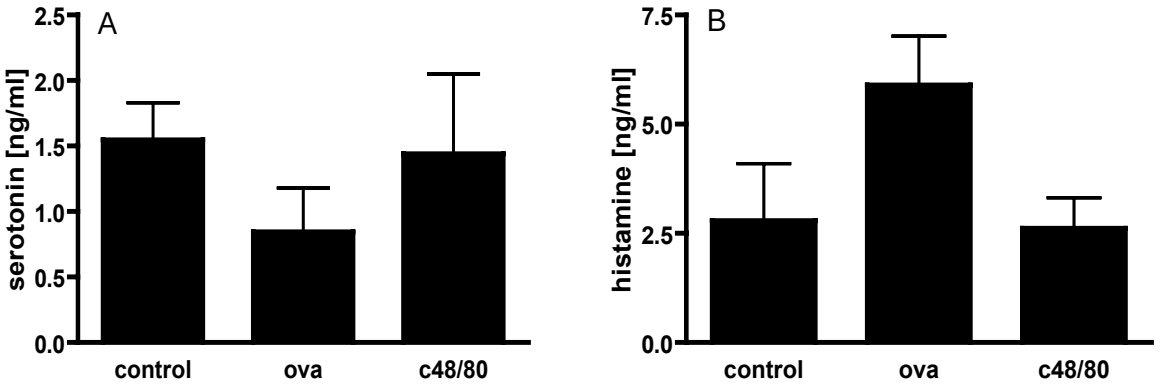

Figure 4.19 Increase of serotonin (A) and histamine (B) levels detected by ELISA in passively sensitized guinea pig PCLS after allergen challenge (ovalbumin) for 5 minutes, and unsensitized PCLS challenged with 100 µg/ml for 5 minutes,. Supernatants were collected, snap-frozen in liquid nitrogen and stored at -80° C until use. Data are mean ± SEM. **, $p < 0.01$ vs. control; *, $p < 0.05$ (one-sided t-test corrected by fdr procedure).

4.3.3 Active sensitization

Another possibility to investigate the EAR and in addition the AHR is the use of actively sensitized animals. These experiments were only performed in mice and rats (in collaboration with A. Braun from the ITEM in Hannover). In contrast to passively sensitized mice, PCLS from actively sensitized mice responded to exposure of allergen with a bronchoconstriction during the first 2 minutes after challenge (Fig. 4.20).

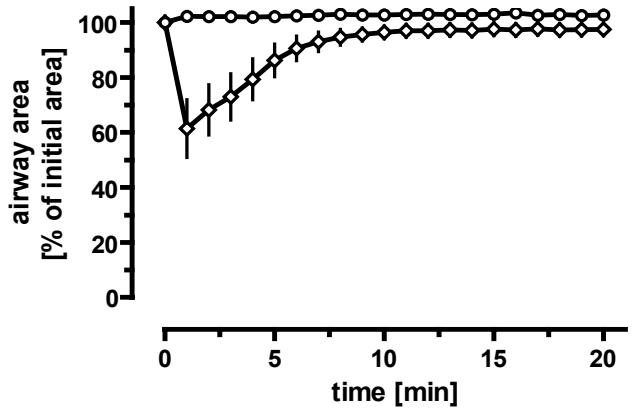

Figure 4.20 Time-course of allergen-induced bronchoconstriction in PCLS from actively sensitized mice (◇) and control mice (○). Animals underwent a 35 day standard sensitization protocol. Data are presented as mean ± SEM from 8 (sensitized mice) and 4 (control mice) independent experiments.

In addition we produced slices from actively sensitized rats subjected to a standard 21 day sensitization protocol. Following allergen provocation, a bronchoconstriction with reduction of airway area to about 40% of the initial area was observed after allergen administration in slices of sensitized animals compared to slices of non-sensitized animals (Fig. 4.21).

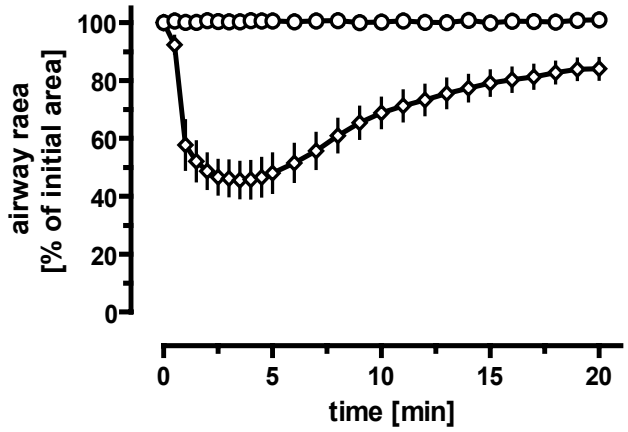

Figure 4.21 Time-course of allergen-induced bronchoconstriction in PCLS of actively sensitized rats (◇) and control rats (○). Animals underwent a 21 day standard sensitization protocol. Data are presented as mean ± SEM from 8 (sensitized rats) and 4 (control rats) independent experiments.

4.3.3.1 Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) is a pathophysiological feature of asthma and characterized by an increase of airway reactivity to various stimuli. We investigated in PCLS from actively sensitized and control animals, whether sensitization modulates airway responsiveness.

4.3.3.1.1 Mice: Airway hyperresponsiveness

PCLS of control and sensitized mice were challenged with methacholine, but no differences were observed (Fig. 4.22A), indicating that sensitization alone was not sufficient to increase airway reactivity in murine PCLS. However, 24 hours after an EAR was provoked with the medium remaining on the slices overnight, airway responsiveness was altered (Fig. 4.22B). A shift to the right of the methacholine concentration-response curve was observed for sensitized airways compared to the control airways, implying a hyporeactivity of sensitized airways in which an EAR had occurred. Responsiveness to adenosine is a particular feature in atopic patients. However, murine PCLS failed to respond to adenosine before, or 24 hours after an EAR (Fig. 4.22C, D), although the concentration of 10 mM adenosine was very high.

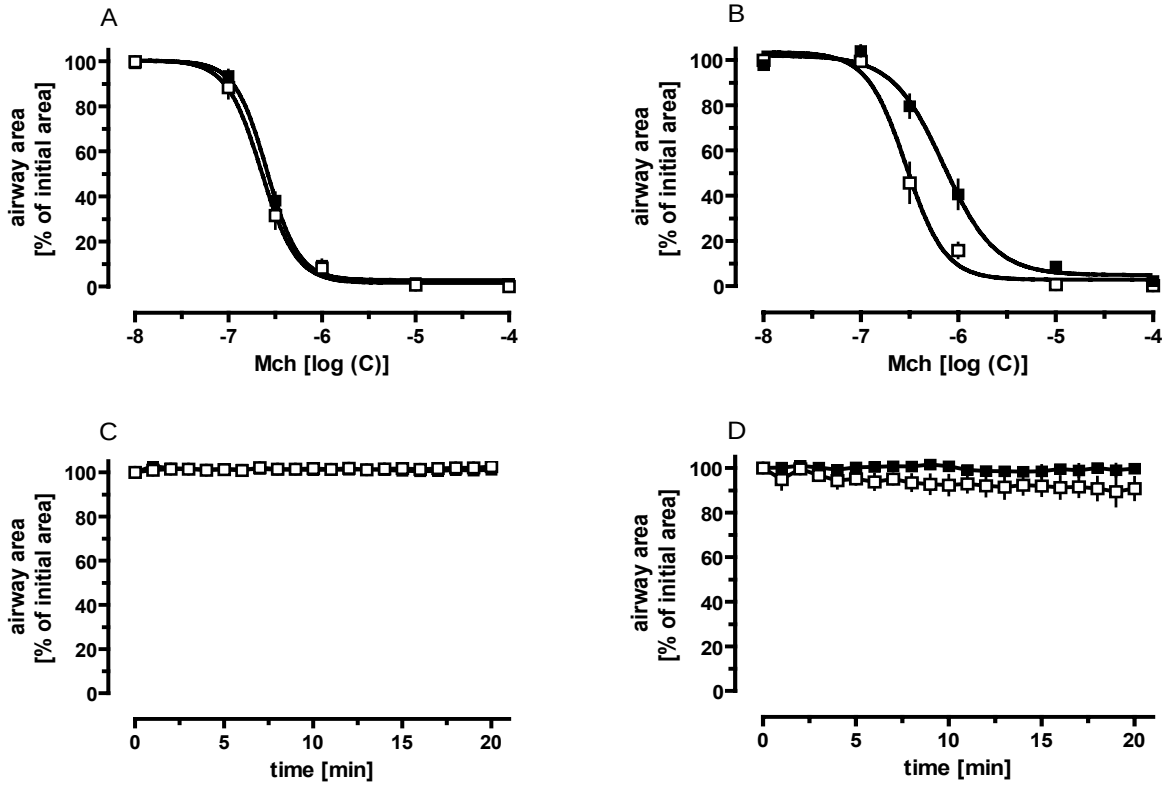

Figure 4.22 Airway responses in PCLS of control (□) and actively sensitized (■) mice. (A) and (B) showed concentration-response curves for methacholine before (A) and 24 hours after (B) an induced EAR. The supernatant after induction of the EAR remained on the slice. The corresponding EC_{50} values were (A) 0.23 µM (control) and 0.26 µM (sensitized mice); (B) 0.29 µM and 0.71 µM, respectively. Data are presented as mean ± SEM from minimum 8 independent experiments. (C) and (D) showed time courses for 10 mM adenosine before (C) and 24 hours after (D) induction of an EAR. Data are from minimum 2 to 16 experiments and are presented as mean ± SEM.

4.3.3.1.2 Rats: Airway hyperresponsiveness

Next, the airway reactivity towards methacholine, adenosine, and to the thromboxane mimetic U46619 was investigated in PCLS from control and actively sensitized rats (Fig. 4.23). For methacholine no differences between control and sensitized airways were detected, suggesting that also in rats, sensitization alone was not sufficient to enhance airway responses. However, the challenge with methacholine 24 hours after an EAR showed an increased reactivity in sensitized airways (Fig. 4.23B). Airways in control rat slices did not respond to adenosine, whereas sensitized airways showed a contraction to 80% of the initial airway area within the first minute after adminiatration followed by a slow relaxation during the following 20 minutes (Fig. 4.23C). Control airways and sensitized airways responded in a similar fashion to U46619 (Fig. 4.23D).

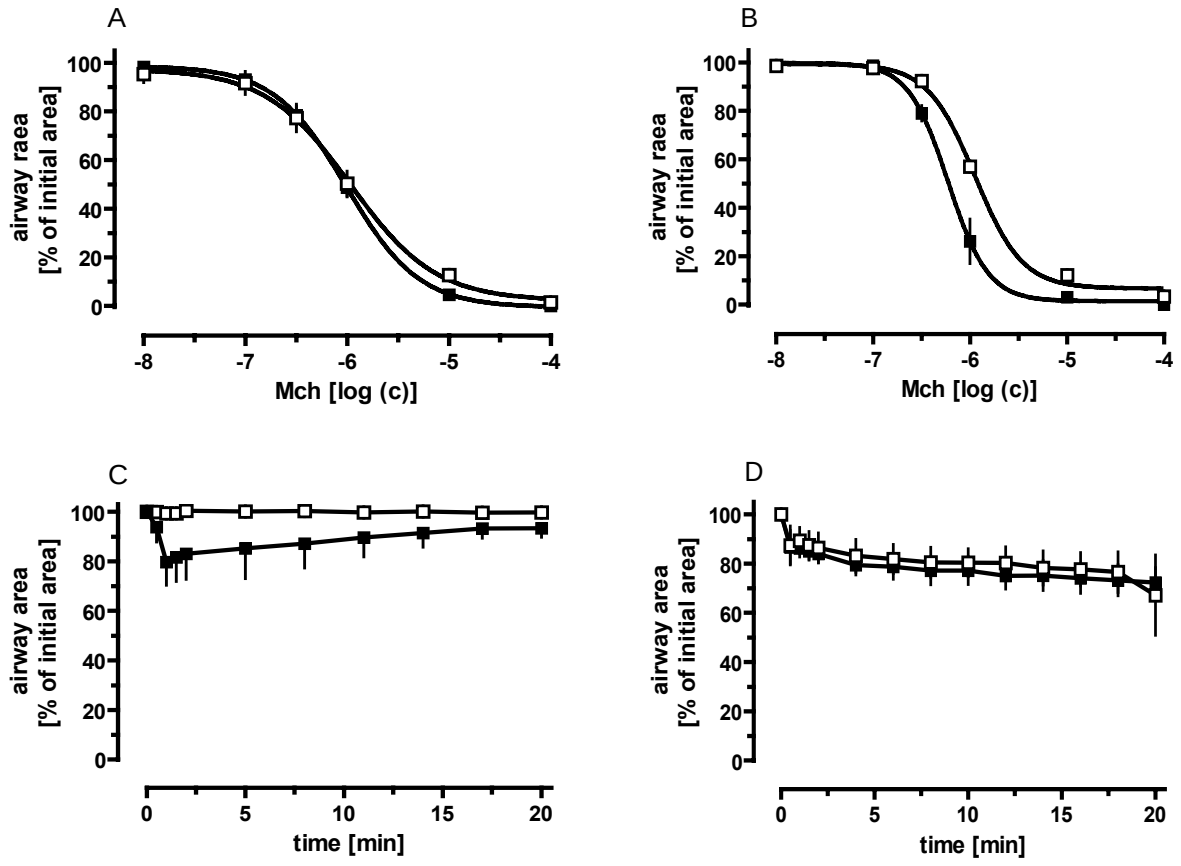

Figure 4.23 Airway responses in PCLS of control (□) and actively sensitized (■) rats. (A) and (B) show concentration-response curves for methacholine before (A) and 24 hours after (B) performance of an EAR. The corresponding EC_{50} values were (A) 1.08 µM (control) and 0.98 µM (sensitized rats); (B) 1.12 µM and 0.59 µM, respectively. Data are presented as mean ± SEM from 4 and 8 independent experiments. (C) and (D) showed time courses for 10 mM adenosine (C) and 50 µM U46619 (D). Data are from at least 3 independent experiments and are presented as mean ± SEM.

4.4 Part III: Mediator signal cascades during the EAR

To investigate the signaling pathways that become activated by mediators of the EAR in the different species, inhibitors of different pathways were examined. These experiments were performed either in passively sensitized PCLS treated with allergen or in unsensitized PCLS stimulated with mediators alone.

4.4.1 Signaling in human PCLS

Pre-incubation of passively sensitized human PCLS with the inhibitor of the phosphatidyl-specific phospholipase C (PI-PLC) L108, the IP_3-receptor inhibitor xestospongin C or the inhibitor of the myosin light chain kinase (MLCK), ML-7 did not significantly reduce pollen extract-induced bronchoconstriction. On the other hand, the Rho-kinase inhibitor Y27632, as well as the two Ca^{2+}-channel antagonists nifedipine (L-type Ca^{2+}-channel antagonist) and mibefradil (T-type Ca^{2+}-channel antagonist) significantly attenuated the allergen-induced bronchoconstriction (Fig. 4.24). The combination of ML-7 and Y27632 failed to show any additive effects.

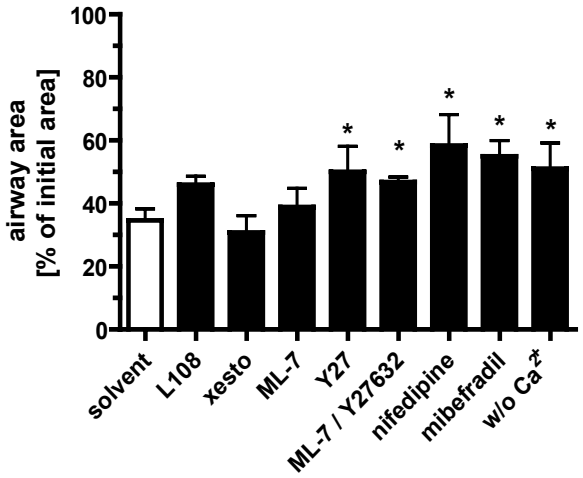

Figure 4.24 Allergen-induced bronchoconstriction in passively sensitized human PCLS, and the effect of various inhibitors. Slices were pre-incubated 20 minutes before administration of the allergen with the respectively inhibitor. The phosphatidyl-specific phospholipase C inhibitor L108 (30 µM), the IP_3-receptor inhibitor xestospongin C (1 µM), the myosin light chain kinase (MLCK) inhibitor ML-7 (35 µM), the Rho-kinase inhibitor Y27632 (10 µM), the L-type Ca^{2+}-channel blocker nifedipine (10 µM) and the T-type Ca^{2+}-channel blocker mibefradil (25 µM) were used to inhibit different signal transduction pathways. Ca^{2+} was removed 2 hours before allergen-challenge. Data are presented as mean ± SEM. **, p< 0.01 vs. solvent; *, p< 0.05 (one-sided t-test corrected by fdr procedure). w/o = without.

Thromboxane and leukotrienes have been identified as the major mediators of the EAR in human PCLS [178]. Therefore, the signal transduction pathways activated by these mediators were investigated, using the same inhibitors as before. U46619 (100 nM), the stable TP-receptor agonist, contracted the airways to 30% of the initial airway area. At higher concentrations of U46619 the inhibitors had no effect. At lower concentrations, the bronchoconstriction was prevented to almost 100% in the case of nearly all inhibitors. For all the mediator shown below, concentration-response curves were performed and a concentration was selected that, reduced the airway area to about 20% - 40% of the initial area to examine the effects of the different inhibitors. At this

point of a concentration-response curve usually the strongest effects are observed. Inhibition of single signal transduction pathways with different inhibitors alone showed only small reductions of the bronchoconstriction, only Y27632 showed a significant effect (Fig. 4.25A). In the case of TP-receptor activation, the Rho-kinase pathway might play the most important role in mediating bronchoconstriction.

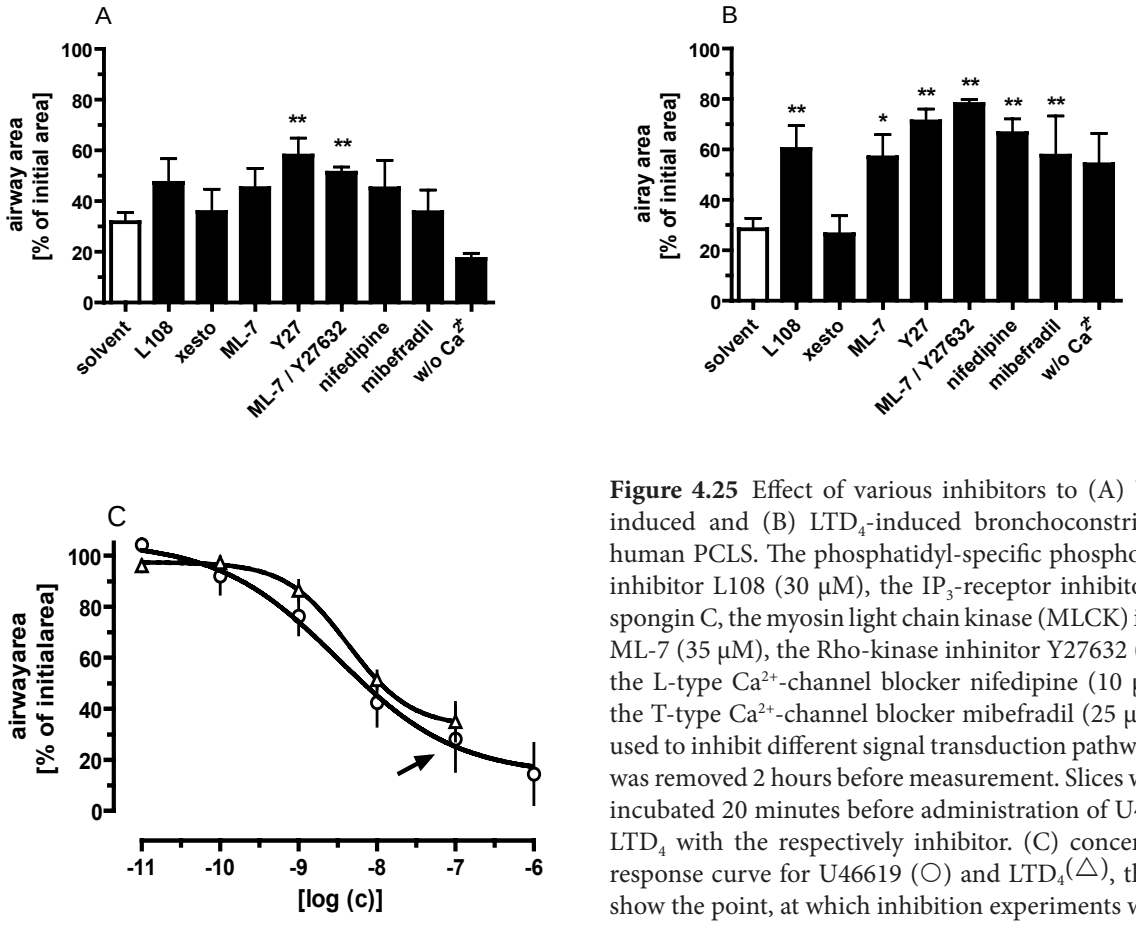

Figure 4.25 Effect of various inhibitors to (A) U46619-induced and (B) LTD_4-induced bronchoconstriction in human PCLS. The phosphatidyl-specific phospholipase C inhibitor L108 (30 µM), the IP_3-receptor inhibitor xestospongin C, the myosin light chain kinase (MLCK) inhibitor ML-7 (35 µM), the Rho-kinase inhinitor Y27632 (10 µM), the L-type Ca^{2+}-channel blocker nifedipine (10 µM) and the T-type Ca^{2+}-channel blocker mibefradil (25 µM) were used to inhibit different signal transduction pathways. Ca^{2+} was removed 2 hours before measurement. Slices were pre-incubated 20 minutes before administration of U46619 or LTD_4 with the respectively inhibitor. (C) concentration-response curve for U46619 (○) and LTD_4(△), the arrow show the point, at which inhibition experiments were performed. Data are presented as mean ± SEM. **, p< 0.01 vs. solvent; *, p< 0.05 (one-sided t-test corrected by the fdr procedure). w/o = without.

LTD_4-induced bronchoconstriction was partly reduced by pre-incubation of the slices with L108 or ML-7. Furthermore Y27632, as well as nifedipine and mibefradil showed beneficial effects. Figure 4.25C shows the inhibitory effects for 100 nM LTD_4. This concentration was also selected from a concentration-response curve, for the same reasons as already mentioned for U46619.

4.4.2 Signaling in GP PCLS

The allergen-induced bronchoconstriction in passively sensitized GP PCLS was significantly attenuated by pre-incubation of the slices with ML-7, or nifedipine. Pre-treatment with L108, Y27632 and mibefradil had small, but not significant effects (figure 4.26). These data indicate that MLCK, as well as extracellular Ca^{2+} entering through the L-type Ca^{2+}-channel into the cytoplasm, play a critical role in mediating the allergen-induced bronchoconstriction in GP PCLS.

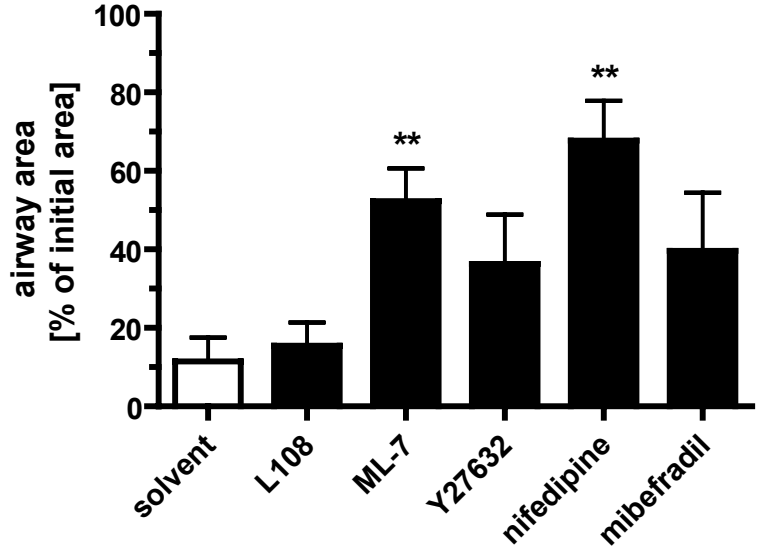

Figure 4.26 Allergen-induced bronchoconstriction in passively sensitized guinea pig PCLS, and the effect of various inhibitors. The phosphatidyl-specific phospholipase C inhibitor L108 (30 μM), the myosin light chain kinase (MLCK) inhibitor ML-7 (35 μM), the Rho-kinase inhibitor Y27632 (10 μM), the L-type Ca^{2+}-channel blocker nifedipine (10 μM) and the T-type Ca^{2+}-channel blocker mibefradil (25 μM) were used to inhibit different signal transduction pathways. Slices were pre-incubated 20 minutes before administration of allergen with the respectively inhibitor. Data are presented as mean ± SEM. **, $p< 0.01$ vs. solvent; *, $p< 0.05$ (one-sided t-test corrected by the fdr procedure)

In order to investigate, whether this is also the case for the mediators that contribute to the EAR, their signal transduction pathways were examined separately. As mediators of the EAR in GP PCLS histamine, thromboxane, and leukotrienes were defined; histamine being the most important (Fig. 4.18A). For the histamine-induced bronchoconstriction only the Rho-kinase inhibitor Y27632 showed a significant effect, whereas the other inhibitors were ineffective (Fig. 4.27A), suggesting that the Rho-kinase pathway is largely responsible. The U46619-induced bronchoconstriction was also significantly attenuated by Y27632; in addition ML-7 and nifedipine showed small effects (Fig. 4.27B). The LTD_4-induced bronchoconstriction was significantly affected by Y27632 and nifedipine. Also mibefradil was slightly effective, indicating an involvement of the Rho-kinase pathway, but also of extracellular Ca^{2+} (Fig. 4.27C). L108 did not influence the bronchconstriction, induced by the different mediators, indicating that the PI-PLC seems to play a minor role in all GP experiments.

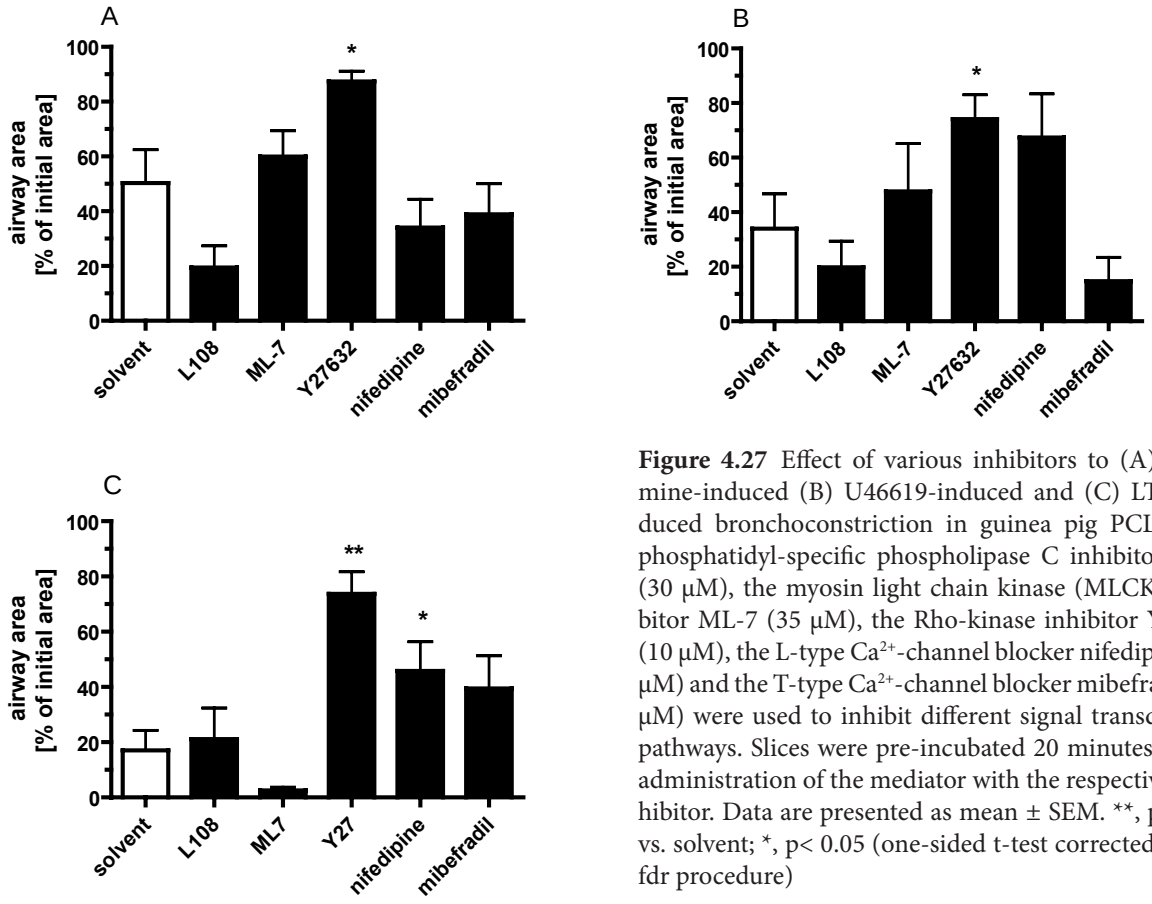

Figure 4.27 Effect of various inhibitors to (A) histamine-induced (B) U46619-induced and (C) LTD_4-induced bronchoconstriction in guinea pig PCLS. The phosphatidyl-specific phospholipase C inhibitor L108 (30 µM), the myosin light chain kinase (MLCK) inhibitor ML-7 (35 µM), the Rho-kinase inhibitor Y27632 (10 µM), the L-type Ca^{2+}-channel blocker nifedipine (10 µM) and the T-type Ca^{2+}-channel blocker mibefradil (25 µM) were used to inhibit different signal transduction pathways. Slices were pre-incubated 20 minutes before administration of the mediator with the respectively inhibitor. Data are presented as mean ± SEM. **, $p < 0.01$ vs. solvent; *, $p < 0.05$ (one-sided t-test corrected by the fdr procedure)

4.4.3 Mediator-induced bronchoconstriction in murine PCLS

Even it is not yet clear which mediators are involved in the bronchoconstriction during the EAR in murine PCLS, we investigated the signal cascades of some potential mediators. In first experiments we examined airway responses to methacholine and the stable thromboxane mimetic (or TP-receptor agonist) U46619. Pre-incubation with ML-7 alone showed only small inhibitory effects for the methacholine-induced bronchoconstriction, but in combination with Y27632 a significant reduction of bronchoconstriction was obtained. Nifedipine and mibefradil showed no significant effect on the bronchoconstriction. The combination of all four inhibitors completely prevented methacholine-induced bronchoconstriction.

As in the other species, Y27632 showed a significant effect for the U46619-induced bronchoconstriction, whereas the other inhibitors played only a minor role. However, a combination of all four inhibitors almost completely prevented U46619-induced airway contraction.

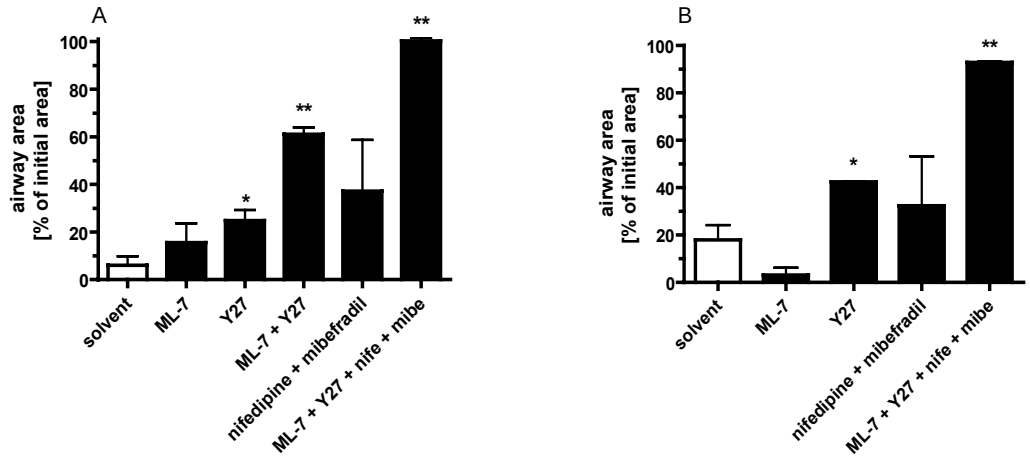

Figure 4.28 Effect of various inhibitors to (A) methacholine-induced and (B) U46619-induced bronchoconstriction in murine PCLS. The myosin light chain kinase (MLCK) inhibitor ML-7 (35 µM), the Rho-kinase inhinitor Y27632 (10 µM), the L-type Ca^{2+}-channel blocker nifedipine (10 µM) and the T-type Ca^{2+}-channel blocker mibefradil (25 µM) were used alone or in combination to inhibit different signal transduction pathways. Data are presented as mean ± SEM. **, p< 0.01 vs. solvent; *, p< 0.05 (one-sided t-test corrected by the fdr procedure)

4.4.3.1 Airway responses in Maxi-K-channel knockout mice

Because Ca^{2+}-sensitive potassium channels were reported to play a role in airway relaxation [203], and may be therefore an important target for asthma therapy, we investigated if potassium channels influence airway responses to different mediators, such as methacholine, serotonin, and U46619. For these experiments we used PCLS of Maxi-K knockout mice and wild type mice to compare their airway reactivity. The experiments with the maxi K knockout mice were performed in collaboration with Dr. Ruth and Dr. Sausbier (Tübingen), who generated the mice. As shown in figure 4.29A maxi-K knockout mice responded weaker to methacholine than wild type mice. In contrast, a stronger response was obtained in maxi-K knockout mice for the serotonin-induced bronchoconstriction compared to wild type mice (Fig. 4.29B). Stimulation with 10 µM U46619 resulted in stronger contraction in maxi-K knockout mice. The maximum airway contraction was reached three minutes after administration of U46619 and remained on that level for 20 minutes in Maxi-K knockout mice, whereas in wild type mice a continuous contraction was observed, with a maximum contraction after 20 minutes.

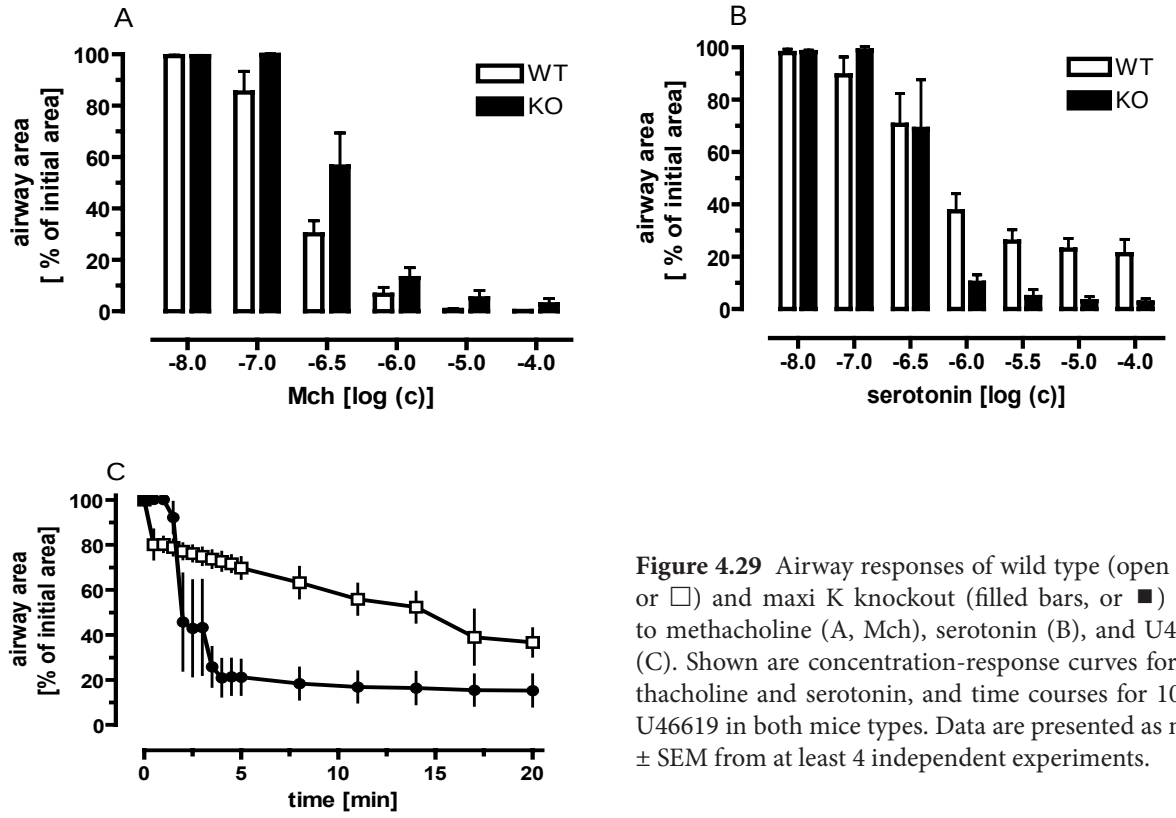

Figure 4.29 Airway responses of wild type (open bars, or □) and maxi K knockout (filled bars, or ■) mice to methacholine (A, Mch), serotonin (B), and U46619 (C). Shown are concentration-response curves for methacholine and serotonin, and time courses for 10 µM U46619 in both mice types. Data are presented as mean ± SEM from at least 4 independent experiments.

4.4.4 Signaling in rat PCLS

Pretreatment with the phosphatidylinositol-specific phospholipase C (PI-PLC) inhibitor L-108 did not significantly affect the allergen-induced bronchoconstriction in passively sensitized rat PCLS, and neither did the IP_3-receptor antagonist xestospongin C. In contrast, inhibition of the MLCK with ML-7 or of the Rho-kinase pathway with Y27632 attenuated the allergen-induced bronchoconstriction. The role of extracellular calcium was examined by inhibiting two different Ca^{2+}-channels. Nifedipine, inhibitor of the L-type Ca^{2+}-channel showed a smaller effect, in contrast to mibefradil, an inhibitor of the T-type Ca^{2+}-channel, that showed a strong inhibitory effect on the allergen-induced bronchoconstriction in rat PCLS (Fig. 4.30).

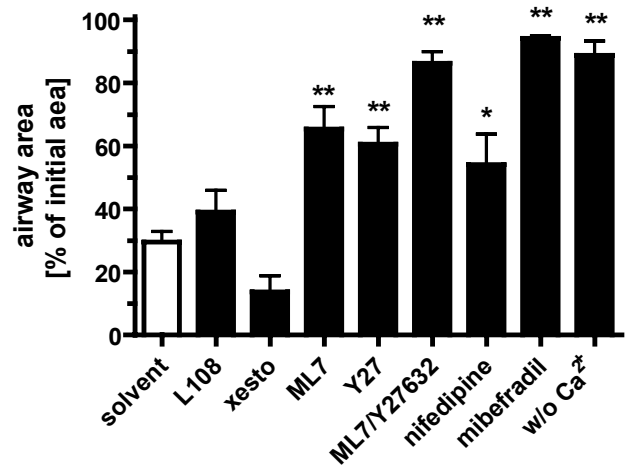

Figure 4.30 Allergen-induced bronchoconstriction in passively sensitized rat PCLS, and the effect of various inhibitors. L108 (30 µM), xestospongin C (1 µM), ML-7 (35 µM), Y27632 (10 µM), nifedipine (10 µM) and mibefradil (25 µM) were used to inhibit different signal transduction pathways. Slices were pre-incubated 20 minutes before administration of allergen with the respectively inhibitor. Data are presented as mean ± SEM. **, $p < 0.01$ vs. solvent; *, $p < 0.05$ (one-sided t-test corrected by the fdr procedure)

The role of calcium on the contraction of airway smooth muscle cells was also investigated in passively sensitized rat slices, labelled with oregon green (5 µM). Cytoplasmatic calcium levels after allergen exposure were monitored by two-photon microscopy, combined with a digital camera recording 4 images per second over a period of 20 minutes. Figure 4.31 shows an increase of cytoplasmatic Ca^{2+} in sensitized PCLS after allergen challenge. Pre-incubation with nifedipine resulted in a delayed, half maximal peak of calcium-increase after allergen challenge compared to controls, whereas mibefradil completely prevented the calcium signal increase (Fig. 4.31A). If extracellular Ca^{2+} was removed two hours before allergen challenge, and the resting extracellular Ca^{2+} was chelated with EDTA, no increase of the calcium signal was observed (Fig. 4.31B).

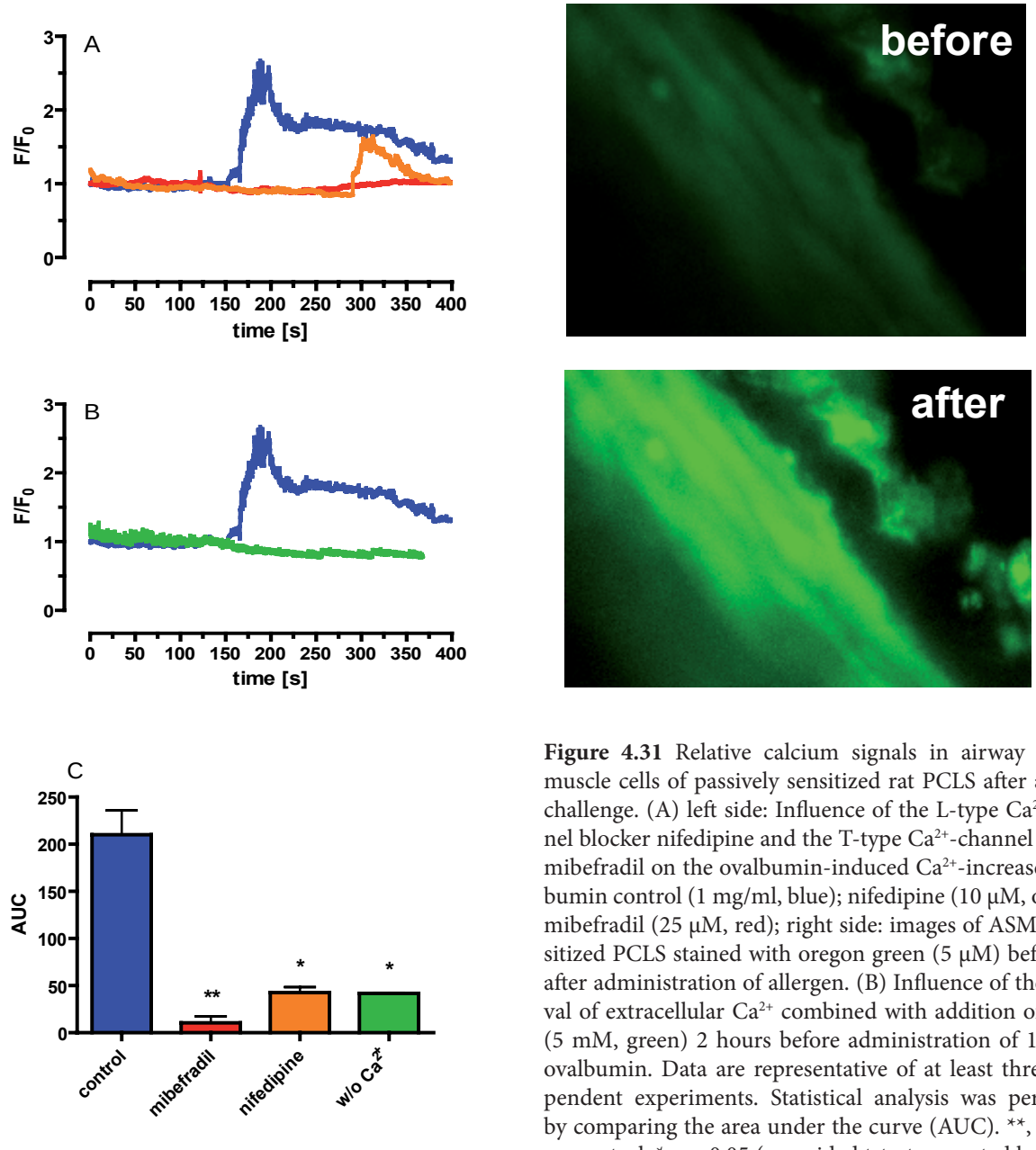

Figure 4.31 Relative calcium signals in airway smooth muscle cells of passively sensitized rat PCLS after allergen challenge. (A) left side: Influence of the L-type Ca^{2+}-channel blocker nifedipine and the T-type Ca^{2+}-channel blocker mibefradil on the ovalbumin-induced Ca^{2+}-increase. Ovalbumin control (1 mg/ml, blue); nifedipine (10 µM, orange); mibefradil (25 µM, red); right side: images of ASM in sensitized PCLS stained with oregon green (5 µM) before and after administration of allergen. (B) Influence of the removal of extracellular Ca^{2+} combined with addition of EDTA (5 mM, green) 2 hours before administration of 1 mg/ml ovalbumin. Data are representative of at least three independent experiments. Statistical analysis was performed by comparing the area under the curve (AUC). **, $p < 0.01$ vs. control; *, $p < 0.05$ (one-sided t-test corrected by the fdr procedure). Data are mean ± SEM. ASM = airway smooth muscle cells.

The main mediator leading to bronchoconstriction during the EAR in rats is serotonin [196]. Therefore we investigated whether the signal transduction pathways involved in serotonin-induced bronchoconstriction resemble those of allergen-induced bronchoconstriction. A challenge with 10 µM serotonin resulted in a bronchoconstriction, reducing the airway area to 37% of the initial area. Neither L108 nor xestospongin C, affected the serotonin-induced airway smooth muscle contraction. Pre-incubation with ML-7 had only a small but significant effect. Pre-treatment with Y27632 resulted in a highly significant reduction of bronchoconstriction, and mibefradil almost completely prevented bronchoconstriction, whereas nifedipine had a smaller effect (Fig. 4.32).

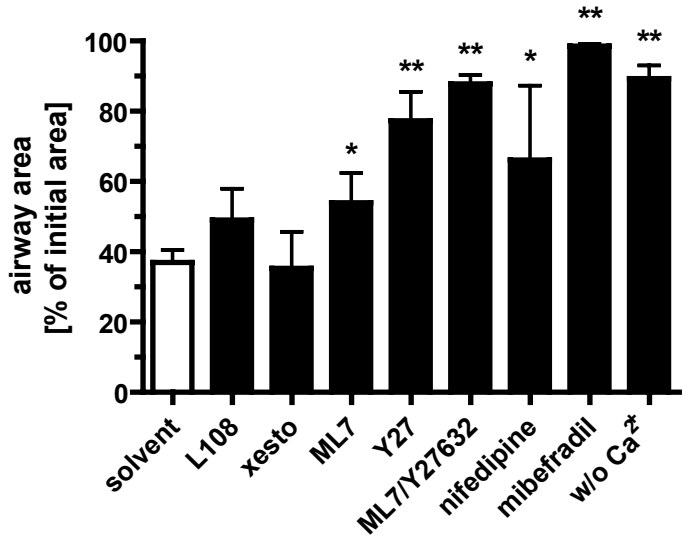

Figure 4.32 Effect of various inhibitors on serotonin-induced bronchoconstriction in rat PCLS. The PI-PLC inhibitor L108 (30 µM), the IP_3-receptor xestospongin C (1 µM), the myosin light chain kinase (MLCK) inhibitor ML-7 (35 µM), the Rho-kinase inhibitor Y27632 (10 µM), the L-type Ca^{2+}-channel blocker nifedipine (10 µM) and the T-type Ca^{2+}-channel blocker mibefradil (25 µM) were used alone or in combination to inhibit different signal transduction pathways. Ca^{2+} was removed 2 hours before challenge with serotonin. Slices were pre-incubated 20 minutes before administration of serotonin with the respectively inhibitor. Data are presented as mean ± SEM. **, $p< 0.01$ vs. solvent; *, $p< 0.05$ (one-sided t-test corrected by Fdr procedure). w/o = without

The calcium signal during serotonin-induced contraction of airway smooth muscle cells was again investigated in rat slices, labelled with oregon green (5 µM). Cytoplasmatic calcium levels after serotonin exposure were monitored by two-photon microscopy, as mentioned above. Figure 4.33 shows an increase of cytoplasmatic Ca^{2+} after serotonin challenge. Pre-incubation with nifedipine halved the calcium-increase after serotonin exposure compared to controls; mibefradil completely prevented the increase in the calcium signal. If extracellular Ca^{2+} was removed two hours before administration of serotonin, no increase of calcium signal was detected.

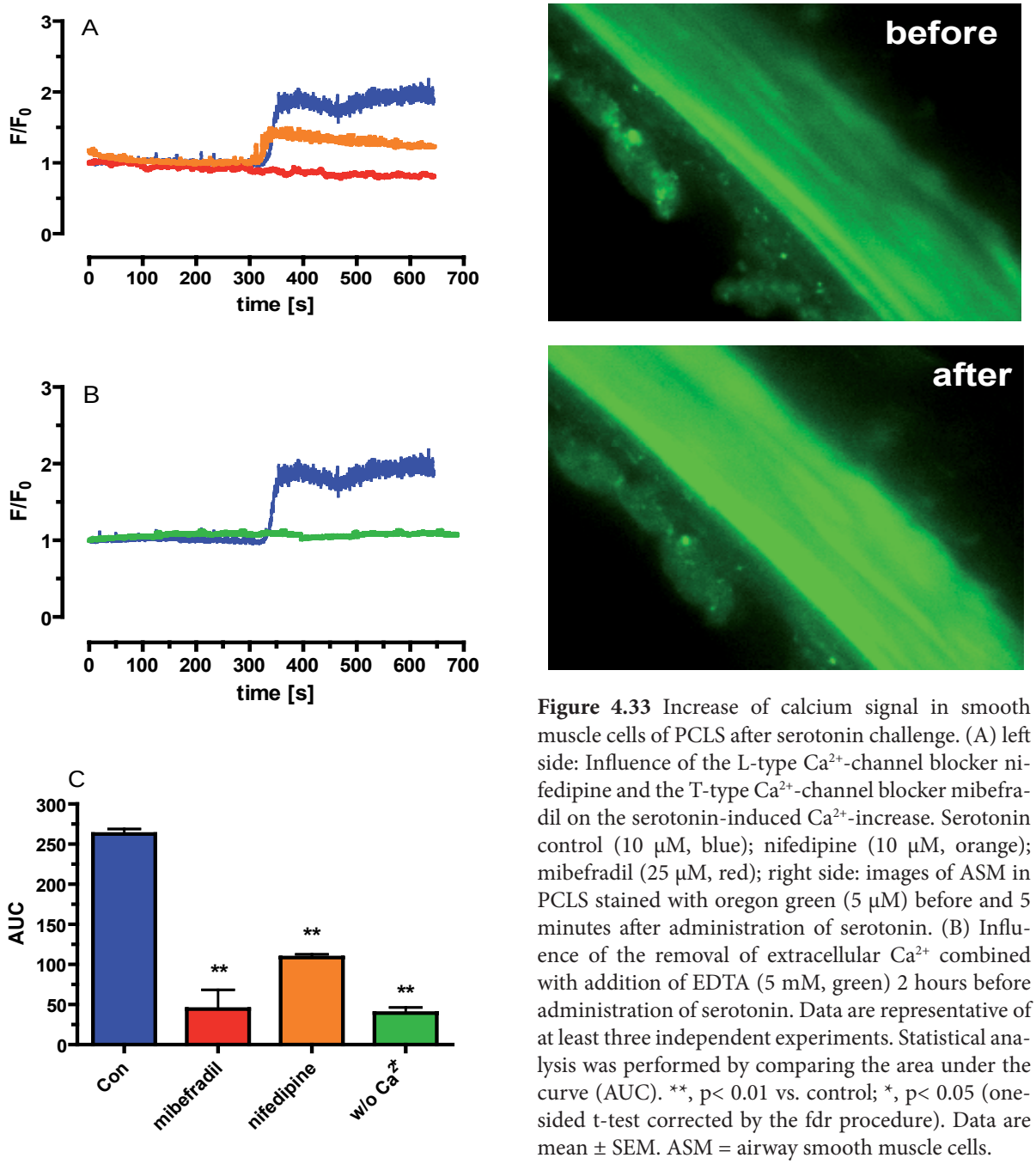

Figure 4.33 Increase of calcium signal in smooth muscle cells of PCLS after serotonin challenge. (A) left side: Influence of the L-type Ca^{2+}-channel blocker nifedipine and the T-type Ca^{2+}-channel blocker mibefradil on the serotonin-induced Ca^{2+}-increase. Serotonin control (10 µM, blue); nifedipine (10 µM, orange); mibefradil (25 µM, red); right side: images of ASM in PCLS stained with oregon green (5 µM) before and 5 minutes after administration of serotonin. (B) Influence of the removal of extracellular Ca^{2+} combined with addition of EDTA (5 mM, green) 2 hours before administration of serotonin. Data are representative of at least three independent experiments. Statistical analysis was performed by comparing the area under the curve (AUC). **, p< 0.01 vs. control; *, p< 0.05 (one-sided t-test corrected by the fdr procedure). Data are mean ± SEM. ASM = airway smooth muscle cells.

Thromboxane was also reported to attenuate the allergen-induced bronchoconstriction, at least in humans and GP. To investigate the signal transduction pathways activated by thromboxane, rat slices were stimulated with a stable TP-receptor agonist U46619 (10 µM) in presence of the same inhibitors as before. The concentration of U46619 chosen, contracted the airways to 40% - 50% of the initial area. The bronchoconstriction was not attenuated by L108, xestospongin C, or ML-7 (Fig. 4.34B). Inhibition of both Ca^{2+}-channel types with nifedipine and mibefradil showed small effects, and pre-treatment with Y27632 significantly prevented the U46619-induced bronchoconstriction, indicating again a strong participation of the Rho-kinase pathway for this stimulus.

The role of the Rho-kinase pathway in the U46619-induced contraction was furthermore investigated

in isolated perfused rat lungs (IPL) that were performed in cooperation with R. Göggel (Borstel) [114]. These experiments showed an increase of airway and vascular resistance after administration of U46619, that were almost completely abolished by Y27632 (Fig. 4.34B,C).

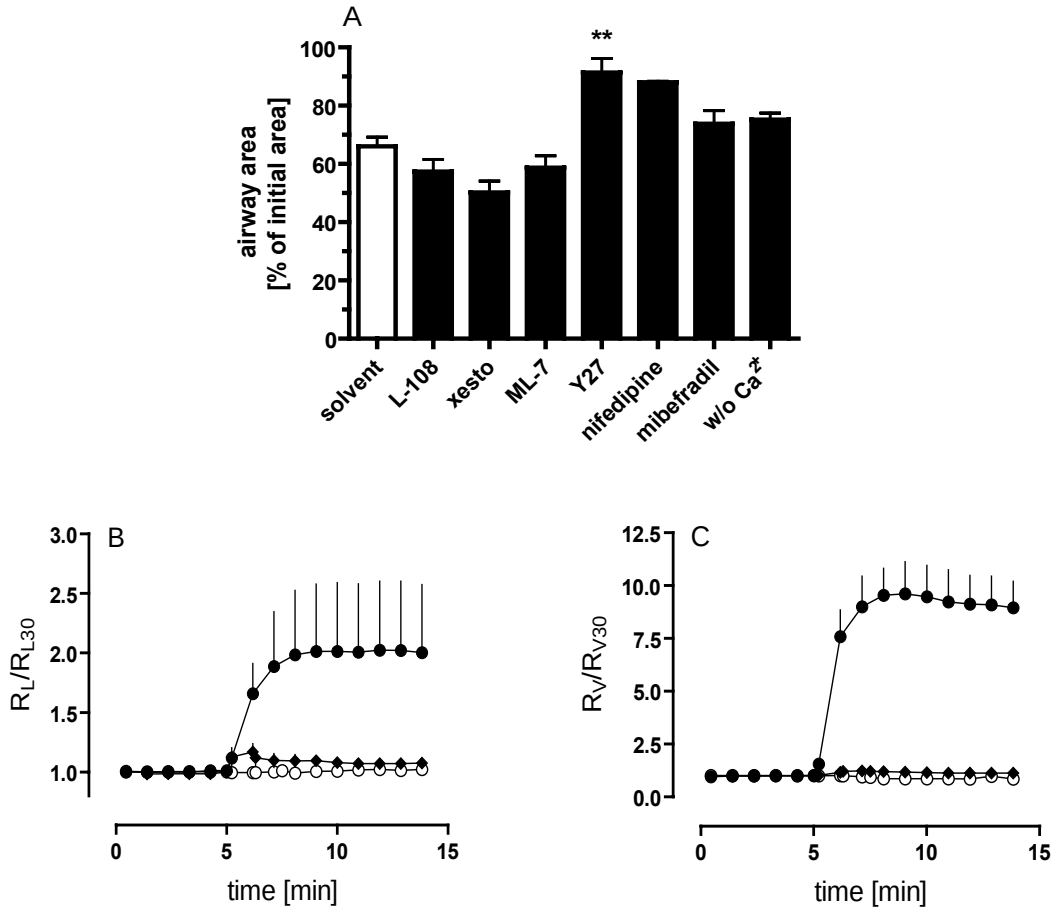

Figure 4.34 Effect of various inhibitors on (A) U46619-induced bronchoconstriction in rat PCLS. The PI-PLC inhibitor, L108, the IP_3 inhibitor xestospongin C, the myosin light chain kinase (MLCK) inhibitor ML-7 (35 µM), the Rho-kinase inhibitor Y27632 (10 µM), the L-type Ca^{2+}-channel blocker nifedipine (10 µM) and the T-type Ca^{2+}-channel blocker mibefradil (25 µM) were used alone or in combination to inhibit different signal transduction pathways. Ca^{2+} was removed 2 hours before challenge. Slices were pre-incubated 20 minutes before administration of 10 µM U46619 with the respectively inhibitor. Data are presented as mean ± SEM. **, $p< 0.01$ vs. solvent; *, $p< 0.05$ (one-sided t-test corrected by the fdr procedure). (B, C) The effect of Y27632 on U46619-induced pressor responses in perfused rat lungs. (B) lung resistance; (C) vascular resistance. U46619 was given as a concentration of 5 nM 10 minutes after administration of Y27632 (10 µM). Y27632 was given a second time 5 minutes after administration of U46619. control: ○, n = 3; U46619: ●, n = 3; U46619 + Y27632: ◆, n = 3. Data are mean ± SEM. The pressor responses in Y27632 treated lungs was significantly diminished compared with lungs treated with U46619 alone and untreated control lungs ($p < 0.05$, one-sided t-test corrected according to Benjamini-Hochberg).

5 Discussion

Asthma is a disease with an increasing incidence worldwide [1], and thus it is more important than ever to have animal models that allow to define the pathogenesis of human asthma. For pharmacological and mechanistic studies relevant to human disease, differences in airway pharmacology between rodent models and human lungs remain an important problem. Because the preparation of precision-cut lung slices (PCLS) is essentially the same in all species, this model provides an excellent way to compare the airway pharmacology of different species. PCLS allow investigation of single airways and vessels under cell culture conditions, and have already been used to elucidate important mechanisms of airway contraction in rat, mouse and human peripheral lungs [181,193,195,196].
In this study we characterized and established PCLS from guinea pigs as a model to study single airway responses and show that GP PCLS resemble to human airways better than do PCLS from mice or rats. Furthermore, the preparation of monkey PCLS was introduced.
Two characteristic features of human asthma are the early allergic response (EAR) and airway hyperresponsiveness (AHR). The present study focussed on airway responses in PCLS of different species after induction of an EAR, the mediators leading to bronchoconstriction during this phase, and the signal transduction pathways involved.

5.1 The model of guinea pig PCLS

In the present study, we describe for the first time the preparation and properties of guinea pig PCLS. The major obstacle in producing good quality slices from guinea pigs was the severe *post mortem* bronchoconstriction that has been documented radiographically [204] and in isolated lungs [205]. This *post mortem* bronchoconstriction has been attributed to the release of substance P from sensory nerves (that relaxes vessels at the same time), but other mediators or direct effects of changing microenvironment (pH, CO_2) on airway muscle may also be involved [206]. Chronic treatment with capsaicin to empty substance P stores from sensory nerves or *in vivo* pre-treatment with morphine avoided the *post mortem* bronchoconstriction [207]. However, such treatments are impractical for routine use, carry some ethical concerns as capsaicin treatment is painful, and may interfere with the purpose of the experiments. Here we report the successful elimination of the *post-mortem* bronchoconstriction by a short initial (3 hours) inclusion of isoproterenol in all the media used for preparation of the PCLS. For the same purpose, β-agonists are also routinely used during the preparation of isolated perfused guinea pig lungs [208]. Once the preparation of the PCLS is finished, isoproterenol treatment can be discontinued, whereupon airways and vessels respond normally to various stimuli showing that the effect of isoproterenol is not sustained. This is also

supported by the bronchorelaxant effects observed upon addition of salbutamol to precontracted, but not to untreated preparations.

Compared to classical models of studying airway functions in vitro, such as tracheal rings or parenchymal strips, PCLS offer many advantages such as auxotonic contraction, economic use of expensive agents, longevity and the possibility to simultaneously study airway and vascular responses; PCLS even permit to differentiate between pulmonary arteries and pulmonary veins in the same slice. This was demonstrated in the present study for endothelin-1, and in related studies using lung explants for histamine and serotonin [209]. In all these studies pulmonary veins responded stronger than pulmonary arteries, corroborating many other findings in rat, sheep and human lungs [210]. In contrast, Cardell et al. using isolated vessels reported that veins are less responsive than arteries [211]. The reason for this difference is unknown, but may relate to differences in the two preparations, the size of vessels studied or the particular guinea pig strain that was used. In any case it should be noted that it is difficult to extrapolate from contractions of individual vessels to total pulmonary resistance *in vivo*.

Guinea pigs are frequently employed for studies in pulmonary pharmacology, because they are thought to possess a pharmacological profile [173,212,213] similar to that observed in humans [179,214]. However, this assumption has never been thoroughly tested in the same laboratory using the same methology. Now having at hand GP and human PCLS we were able to check this assumption and to define similarities and differences between both species with relevance to human asthma.

Comparison between GP and human airways was performed by examining their airway responsiveness towards a variety of endogenous mediators (table 5.1). The EC_{50} values for human and GP bronchoconstriction are almost identical for LTD_4 and methacholine. Airways of both species responded to thromboxane and histamine, with human airways being more sensitive to thromboxane, and GP airways being more sensitive to histamine. The most significant difference was obtained for serotonin which was quite effective in GP and completely ineffective in humans. Overall, the comparison between GP and human airways showed that GP are not a perfect match but do nevertheless provide a reasonable agreement to humans. This is certainly true in comparison to mouse and rat airways that do or only weakly respond to leukotrienes and histamine [181,196], both of which mediators are thought to play a role in human asthma.

Table 5.1 Median effective concentrations (EC_{50}) and logarithmic effective concentrations (pD_{50}) for agonist-induced airway contractions in guinea pig and human PCLS.

Agent	PCLS GP EC_{50} [nM]	PCLS human EC_{50} [nM]]	PCLS GP pD_{50}±SE [M]	PCLS human pD_{50}±SE [M]
LTD_4	1.8	5.0	8.7 ± 0.10	8.3 ± 0.10
U46619	16	1.3	7.8 ± 0.10	8.8 ± 0.10
Serotonin	69	no reaction	7.2 ± 0.10	no reaction
Histamine	217	2710	6.7 ± 0.10	5.6 ± 0.10
Methacholine	231	234	6.6 ± 0.10	6.6 ± 0.10

5.2 The early allergic response in PCLS

A causal therapy to prevent the strong bronchoconstriction during the EAR is a major goal in asthma research. To develop new therapeutic strategies, it is important to understand the underlying mechanisms. For studying those mechanisms, animal models in which an EAR can be induced are required.

As in PCLS from rats and humans [178,196] also in GP PCLS an EAR can be evoked by different stimuli as shown in this study. On the one hand by provocation with allergen in passively sensitized PCLS, on the other hand by C48/80, a chemical stimulus leading to mast cell degranulation. This mast cell degranulation, initiated either immunologically or chemically resulted in a comparable kinetic of bronchoconstriction, which was characterized by an immediate contraction (phase 1) two minutes after allergen challenge, and a prolonged contraction that resolved only slowly (phase 2) within 20 minutes. Histamine was shown to be responsible for the immediate contraction within the first two minutes, whereas the prolonged contraction is mediated by thromboxane and leukotrienes (Fig. 4.11C and 4.18B).

In humans, compared to GP, thromboxane and leukotrienes contribute to the EAR [178]. The major difference between GP and humans relates to histamine which is clearly more important in guinea pigs. Thromboxane, leukotrienes and histamine are all potent bronchocontrictors, and therefore it is expected that the blockade of one mediator at a time will only have a small effect. Indeed, this was our finding, even though in GP significant protection could be obtained with the H_1-receptor antagonist alone. The combination of the H_1-receptor antagonist with either TP- or $CysLT_1$-receptor

antagonist was particularly effective. In general, these results are in line with previous findings in other guinea pig preparations such as the perfused lung [213] and isolated tracheas [215] or parenchymal strips [212], that also show that histamine, leukotrienes and cyclooxygenase (COX) products contribute additively to the EAR. Those studies, however, employed COX inhibitors rather than TP-receptor antagonists; in comparison, it appears that when given alone, COX inhibitors are more effective than TP-receptor antagonist. This may indicate that other COX products such as PGE_2 (mediating bronchoconstriction via EP_1- receptors) and $PGF_{2\alpha}$ also involved in the EAR. An important difference between our study in PCLS and the discussed organ baths studies is that the latter experiments were all performed in lung tissue from actively sensitized animals. It is therefore possible that in those studies some COX products came from infiltrated eosinophils and other inflammatory cells. Nonetheless, all these studies show that the EAR can only be prevented if at least two of three bronchoactive mast cell mediators are blocked.

None of the mediators that play a role in GP and humans, are involved in passively sensitized PCLS from rats, where the EAR is almost exclusively mediated by serotonin (Fig. 4.14) as has been described before by Wohlsen *et al.* [196]. In actively sensitized rats, though, in addition to serotonin, a role of LTD_4 was described for the EAR [216].

In the mouse the mechanism of the EAR appears to be even more different as in the other species examined. In contrast to PCLS from guinea pigs, rats, and humans, murine PCLS could not be passively sensitized and they did not respond to non-toxic concentrations of the mast cell degranulator C48/80. The bronchoconstriction at very high concentration of C48/80 in murine PCLS is therefore most likely a mast cell-independent mechanism. Investigations with mast cell-deficient mice that responded in the same way as wild type mice to the chemical stimulus strengthen this assumption.
Similar observations were made by Crosby *et al.*, demonstrating that in sensitized mast cell-deficient mice an airway response after ovalbumin challenge occurs, whereas in sensitized mice deficient for B and T cells an airway response to ovalbumin challenge was absent. The transfer of ova-specific IgG, but not IgE before allergen challenge was capable to induce the EAR also in lymphocyte-deficient mice [217]. Another study showed that sensitization and challenge with allergen results in histological changes of the airway epithelium and lung dysfunction also in mice deficient of IgE [218,219]. Taken together, these data indicate that the EAR in mice is immunologically dependent on allergen-specific IgG, but independent of mast cell degranulation and IgE.
In contrast passively sensitized murine PCLS, we have shown that a contractile airway response to allergen occurs in PCLS from actively sensitized mice. Since the EAR in murine PCLS seems to be mast cell-independent, a role of eosinophils seems possible, also because during the procedure of sensitization the number of eosinophils in the lung is increased [220]. In humans a degranulation of eosinophils was triggered by binding of IgG to its receptor on the surface [221], and also by the chemical stimulus C48/80 [222]. Once degranulated, eosinophils release mediators like PAF, LTC_4,

and COX metabolites, which could be responsible for the bronchoconstriction during EAR seen in PCLS from actively sensitized mice. However, a contribution of neutrophils, and lymphocytes can also not be excluded.
Table 5.2 gives an overview about the mediators responsible for the bronchoconstriction during the EAR in different species after the stimulation either with allergen in passively sensitized slices or with C48/80 as a chemical mast cell degranulator.

Table 5.2 Mediators involved in the EAR in PCLS from different species

Agent	rat	guinea pig	human	monkey
Serotonin	+ [196]	-	-	No EAR observed
Histamine	-	+	(+)	"
Thromboxane	-	+	+ [178]	"
Leukotrienes	-	+	+ [178]	"

+ = major mediator; (+) = minor mediator; - = no mediator

5.3 PCLS from non-human primates

Apart from the establishment of guinea pig PCLS, in this study we introduced for the first time the preparation of monkey PCLS. One would expect that monkeys being close to humans on the phylogenetic tree, resemble the human situation better than do other species. However, our results demonstrate that already between the three investigated monkey species - cynomolgus monkey, baboon, and rhesus monkey - considerable differences in airway reactivity were observed. Whereas cynomolgus monkeys and baboons responded in a concentration-dependent manner to methacholine, histamine and serotonin, the rhesus monkey showed no airway response to the latter stimulus, a finding also observed in human PCLS. This may indicate a higher comparability of rhesus monkey and human PCLS. No airway contraction to LTD_4 was detected in PCLS of cynomolgus monkeys, but baboons and rhesus monkeys showed a bronchoconstriction. An airway reactivity of rhesus monkeys to LTD_4 was also described by Patterson *et al.*, who designed a monkey model to investigate the effect of a specific LTD_4 antagonist [223]. Turner *et al.* showed a reduction of airway hyperresponsiveness and antigen-induced pulmonary inflammation in atopic cynomolgus monkeys, when they were pre-treated with a LTD_4 receptor antagonist [224]. LTD_4 could therefore be considered as a mediator playing not only a role in bronchoconstriction, as

described for humans [179,225-227], but also in AHR and inflammation of the airways as shown in this monkey model of asthma [224].
In order to investigate whether an EAR in monkey PCLS can be evoked with pollen extract as antigen, PCLS from monkeys were passively sensitized overnight with serum from a human atopic donor, containing the specific IgE molecules. The subsequent challenge with the extract of timothy grass pollen did not elicit a strong bronchoconstriction in PCLS from baboons or rhesus monkeys. In contrast, the challenge of sensitized human PCLS with allergen always results in a contraction of the airways within the first 5 minutes. That no EAR was seen in monkey PCLS, is possibly explained by the fact that IgE receptors in monkeys do not recognize human IgE. All three monkey species, investigated in this study belong to the non-human primates. In other primate models of airway hyperresponsiveness or airway inflammation, different allergens such as Ascaris suum or house dust mite antigens were used to sensitize the animals [224,228-230]. Monkey models have been performed in rhesus monkeys, sensitized to Ascaris suum antigen [231,232]. Some monkeys also show a natural allergy to this antigen, used by Michoud *et al.* to investigate wether sensitive animals respond stronger to histamine and methacholine than unsensitive animals [233]. No differences in reactivity were observed between allergic and nonallergic monkeys in response to both stimuli. However, the breathing pattern changed into a rapid shallow breathing when the ascaris antigen or histamine was administred to ascaris-sensitive monkeys [233]. Allergy to the ascaris antigen seems to be a naturally occurring allergy in monkeys, induced by infection with Ascaris suum [234], whereas allergy to grass pollen naturally occurs in humans. As described for C48/80-induced bronchoconstriction in humans [70], also in monkeys responder and non responder to allergen exist [235]. That could be another reason, why no airway response in our passively sensitized PCLS occurs, but this has to be further investigated in the future. Taken together, as indicated by the first few data, PCLS from monkeys, even from non-human monkeys, offer a possibility to study airway responses with resemblance to humans, even if no EAR was obtained under these conditions.

5.4 Mast cell degranulation

Mast cells play a central role in the EAR, by releasing a wide range of potent mediators that are responsible for bronchoconstriction, airway hyperresponsiveness and attraction of inflammatory cells. We have used two different stimuli, to induce mast cell degranulation, and thereby the release of mediators. On the one hand passive sensitization of PCLS and stimulation with allergen as an immunological stimulus, and on the other hand the use of C48/80, a chemical mast cell degranulator. Both stimuli led to bronchoconstriction in GP, rat and human PCLS, but the amount of preformed mediators released upon the different stimuli differs. In rat PCLS the levels of serotonin and histamine in the supernatant were 2-fold higher after C48/80, than after allergen

challenge. Serotonin levels in human PCLS increased to the same effect after both stimuli, whereas histamine levels showed no significant differences between control conditions and stimulation, neither immunologically nor chemically. In GP PCLS, serotonin levels were not increased, neither with allergen or with C48/80, and histamine levels showed only a small increase after ovalbumin challenge. These findings lead to the assumption that mast cells possess certain compartments that are activated and degranulate different upon an immunological or a chemical stimulus. It has been described before that chemical stimulation induces a pattern of mediator release different from those associated with IgE-dependent mast cell activation [94]. This might be one explanation for the release of different amounts of preformed mediators. Another possible explanation is mast cell heterogeneity. Different types of mast cells are known, such as MC_T and MC_{TC} in humans, and CTMC and MMC in rats and mice [94]. Maybe the response of the different mast cell types varies depending on the stimulus. For example, Church et al. described that only mast cells of the MC_{TC} type release histamine after C48/80, whereas MC_T mast cells are unresponsive to this stimulus [236]. Liu et al showed a response of CTMC and MMC mast cells to C48/80 inhalation in rats [237]. A third possibility for the different release of preformed mediators is that the extent of the exocytosis is dependent on the stimulus. In particular, the granules may release only a part of their content, or empty completely. Hide et al. using light and electron microscopy showed differing morphological changes accompanying degranulation on the one hand elicited by C48/80 and on the other hand by the effector combination Ca^{2+} plus GTP-γ-S. While the effector combination has the characteristics of an all-or-none process, C48/80 induces a partial degree of exocytosis [238].

5.5 Airway hyperresponsiveness in PCLS

An important feature of asthma is airway hyperresponsiveness (AHR), an increased airway reactivity to a variety of provocative agents, including chemical mediators (methacholine, adenosine, histamine) and physical stimuli, such as exercise or ventilation of cold air. Many animal models exist to study this disorder of airway reactivity. In this study, we investigated, whether in PCLS of sensitized animals, i.e. mice and rats, an AHR is possible.

We used murine PCLS from sensitized Balb/c mice and non sensitized control mice, to investigate whether hyperresponsiveness develops in the context of sensitization. PCLS from sensitized and non-sensitized mice were treated with methacholine to induce airway contraction, but no differences were observed between both mice. In contrast, a weaker response was observed for methacholine in sensitized PCLS, after an EAR has been performed in the same PCLS 24 hours before.
Since adenosine was reported to induce an AHR in asthmatic patients only, we investigated its effect on murine PCLS, but no airway response was detected, neither in PCLS from sensitized

nor in PCLS from control mice. Also no bronchoconstriction was observed in PCLS treated with adenosine 24 hours after an EAR had been performed in the same slice. Furthermore, *in vivo* no AHR was observed after an appropriate analysis (data not shown). The mechanisms of AHR in mice are still unclear, but in one study from Leigh *et al.* it was shown that sustained AHR and airway remodelling occurr in mice after chronic allergen exposure [239]. They further demonstrated that IL-4 and IL-3 knockout mice were protected from developing a sustained AHR compared to wild type or IL-5 knockout mice, which showed a strong AHR. Thus, IL-4 and IL-3 may play a critical role in the development of AHR in mice [239].

Whereas in murine PCLS no AHR was detected, in PCLS of sensitized rats an increased reactivity towards methacholine after an EAR was observed. One explanation for the increased reactivity of airways, 24 hours after an EAR in the same slice has been occurred, is the release of mediators, i.e. cytokines from mast cells after activation that might induce a stronger response to following stimuli. TNFα and IL-1, two proinflammatory cytokines released by mast cells are described to increase airway resistance in the isolated perfused rat lung [241]. In that study thromboxane was determined to mediate the cytokine-induced bronchoconstriction [241]. An enhanced production of TNFα has been demonstrated after crosslinking of the FcεRI in human skin mast cells [242]. It is furthermore described, that preformed TNFα is rapidly released from activated mouse mast cells, and newly synthesized TNFα is released over a period of hours after cell activation [243,244]. Mast cells can also release IL-4 in response to FcεRI-dependent activation [245], which is reported to be found at sites of allergic inflammation and may play a role in the shift to a Th2 cell differentiation [246,247]. Finally, in the PCLS of sensitized animals an AHR was only observed, after an EAR has been performed before, indicating a role for cytokines, such as TNFα, IL-1, and IL-4 to induce AHR in this model.

Adenosine contracted the airways in PCLS from sensitized rats, whereas absolutely no airway response was detected in PCLS of control animals. L. Wollin *et al.* has demonstrated that sensitized rats in vivo show an AHR towards adenosine after challenge with ovalbumin, this effect was now also reproduced with the PCLS [240]. Therefore PCLS from actively sensitized rats might be a model to study an increased airway reactivity compared to PCLS from untreated control animals.

5.6 Mechanisms of bronchoconstriction

The contraction of airway smooth muscle cells, leading to bronchoconstriction during an asthmatic attack, is mediated by different signal transduction pathways [248]. A better understanding of the mechanisms for regulation of smooth muscle contraction might lead to improved treatments for an occurring bronchoconstriction in asthma patients. Some experimental models exist to study single signal transduction pathways, but the pathways that are important for the signaling in human bronchoconstriction are only poorly defined.

Clearly established is the role of calcium as an intracellular messenger that triggers muscle contraction [249]. An increase of the intracellular Ca^{2+}-level is thought to activate myosin light chain kinase (MLCK), followed by activation of the 20 kDa light chain of myosin (MLC_{20}) and contraction of the smooth muscle cell. In recent years, many studies have shown that not only an enhancement of the intracellular Ca^{2+}-level and phosphorylation of MLCK results in smooth muscle contraction, but also the inhibition of the myosin light chain phosphatase (MLCP) *via* the Rho-kinase pathway [250].

In the present study different signal transduction pathways and their participation for bronchoconstriction during EAR, were investigated. An important observation was that the bronchconstriction during EAR in different species is mediated by different mediators. Therefore it was expected that different signal transduction pathways are activated by the different mediators, and that this is also species-dependent.

In contrast to the other investigated species, the allergen-induced bronchoconstriction in mice appears to be mast cell independent. Nonetheless, in this species we investigated the signaling pathways for single potent bronchoconstrictors, the stable acetylcholine derivate methacholine and the thromboxane mimetic U46619, using inhibitors of the "classic" pathway via MLCK, of the Rho-kinase pathway, or Ca^{2+}-channel inhibitors. With both stimuli a reduction of bronchoconstriction was observed, if the Rho-kinase pathway was inhibited. An almost completely prevention of bronchoconstriction was obtained in combination with Ca^{2+}-channel inhibitors. These results indicate a strong role for the Rho-kinase pathway and for extracellular Ca^{2+}, mediating bronchoconstriciton in mice, at least for thromboxane and methacholine.

In rats, allergen as well as serotonin-induced bronchoconstriction was completely abolished by mibefradil, indicating a prominent role for extracellular Ca^{2+} entering *via* the T-type Ca^{2+}-channel; but also the MLCK, and the Rho-kinase pathway played a role. Comparing the inhibition experiments of the allergen and the serotonin-induced bronchoconstriction, similar inhibition profiles were observed for both stimuli (Fig. 4.30 and 4.32). This is another indication that serotonin is the main mediator during EAR in rats, because very similar effects with several inhibitors were obtained for both allergen and serotonin-induced airway contraction.

Most relevant to human asthma are of course human PCLS, to which from all these animal models, the best resemblance was observed with GP PCLS; here the same mediators as in human PCLS appear to be responsible for bronchoconstriction. The analysis of the signal transduction pathways of human and GP PCLS showed that the allergen-induced bronchoconstriction in passively sensitized human PCLS was attenuated by inhibition of the Rho-kinase pathway and extracellular Ca^{2+}. However, the inhibitors only partly reduced the bronchoconstriction, and no complete prevention was observed (Fig. 4.24). These results suggest that the EAR in human PCLS is dependent on extracellular Ca^{2+} and the Rho-kinase pathway, but further signal transduction pathways may be activated as well.

In GP PCLS the inhibition of the MLCK and the L-type Ca^{2+}-channel, but not of the Rho-kinase pathway significantly diminished bronchoconstriction by allergen challenge (Fig. 4.26), indicating

that the "classic" pathway *via* MLCK phosphorylation and extracellular Ca^{2+} mediate the ovalbumin-induced bronchoconstriction in GP.

The LTD_4-induced bronchoconstriction in human PCLS was, with exception of the IP_3-receptor antagonist, significantly attenuated by all inhibitors (Fig. 4.25B), but again no complete prevention of bronchoconstriction was observed with any single inhibitor alone. These findings suggest that LTD_4-induced bronchoconstriction depends on PI-PLC, MLCK, the Rho-kinase pathway and extracellular Ca^{2+}, but not on intracellular Ca^{2+} released via IP_3-receptors. It was discussed, that LTD_4 perhaps mediates the bronchoconstriction not only *via* activation of $CysLT_1$-receptors, but also *via* formation of thromboxane. However as described by Wohlsen *et al.* inhibition of the TP-receptor does not reduce the LTD_4-induced bronchoconstriction in human PCLS [178]. In line with our findings, in experiments with dissected human intralobuar bronchioles a partly reduction of the response to LTD_4 was obtained by inhibition of the L-type Ca^{2+}-channel with nifedipine [251]. The inhibition of IP_3-receptors with 2-APB showed no effect on the LTD_4-induced bronchoconstriction [251]. These data are in line with our results, i.e. that internal Ca^{2+} release via IP_3-receptors plays no role for the LTD_4-induced bronchoconstriction in human PCLS (Fig. 4.25B). Snetkov *et al.* showed that also a release of Ca^{2+} from intracellular stores provoked with caffeine, does not cause directly any contractions in human small bronchioles [251]. T-type Ca^{2+}-channels have been reported to be activated by diacylglycerol (DAG) [258]. The fact, that L108 reduced LTD_4-induced bronchoconstriction in human PCLS could imply that LTD_4 stimulates PI-PLC mediated formation of DAG, and thus activation of T-type Ca^{2+}-channels, and influx of extracellular Ca^{2+}. In GP PCLS only the Rho-kinase inhibitor and the L-type Ca^{2+}-channel inhibitor were effective, indicating a dependence on the Rho-kinase pathway and extracellular Ca^{2+}. In contrast to human PCLS, it has been reported that in GP airways the bronchoconstriction induced by LTD_4 is partly mediated by thromboxane [252,253]. Aizawa *et al.* showed on tracheal strips compared to parenchymal strips from the GP, that in central airways (tracheal strips) LTD_4 acts directly on smooth muscle, but in peripheral airways (parenchymal strips) LTD_4 induces bronchoconstriction both directly and indirectly *via* thromboxane formation [252]. The effect of Y27632 on the LTD_4-induced bronchoconstriction in PCLS from GP might be therefore due to inhibition of the thromboxane-mediated part of the contraction. On the other hand, perhaps the direct induction of bronchoconstriction by LTD_4 in GP PCLS was attenuated by inhibition of the L-type Ca^{2+}-channel. The histamine-induced bronchoconstriction in GP PCLS was significantly attenuated only with the Rho-kinase inhibitor (Fig. 4.27A). In line with that, Tokuyama *et al.* showed in isolated perfused GP lungs that increases in lung resistance (RL) caused by LTD_4 or histamine were attenuated by treatment with Y27632 [254].

Besides the activation of different signal transduction pathways by the main mediators of the EAR in the different species, there was one similarity that was observed in all species. In all cases, the U46619-induced bronchoconstriction was significantly diminished by preincubation with the Rho-kinase inhibitor Y27632. This indicates a central role of the Rho-kinase pathway in mediating the

TP-receptor triggered bronchoconstriction in PCLS, that it appears to be independent of PI-PLC, IP_3 and extracellular Ca^{2+} (Fig. 4.25A, 4.27B, and 4.34). These data are corroborated by findings in the isolated perfused rat lungs, where Y27632 completely abolished U46619-induced pressor responses, both in airways and vessels [114] (Fig. 4.35). Furthermore, the effects of U46619 in GP tracheae and main bronchi preparations were inhibited by Y27632 [255].
Taken together, different signal transduction pathways are activated by different mediators, - to further complicate matters - this is also species dependent. Only the U46619-induced bronchoconstriction is mediated via the Rho-kinase pathway in mice rats, humans and GP. The release of Ca^{2+} from intracellular stores via IP_3 appears to play a minor role in mediating bronchoconstriction induced by the different mediators.

The combination of PCLS and two-photon microscopy allows further to study Ca^{2+}-signaling in airway smooth muscle cells. With this technique it is possible to investigate airway contraction on the one hand and intracellular free Ca^{2+}-level increases after stimulation on the other hand within the same model. In contrast to confocal microscopy, two-photon microscopy offers the possibility to measure Ca^{2+} signals not only in one cell layer, but also up to 150 µm deep in the tissue of the PCLS. Investigations with rat PCLS either passively sensitized or unsensitized and treated with serotonin showed an increase of the intracellular Ca^{2+} levels after stimulation (Fig. 4.31 and 4.33). As an increase of intracellular Ca^{2+} is responsible for contraction of smooth muscle cells, these data are in line with the results obtained in the contraction experiments, where the airways in PCLS contract in response to allergen or serotonin. Pre-incubation of the slices with mibefradil completely prevented the response in both cases. No airway contraction and no increase of the Ca^{2+} level in airway smooth muscle cells were observed anymore. The L-type Ca^{2+}-channel inhibitor nifedipine only partly reduced bronchoconstriction on the one hand, and increase of the Ca^{2+}-signal on the other hand. If extracellular Ca^{2+} was removed before stimulation, no response was observed, neither bronchoconstrition nor Ca^{2+}-level increase in airway smooth muscle cells was determined (Fig. 4.31 and 4.33). Because we were able to inhibit the allergen or serotonin-induced bronchoconstriction and Ca2+-signaling with mibefradil or the remove of extracellular Ca2+, the allergen and serotonin-induced Ca2+-signaling appears to directly mediate bronchoconstriction in rat PCLS.

5.7 Role of the Maxi-K channel in bronchoconstriction

Calcium-sensitive potassium channels (Maxi-K channels) are reported to play an important role in regulating the tone of airway smooth muscle cells [203,256,257]. It has been suggested that their activation might be useful in asthma therapy to inhibit contraction of the airways.

K^+-channels are important for maintenance of resting membrane potential for repolarization of action potentials and other depolarizing stimuli. The concentration of K^+ ions inside cells is much higher (150 mM) than outside (3-5 mM). The opening of K^+-channels lead to an outflow of K^+ ions along the electriochemical gradient, resulting in hyperpolarization of the cell membrane to -80 mV from the resting value of about -60 mV. As a consequence the excitability of the cell to contractile stimuli is reduced [257]. Therefore one would expect that animals, lacking potassium channels respond stronger to contractile stimuli compared to control animals.
In the present study we used maxi-K knockout and control mice, to compare airway contraction towards various stimuli. Concentration-response curves for methacholine showed a weaker contractile response in the maxi-K knockout mice compared to wild type mice (Fig. 4.29A). Bronchoconstriction towards serotonin and U46619, however, were stronger in the knockout mice compared to the wild type (Fig. 4.29B,C). Data from isolated perfused lungs confirm the results obtained for methacholine. The respiratory capacity was significantly decreased in wild type mice compared to maxi-K knockout mice (unpublished data). M. Sausbier and colleagues detected an increased synthesis of cGMP in maxi-K knockout mice, which is maybe responsible for the weaker airway contraction towards methacholine (unpublished data).
Taken together, the maxi-K channel seem to play a role for airway relaxation in murine PCLS, but this is maybe dependent on the stimulus.

5.8 Conclusions

PCLS offer the opportunity to study several aspects concerning airway reactivity in response to various stimuli. The establishment of PCLS from guinea pigs and monkeys in addition to those of rat, mice and humans, provides the possibility to compare airway and vessel responses from different species to a variety of stimuli by using the same method. Animal models are important and offer new insights into various areas of a disease, but all animal models have their limitations. GP are shown to resemble the human situation more than do mice or rats, therefore PCLS from guinea pigs are a valuable model to study the EAR and maybe further airway pharmacology with regard to humans.

6 Summary

Species differences are a critical point for the extrapolation of data from animal models to the human situation. This appears to be particularly true for asthma. The model of precision-cut lung slices (PCLS) offers the possibility to analyze airway and vessel responses by digital video microscopy in different species such as rat, mouse and even human. In the present study the model of PCLS was established for guinea pigs and monkeys, animals that may resemble human airway responses better than do rodents.

The early allergic response (EAR) is one characteristic feature of human asthma, characterized by mast cell degranulation and subsequent bronchoconstriction. Thromboxane, leukotrienes and histamine were identified as the mediators responsible for airway contraction during the EAR in guinea pig and human PCLS, whereas in PCLS from rats the major mediator is serotonin. In contrast to the other species, murine PCLS showed no airway contraction after passive sensitization. In actively sensitized mice, however, allergen provocation induced a bronchoconstriction, but this appeared to be mast cell-independent, calling into question the suitability of the mouse model to study the acute asthmatic response. In monkey PCLS no EAR was observed, when the slices are passively sensitized with human serum. Thus, concerning the mediators responsible for the bronchoconstriction during an EAR, the best resemblance to humans was observed with guinea pig PCLS.

The PCLS model also allows investigation of airway hyperresponsiveness (AHR), another hallmark of asthma. However, an AHR was only observed in PCLS from actively sensitized rats in which an EAR has been performed 24h before. The mechanisms of this response are unknown, but this interesting finding suggests a new model for studying the mechanisms of AHR in asthma.

A major part of this thesis was focussed on the signalling pathways involved in airway smooth muscle contraction in the various species under various conditions. The present findings suggest that in guinea pig airways the early allergic bronchoconstriction depends on the myosin light chain kinase (MLCK) and extracellular Ca^{2+}, entering *via* L-type Ca^{2+}-channels. In humans, PCLS extracellular Ca^{2+} and the Rho-kinase pathway are involved. However, in both species inhibition of a single pathway attenuated the bronchoconstriction only to a small degree, indicating that further (and maybe unknown) signal transduction pathways may become activated. In rats, inhibition of the T-type Ca^{2+}-channels or removal of extracellular Ca^{2+} almost completely prevented the bronchoconstriction during an EAR, again suggesting a major role for extracellular Ca^{2+}. This conclusion was further supported by direct Ca^{2+}-measurement in single airway smooth cells by the means of two-photon microscopy. The investigation of signalling pathways of bronchoconstriction triggered by thromboxane showed a participation of the Rho-kinase pathway in all species. However, in most cases other signalling pathways contribute as well indicating that airway smooth muscle contraction is a complex, but robust process that is controlled at different levels.

Taken together, guinea pig PCLS have been established as a model to study airway responses with relevance to humans. In addition, the novel combination of PCLS with two-photon microscopy offers the opportunity to investigate signaling responses of single cells in their intact microenvironment. With respect to asthma therapy our studies suggest that the signal transduction pathways of bronchoconstriction may be too complex for developing useful asthma drugs targeted at this level. However, studies also indicate that reliable and complete protection against allergen-induced bronchoconstriction can be obtained if several receptors (i.e. TP, CysLT, H_1) are blocked simultaneously, a concept that is currently examined in a clinical trial.

7 Deutsche Zusammenfassung

Speziesunterschiede sind ein kritischer Punkt bei der Datenübertragung von Tiermodellen auf die humane Situation. Besonders beim Asthma scheint das deutlich zu sein. Das Modell der präzisions-geschnittenen Lungenschnitte bietet die Möglichkeit mittels Videomikroskopie Atemwegs- und Gefäßreaktivität in unterschiedlichen Spezies, wie Ratte, Maus, und auch Mensch, zu untersuchen. In der vorliegenden Arbeit wird die Etablierung des Modells der präzisions-geschnittenen Lungenschnitte von Meerschweinen und Primaten gezeigt. Diese Tiere spiegeln humane Atemwegsreaktivität eventuell eher wieder, als es Nagetiere tun.

Die allergische Frühphase ist ein charakteristisches Merkmal des Asthma bronchiale, gekennzeichnet durch eine IgE-vermittelte Mastzelldegranulation und anschliessende Kontraktion der Atemwege. Es wurde gezeigt, dass Thromboxan, Leukotriene und Histamin in Lungenschnitten von Meerschweinen und Menschen verantwortlich für die Atemwegskontraktion sind. In Lungenschnitten von Ratten ist es hingegen Serotonin. Im Gegensatz zu den anderen Spezies, zeigen Lungenschnitte von der Maus keine Atemwegskontraktion nach passiver Sensibilisierung. In aktiv sensibilisierten Mäusen löst eine Allergenprovokation jedoch eine Atemwegskontraktion aus. Diese scheint aber Mastzell-unabhängig zu sein, und stellt damit die Eignung des Mausmodels zur Untersuchung der allergischen Frühphase in Frage. In Lungenschnitten von Primaten konnte keine allergische Frühphase beobachtet werden, wenn die Lungenschnitte mit humanem Serum passiv sensibilisiert wurden. In Bezug auf die Mediatoren, die verantwortlich für die Atemwegskontraktion während der allergischen Frühphase sind, kann man sagen, dass das Meerschwein die humane Situation am ehesten widerspiegelt.

Das Modell der Lungenschnitte erlaubt auch die Untersuchung von Atemwegshyperreagibilität, ein weiteres Kennzeichen des Asthma bronchiale. Sie konnte jedoch nur in Lungenschnitten von aktiv sensibilisierten Ratten beobachtet werden, an denen 24 Stunden zuvor eine allergische Frühphase ausgelöst wurde. Die Mechanismen für diese Hyperreaktivität sind noch nicht bekannt, aber die interessanten Daten deuten auf ein neues Modell hin, um die Mechanismen der Atemwegshyperreagibilität im Asthma näher zu untersuchen.

Ein großer Teil der Arbeit war auf die Signaltransduktionswege gerichtet, die bei der Atemwegskontraktion in den verschiedenen Spezies aktiviert werden. Die aktuellen Daten zeigen, dass beim Meerschwein die Atemwegskontraktion während der allergischen Frühphase abhängig ist von der Myosin-Leichtkettenkinase und extrazellulärem Ca^{2+}, welches über den L-typ-Ca^{2+}-Kanal ins Zytoplasma strömt. Bei Lungenschnitten vom Menschen ist ebenfalls extrazelluläres Ca^{2+} und auch der Rho-kinase Signaltransduktionsweg involviert. In diesen beiden Spezies führt die Hemmung jeweils nur eines Signalweges nur zu einem leichten Schutzeffekt, was darauf schliessen lässt, dass eventuell noch andere, bisher vielleicht noch nicht bekannte Signaltransduktionswege aktiviert werden. In Lungenschnitten von Ratten konnte gezeigt werden, dass die Hemmung des T-typ Ca^{2+}-Kanals oder die Wegnahme von extrazellulärem Ca^{2+} eine allergen-induzierte Atemwegskontraktion fast komplett verhindert, was wiederum zeigt, dass extrazelluläres Ca^{2+} eine

große Rolle zu spielen scheint. Diese Feststellung wird weiter untermauert durch direkte Ca^{2+}-Messungen in Glattmuskelzellen der Atemwege mittels Zwei-Photonen Mikroskopie.
Für eine Thromboxan-vermittelte Atemwegskontraktion konnte in allen Spezies die Beteiligung des Rho-kinase Weges gezeigt werden.

Das Modell der Präzisionslungenschnitte vom Meerschwein wurde etabliert als ein Modell, mit dem es möglich ist, Atemwegsreaktivität im Hinblick auf den Menschen zu untersuchen. Desweiteren bietet die neue Kombination von Lungenschnitten und Zwei-Photonen Mikroskopie die Möglichkeit, Signalprozesse einzelner Zellen in einer intakten Mikroanatomie zu untersuchen.
Mit Bezug zur Asthmatherapie, deuten unsere Studien daraufhin, dass die bei einer Atemwegskontraktion aktivierten Signaltransduktionswege zu komplex sind, um Asthmamedikamente auf diesem Level einzusetzen.
Studien haben aber auch gezeigt, dass ein vertrauenswürdiger und kompletter Schutzeffekt gegen eine Allergen-induzierte Atemwegskontraktion besteht, wenn mehrere Rezeptoren gleichzeitig geblockt werden, z.B. der Thromboxan-Rezeptor, $CysLT_1$-Rezeptor und H1-Rezeptor. Dieses Konzept wird gerade in einer klinischen Studie getestet.

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